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Solanum tarijense reaction to *Phytophthora infestans* and the role of plant defence molecules

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Abstract

Late blight is the main disease of potatoes around the world. Because of the difficulties in applying effective control methods, the introduction of resistant cultivars represents a safe strategy. The potato species *Solanum tarijense* represents an attractive resistance source as its adaptation to long days is promissory, producing tubers of good size and aspect. Deposition of structural compounds like lignin and callose were described as a non-specific resistance mechanism. In this work, we measured polyphenoloxidases (PPO) and peroxidases (POX) activities and the accumulation of phenols, lignin and callose and their correlation with the resistance levels of *S. tarijense*. Clones Oka 6320.9 and Oka 5632.11 showed low infection rates and these were correlated with a higher accumulation of phenols, lignin and callose and a strong induction of PPO and POX activities. However, in highly infected clones, a lower or no accumulation of these compounds was observed. These results demonstrate a correlation between the amount of defence molecules and the resistance levels according to the detached-leaf assay. However, more field experiments are required to validate these results.

Key words: potatoes — horizontal resistance — phenols — polyphenol-oxidases — peroxidases — callose — lignin

Traditionally, genetic resistance of potato against late blight is classified into two different types: the qualitative (race-specific) and quantitative (race-non-specific) resistance. Qualitative resistance is mediated by *R* genes that lead to a race-specific hypersensitive response. These *R* genes only provide short-lived resistance in the field as new virulent races of the pathogen rapidly overcome the resistance encoded by single race-specific resistance genes (Fry and Goodwin 1997, Wang et al. 2005). In contrast, quantitative resistance is controlled by many interacting genes that do not prevent infection, but slow down the development of the pathogen at individual infection sites on the plant, and hence, lasts longer (Colon et al. 1995, Wang et al. 2005).

In Argentina, *Solanum tarijense* (*trj*) is one interesting species, as its adaptation to long days is promissory and it produces tubers of good size and aspect (Huarte 2002). This species is a wild potato endemic to South America, with predominant geographical distribution in the central and southern areas of Bolivia and the northern region of Argentina (Hawkes 1990, Labarte et al. 2003). From a potato-breeding point of view, the importance of this species is due to the fact that it is a natural reservoir of genes controlling resistance against major potato diseases and pests (Buso et al. 2003).

Deposition of structural compounds, which are thought to have a function in cell wall strengthening, can be seen as a non-specific resistance mechanism. During compatible and incompatible *Phytophthora infestans*–potato interactions, wall appositions with accumulated callose have been found (Vleeshouwers et al. 2000, Hamiduzzaman et al. 2005). In addition, lignification, which is considered as a general response to pathogen attack in several plant species, appears to play a role in resistance to *P. infestans* (Friend 1973, Vleeshouwers et al. 2000, Wang et al. 2008). On the contrary, peroxidases (POX) and polyphenol-oxidases (PPO) catalyse the formation of lignin and other oxidative phenols that contribute to the formation of defence barriers for reinforcing the cell structure (Bashan et al. 1985). It has been reported that the expression of PR proteins, PPO, phytoalexins and phenols differs with the level of resistance of the different potato clones. Clones with a high resistance level to *P. infestans* showed an early expression and larger accumulation of these compounds (Tonón et al. 1998, Andreu et al. 2001, Conrath et al. 2001).

Previous results showed that *trj* clones were significantly less infected than cultivars 'Bintje' and 'Pampeana-Instituto Nacional de Tecnología Agropecuaria' (INTA) (Wolski et al. 2009) and the activity of pathogenesis related proteins, including glucanases, chitinases, peroxidases and polyphenoloxidases was higher, constitutively, in *trj* than in 'Bintje' and 'Pampeana INTA' (Wolski et al. 2009). Therefore, in the present study, the activity of POX, PPO and the relative content of lignin, callose and phenols of plants were measured. The responses in field experiments and detached-leaf assay of a set of *trj* clones displaying differential behaviour to *P. infestans* were determined. The study of the non-specific resistance in new clones is a pathway to improve the genetic resistance to *P. infestans*.

Materials and Methods

Biological material: A strain of *P. infestans* (race 2, 3, 6, 7, 8, 9; mating type A2), was grown on rye-agar medium (Agar bacteriological, Agar Nº1, LP0011, OXOID Ltd, Basingstoke, England) and on potato tuber slices. Slices were incubated in closed plastic boxes with filter paper soaked in water (approximately 90% relative humidity), and were maintained in darkness at 18°C. After 7 days, the mycelium that developed on the slices was washed with distilled water, then filtered through 20 μm Nalgene filters and placed for 2 h at 4°C for the release of zoospores. The concentration of the inoculum was determined by counting the sporangia in a Neubauer chamber.

Potato cultivars of *Solanum tuberosum* L., 'Pampeana INTA' and 'Bintje' and *Solanum tarijense* (*trj*) clones (Oka 6320.9, Oka 5632.11, Oka 5632.12 and Hof 1717.10) were provided by the Balcarce Experimental Station of the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina. *Solanum tuberosum* L. cv. 'Pampeana INTA' (MPI 59.789/12 × 'Huinkul MAG') is a cultivar from the Argentine Breeding Programme (INTA-Balcarce).

Field evaluation: Potato clones of *trj* and 'Bintje' (susceptible) and 'Pampeana INTA' (moderately resistant) cultivars were planted in randomized complete blocks with four replicates. Five plants per experimental unit (clone) were planted in one contiguous row. Two flanking rows were planted with 'Pampeana INTA'. 'Bintje' and 'Pampeana INTA', without major genes, were used as reference cultivars. The plants were inoculated 40 days after planting with a sporangia suspension of *P. infestans*. The concentration was adjusted to 9.0×10^3 sporangia/ml. Supplementary watering was applied in order to give the appropriate conditions of humidity for the development of the disease. The percentage of affected foliage was determined for each plot using the scale of the International Potato Centre, Perú (https://research.cip.cgiar.org/typo3/web/index.php?, May 2009). Four weekly readings were carried out beginning 7 days after the inoculation. The Area Under the Disease Progress Curve (AUDPC) was determined for each clone (Shaner and Finney 1977, Campell and Madden 1990) as $\mathrm{AUDPC} = \Sigma_{i=1}^{n-1}[(X_{i+1} + X_i)/2][t_{i+1} - t_i]$, where X_i is the tissue percentage affected at the observation i; $[t_{i+1} - t_i]$ is the time in days between two observations; and n is the total number of observations. The trials were carried out during the 2007/2008 cycle.

Detached-leaf assay: Foliar infection with late blight was assessed in individual potato plants. The plants were cultivated in a growth chamber. During plant growth, the temperature ranged between 15–24°C and natural daylight was supplemented by high-pressure sodium lamps (400 W) in 14–10 h day–night cycle. Plants were irrigated with a sprinkler system when needed. The experiment was repeated three times. Each experiment consisted of three replicates of 10 plants each.

The foliage of plants after 30 days of emergence, period of maximum expression of resistance (J. Landeo, International Potato Centre, Perú, personal communication), was cut and the leaves placed in 9-cm Petri dishes on 2% water-agar. The leaves were inoculated with *P. infestans* sporangial suspension (9.0×10^3 sporangia/ml) under laboratory conditions ('detached-leaf' assay) (Goth and Keane 1997). The plants were then placed in a growth chamber at 18–20°C and 80% relative humidity during the rest of the experiment. Disease development was recorded at 24, 48, 72 and 96 h after inoculation and was assessed using image analysis software (APS ASSESS 2 software; Lakhdar Lamari, Winnipeg, Canada) for quantification of infected area (lesion area/total leaf area). In addition, uninfected leaves and leaves inoculated with *P. infestans* were collected at different times (0, 24, 48 and 72 h) and maintained at −20°C for biochemical determinations.

Extraction of POX and PPO: Uninfected and infected leaves from each clone and cultivar were homogenized with liquid nitrogen using a mortar and pestle. The homogenized tissue was rinsed with sodium acetate 50 mM, pH 7.2 and activated charcoal, and filtered through a 0–20 mm nylon filter. For each gram of tissue 4 volumes were added. The tissue extract was centrifuged at 12 000 × ***g*** for 20 min. All operations were carried out at 0–4°C. The supernatant was transferred to a 1.5-ml vial and stored at −20°C.

POX assay: Peroxidases activity was measured as was described by Yao and Tian (2005) with some modifications. The reaction mixture consisting of 0.5 ml of crude extract and 2 ml of 60 mM guaiacol as substrate in 100 mM sodium phosphate, pH 6.4, then the mixture was incubated for 5 min at 30°C. The increase in absorbance at 460 nm was spectrophotometrically assayed after the addition of 1 ml H_2O_2 (24 mM). Enzymatic activities were defined as the increase of absorbance, and one unit was defined as the increase in one absorbance unit per minute under the conditions of the assay.

PPO assay: Polyphenoloxidase (cathecol oxidase) activity was determined using the method described by Chen et al. (2000). Two hundred microlitres of the leaf extract were mixed with 700 µl of phosphate 50 mM, pH 7.2. The rate of increase in absorbance at 420 nm was measured for 1 min after the addition of 100 µl 0.2 M cathecol. Enzymatic activities were defined as the increase of absorbance, and one unit was defined as the increase in one absorbance unit per minute under the conditions of the assay.

Extraction and quantification of phenols: Infected and uninfected leaves from each clone and cultivar were homogenized with liquid nitrogen using mortar and pestle. The homogenized tissue was rinsed with 80% (v/v) aqueous methanol (Cvikrová et al. 1993, Torti et al. 1995). For each gram of tissue 10 volumes were added. The tissue extract was centrifuged at 12 000 × ***g*** for 20 min. All operations were carried out at 0–4°C. The supernatant was transferred to a 1.5-ml vial and stored at −20°C. The concentration of soluble phenols was determined with Folin–Ciocalteau reagent and was expressed as micrograms of chlorogenic acid per gram of fresh weight (Bray and Thorpe 1954).

Extraction and determination of lignin: Lignin was extracted according to the method described by Bruce and West (1982) and was quantified in the alcohol insoluble residue (AIR), as thioglycolic acid derivatives following alkali hydrolysis (Campbell and Ellis 1992). Results were expressed as the increase in absorbance 280 nm/µg of AIR. Lignin content was also assayed by digestion of AIR material with 25% acetyl bromide in acetic acid (Sasaki et al. 1996).

Extraction and determination of callose: Callose extraction and determination was assayed as described by De Ascensao and Dubery (2000). Infected and uninfected leaves from each clone and cultivar were homogenized with liquid nitrogen by means of a mortar and pestle. The homogenized tissue was rinsed with 80% (v/v) aqueous methanol and centrifuged at 12 000 × ***g*** for 20 min. For each gram of tissue 10 volumes were added. Precipitates were treated with 1 M NaOH at 80°C for 15 min to solubilize callose. Callose content was expressed as pachyman equivalents per gram fresh weight.

Statistical analysis: Data records were evaluated through analysis of variance procedures and by linear regression procedures using the routines included in SAS/STAT (SAS Institute 1990). For comparison of means least significant differences (LSD) were used at P = 5%.

Results

Field and detached-leaf assays

Results from detached-leaf assays (Fig. 1) showed that potato clones can be separated in two groups based to their behaviour to late blight. One group is formed by the *trj* clones Oka 6320.9 and Oka 5632.11. These clones showed significantly less infection (about 5% of the leaf infected area at 96 h after inoculation) than the group formed by the *trj* clones Oka 5632.12 and Hof 1717.10 (between 55% and 75% of the leaf infected area at 96 h). Significant differences were observed among potato clones (Table 1).

Results obtained from field trial showed the same pattern as observed in the detached-leaf assays. Hof 1717.10 showed the highest AUDPC values (1749), whereas Oka 6320.9 (1173.75) showed the best performance against *P. infestans* (Table 1). Oka 5632.11 also showed a good performance (1413.75 AUDPC), but significantly different from 'Pampeana INTA' (1343.4 AUDPC). Oka 5632.12 also showed a high AUDPC

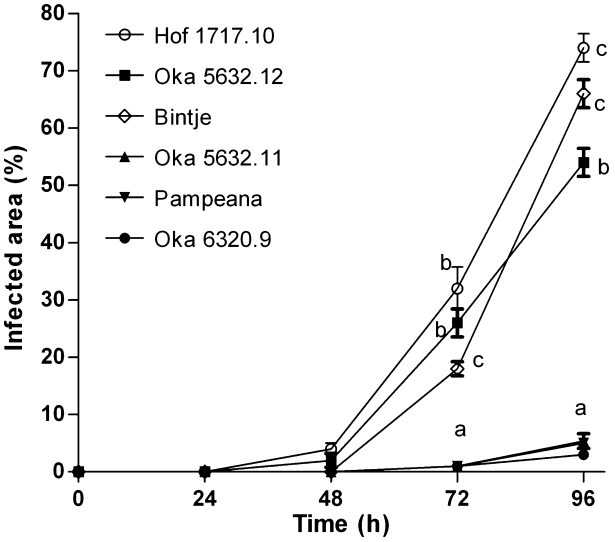

Fig. 1: Detached-leaf assay. Detached leaves were inoculated with *Phytophthora infestans* under laboratory conditions. Disease progress was evaluated by measuring the infected area at different times postinoculation. Each point represents the mean value ± SD of three independent experiments. Same letters are not statistically different at 5% according to Tukey's test

value (1719.6), but no significant differences were observed when compared with 'Bintje' (1510.33) (Table 1).

PPO and POX activities in *trj* clones

Potato clones, which showed less infection in the field trial and in the detached-leaf assay, also showed high levels of PPO activity (Fig. 2). After the inoculation with *P. infestans*, PPO activity approximately increased twice, at 24 h for *trj* clones Oka 6320.9, Oka 5632.11 and 'Pampeana INTA'; the activities were 16-, 12- and 3-fold higher, respectively, than for the other clones and cultivars (Fig. 2). The maximum activities were detected between 24 and 48 h postinoculation. On the contrary, clones Oka 5632.12, Hof 1717.10 and 'Bintje' did not show an increase on PPO activity at least until the 72 h postinoculation. In addition, those clones with lower infection in detached-leaf and field assays also showed higher levels of PPO activity at time zero (without inoculation with *P. infestans*) (Fig. 2).

For the POX activity, a similar pattern was observed, where *trj* clones Oka 6320.9 and Oka 5632.11 showed the highest activity (Fig. 2). However, the maximal activity was

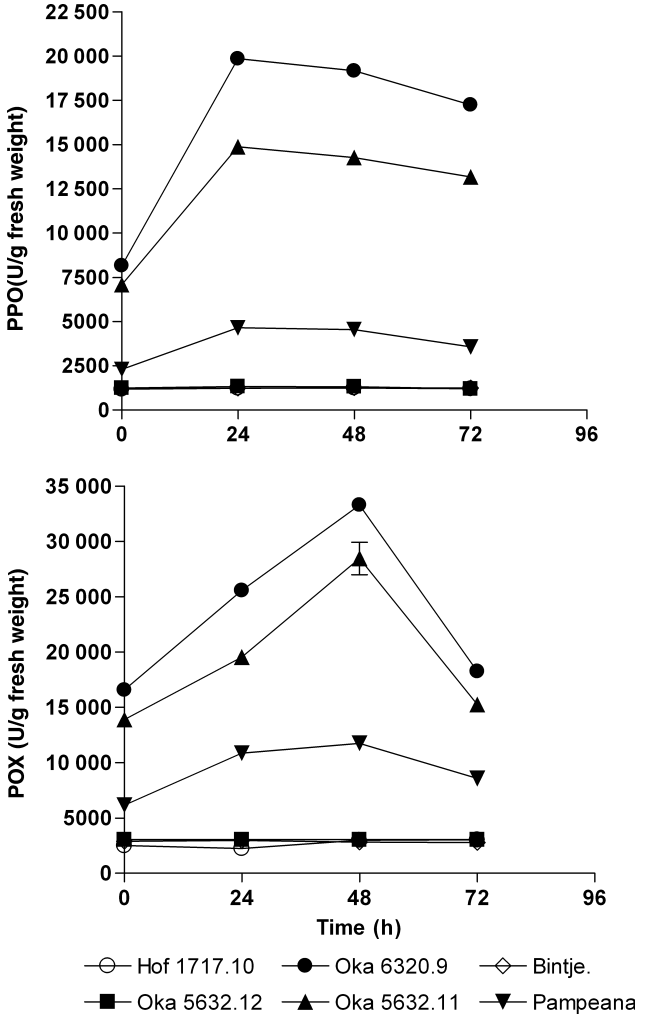

Fig. 2: PPO and POX activities in *trj* clones inoculated with *Phytophthora infestans*. PPO and POX activities were measured in leaves infected with *P. infestans*. Uninfected leaves (time zero) were used as controls. Each point represents the mean value ± SD of three independent experiments, with three replicates per experiment

observed at 48 h postinoculation (Fig. 2). In addition, the constitutive levels of POX were higher in these clones than in 'Pampeana INTA', 'Bintje', and *trj* Oka 5632.12 and Hof 1717.10.

The activities of PPO and POX decreased after 72 h postinoculation.

Table 1: Maximum values of *Phytophthora* infection levels in field and detached-leaf assays and amounts of defence-related compounds and activities

Clones	Infected area (%)	AUDPC	PPO (U/g FW)	POX (U/g FW)	Phenols (mg Ferulic/g FW)	Lignin (A_{280} nm/μg AIR)	Callose (mg Pachyman/g FW)
Hof 1717.10	74 ± 5.5d	1749 ± 412.7c	1250 ± 37.3a	2979.6 ± 102.8a	309.6 ± 19.33b	2 ± 0a	2 ± 0a
Oka 5632.12	54 ± 5.5b	1719.6 ± 172.9c	1324.6 ± 60b	3033.3 ± 111a	360.4 ± 13.9c	50 ± 7.07b	4 ± 0a
Oka 5632.11	5.4 ± 2.9a	1413.75 ± 130.6b	14268 ± 13.12d	28461.6 ± 3297.9c	1232.8 ± 23.6e	231 ± 16c	449 ± 45c
Oka 6320.9	3 ± 1.87a	1173.75 ± 281a	19169 ± 35.7e	33278.6 ± 425.3d	1532.4 ± 23f	285 ± 7.1d	545 ± 25.5d
Bintje	66 ± 5.5c	1510.33 ± 75.2bc	1260.7 ± 50.3a	2821.6 ± 489.1a	256.4 ± 23a	4 ± 0a	6 ± 0a
Pampeana	5 ± 0a	1343.4 ± 176.3a	4538 ± 45.15c	11738.2 ± 461.5b	1128.8 ± 18.6d	219 ± 22.5c	345 ± 25.5b

Values with the same letters are not statistically different at the 5% error level according to means least significant differences (LSD) tests.

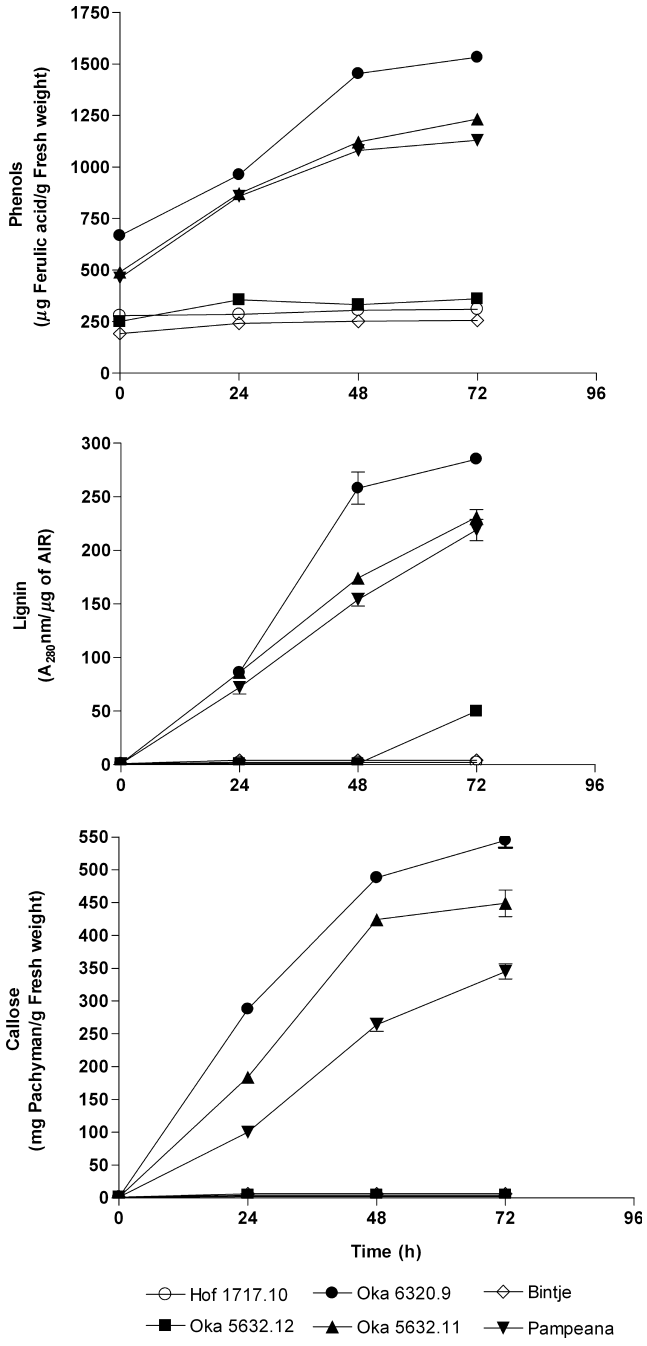

Fig. 3: Differential accumulation of soluble phenols, lignin and callose in *trj* clones inoculated with *Phytophthora infestans*. Accumulation of soluble phenols, lignin and callose were measured in potato leaves inoculated with *P. infestans*. Uninfected leaves (time zero) were used as controls. Each point represents the mean value ± SD of three independent experiments, with three replicates per experiment

Phenols, lignin and callose content

In addition, a strong accumulation of soluble phenols, lignin and callose was induced by *P. infestans* in those *trj* clones that showed a higher performance in the field and in detached-leaf assays (Fig. 3).

The accumulation of soluble phenols was higher in *trj* Oka 6320.9 and Oka 5632.11 and 'Pampeana INTA', than in 'Bintje', *trj* Oka 5632.12 and Hof 1717.10 (Fig. 3). For cultivar 'Bintje' and *trj* clones Oka 5632.12 and Hof 1717.10, soluble phenols did not increase after the inoculation with *P. infestans* (Fig. 3).

Lignin accumulation also increased rapidly in *trj* Oka 6320.9 and Oka 5632.11, and cultivar 'Pampeana INTA', but in the more susceptible clones and cultivars only after 72 h a slight increase was observed (Fig. 3).

Callose accumulation, after the inoculation with *P. infestans*, showed similar results for soluble phenols: the accumulation of callose was much higher in *trj* Oka 6320.9 and Oka 5632.11 and cultivar 'Pampeana INTA', than in the rest of cultivars and clones, in which no accumulation of callose was detected using this method (Fig. 3).

In addition, the initial content of soluble phenols was higher in 'Pampeana INTA', and *trj* Oka 6320.9 and Oka 5632.11, at time zero (without inoculation) (Fig. 3). These results showed that these clones have higher constitutive levels of soluble phenols than the clones Hof 1717.10 and Oka 5632.12. However, all the clones and cultivars showed almost the same amount of lignin and callose at time zero (Fig. 3).

Table 2: Correlation analysis between *Phytophthora* resistance levels and amounts of defence related compounds and activities at 72 h postinoculation

Compound or activity	Assay	Squared correlation (r^2)
PPO	Detached leaf	0.59
PPO	Field	0.6133
POX	Detached leaf	0.6641
POX	Field	0.7098
Phenols	Detached leaf	0.93
Phenols	Field	0.788
Lignin	Detached leaf	0.97
Lignin	Field	0.744
Callose	Detached leaf	0.94
Callose	Field	0.76

For correlation analysis maximal values were used.

Correlation between resistance levels and amount of plant defence molecules

The correlation between resistance levels observed in field and detached-leaf assays and the activities and accumulation of phenolic compounds in the plant was also analyzed (Table 2). At 72 h postinoculation, a strong correlation among phenols, lignin and callose accumulation and detached-leaf and field assays was observed, where *trj* clones and cultivars with lower percentage of infected area showed higher accumulation of these compounds after infection with *P. infestans*. PPO and POX activities also showed a correlation with the detached-leaf assay (Table 2).

Discussion

Although a rich pool of resistance sources is available in wild *Solanum* species, little is known about the physiological and molecular basis of the various resistant genotypes (Vleeshouwers et al. 2000). For this reason, it is necessary to characterize their behaviour to late blight. In this work, we showed that there is a strong correlation among molecules involved in plant defence responses and *Phytophthora* resistance levels of *trj* clones.

It is well known that PPO and POX enzymes are involved in plant defences. PPO catalyses the formation of highly

reactive quinones and these ones may possess direct antibiotic and cytotoxic activities to pathogens (Mayer and Harel 1979, Li and Steffens 2002). In addition, it has been reported that transgenic tomato plants that overexpressed a PPO from potato exhibited an important increase in resistance to *Pseudomonas syringae* (Li and Steffens 2002). These results emphasize the importance of PPO in restricting plant disease development. On the contrary, POX protects the plant tissue from the oxidative damage taking place during the attack of pathogens, the products of POX can have antimicrobial and antiviral activity, and they are also involved in the formation of lignin (Avdiushko et al. 1993). After the inoculation with *P. infestans*, the clones Oka 5632.11 and Oka 6320.9 showed a strong increase of PPO activity, whereas the rest of the clones did not show changes in the activity. The same results were observed for POX activity and these agree with those presented by Lozoya-Saldaña et al. (2007), which showed that resistant potatoes had an early increase of POX activity. A similar behaviour was also reported for pepper and tomato (Mozzetti et al. 1995, Zacheo et al. 1995).

Phenolic compounds were constitutively higher in *trj* clones Oka 5632.11 and Oka 6320.9 than in Oka 5632.12 and Hof 1717.10. Besides, the clones with best performance to *P. infestans* showed a strong increase in the accumulation of phenols (Fig. 3). It was already reported by Rubio-Covarrubias et al. (2006) that the concentration of phenolic compounds was correlated with the resistance levels of different potato cultivars. In addition, Andreu et al. (2001) showed that the accumulation of phenolic compounds was relatively lower in 'Bintje' than in 'Pampeana INTA' cultivars, as also shown here.

Lignin and callose content increased significantly in the leaves of the best performing clones upon the infection with *P. infestans* (Fig. 3). The accumulation of lignin in resistant potato plants of *S. tuberosum* and *S. phureja* was shown by Evers (2002). The increase of callose and lignin could correspond to the establishment of a physical barrier for the introgression or spreading of the pathogen in resistant plants (Mauch-Mani and Slusarenko 1996, Vleeshouwers et al. 2000). Lignification on hyphae has been shown previously for the oomycetes *Peronospora parasitica* in Arabidopsis (Mauch-Mani and Slusarenko 1996), and *Bremia lactucae* in lettuce (Bennett et al. 1996).

Biochemical results of the present study showed a strong correlation with the performance of *trj* clones in the detached-leaf assay (Table 2). It is known that POX enzymes are involved in the polymerization of lignin (Ralph et al. 2004), some studies showed that the reduction in POX expression produces also a reduction in lignin content (Talas-Ogras et al. 2001, Li et al. 2003). In soybean, the treatment with a *Phytophthora sojae* elicitor resulted in the increase of POX activity concurrent with the accumulation of phenolic polymers, including lignin-like polymers (Graham and Graham 1991). In addition, Wang et al. (2005) showed through the utilization of cDNA microarrays that many genes that encode enzymes participating in the defence-related metabolic pathways such as the biosynthesis of phenylpropanoids and alkaloids were activated by *P. infestans* in potato clones with quantitative resistance to this pathogen.

It is important to distinguish the role of insoluble phenols, like lignin, which strengthen the cell wall and soluble phenolic compounds with antimicrobial activity (Rubio-Covarrubias et al. 2006). In this regard, based on cytological observation, Vleeshouwers et al. (2000) did not find any correlation between the deposition of phenolic globules on leaf cells and resistance levels of different *Solanum* species.

A better understanding of changes that occur in the plant's defences upon infection with the pathogen will help to develop new tools for breeding programmes. Constitutive and induced deposition of phenolic compounds, lignin and callose on the cell walls is thought to be a resistance mechanism against invasion by the pathogen in many plant species (Rubio-Covarrubias et al. 2006). The results presented here showed a correlation between the amount of defence molecules and the resistance levels according to the detached-leaf assay. However, more field experiments are required to validate these results.

Finally, defence molecules studied in this work could be used as biochemical markers to assist breeders in the selection of horizontally resistant cultivars.

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