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Biochemical Markers involved in Horizontal Resistance to *Phytophthora infestans* in Potatoes

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ABSTRACT

Potato Late Blight is the main pathogen which affects potatoes. Due to unavailability of effective chemical control methods and the intensive use of fungicides, the introduction of resistant cultivars represents a safe strategy. The sources of horizontal resistance to Late Blight in the Argentinean Potato Breeding Programs have been varied. One of the interesting species to evaluate is *Solanum tarijense* since its adaptation to long days is promissory, producing tubers of good size and aspect. In this study, the enzymatic activity of different PR proteins in the specie *S. tarijense* was measured. These clones were more resistant to Late Blight than 'Bintje' and 'Pampeana INTA' cultivars. The activity of glucanases, chitinases, peroxidases and polyphenoloxidases were 15, 1.5, 2 and 3 times higher respectively, in *S. tarijense* than in 'Bintje' and 'Pampeana INTA'. These results suggest that some PR proteins can be used as biochemical markers to assist breeders in the selection of horizontal resistant cultivars.

Keywords: late blight, pathogenesis-related proteins, polyphenoloxidases, potato, potato breeding
Abbreviations: PAL, phenylalanine ammonia-lyase; **POX**, peroxidases; **PPO**, polyphenoloxidases; **PR**, pathogenesis-related proteins

INTRODUCTION

The control of fungal diseases is generally based in the application of pesticides at high doses and frequencies. In Argentina, the control of Late Blight, caused by the oomycete *Phytophthora infestans* is also performed by means of the use of fungicides in a preventive way. The amounts of fungicides used are considered high under international standards, with the rising risks of contamination to the environment and people, besides increasing the production costs. The surge of fungal populations with more aggressive strains of the pathogen, more virulence factors, with the possibility of sexual mating (type A1 and A2) and oospore formation, and with a growing resistance to the systemic fungicides. Late Blight is present in all the production areas in Argentina and it has been proven that resistance to the fungicide metalaxyl is present in 100% of the collected strains of the A2 mating type (Mantecon *et al*. 1995), which conformed 85% of the populations of *P. infestans* (Van Damme *et al*. 1998; Distel and Huarte 2000). For this reason the search of genetic resistance to Late Blight constitutes a valid tool for the integrated management of the crop.

Genetic improvement is generally orientated to the incorporation of resistance genes in a process which concentrates those genes on different genetic backgrounds. Cultivars with vertical resistance, governed by few genes, or cultivars with quantitative resistance (horizontal resistance), governed by many genes with small effects are used. Of both resistance types the second is preferred, as it is considered to be durable and is not affected by changes in the pathogen (Malcolmson and Black 1966; Black 1970; Wang *et al*. 2005). Horizontal resistant cultivars have several combinations of genes; none of which gives absolute resistance, but together may reduce the development of the disease, allowing tolerance to the infection and exercising a smaller selection pressure for the evolution of new races of the pathogen (Johnson 1984). Besides showing good resistance aptitudes or tolerance to the disease, the new cultivars should show other promising characteristics of economic importance. These cultivars are traditionally bred through crossing, subsequently selected under favorable conditions for disease expression and evaluated using appropriate procedures. In Argentina, tetraploid and diploid material has been evaluated with different results. Some diploid species like *Solanum chacoense* have demonstrated to possess a good level of quantitative resistance but their tuber characteristics are not very attractive (Micheletto *et al*. 2000). Other interesting Argentinean species are *Solanum tarijense* and *Solanum gourlayii*, since their adaptation to long days is promissory and produce tubers of good size and aspect (Huarte 2002). *S. tarijense* is a wild potato species that is distributed in Bolivia and in the Northwest of Argentina, particularly is located in Salta province, from the 2000 to 3100 mosl, in arid environments of the Andean region.

The biochemical mechanisms of defense, related with the resistance to disease in plants, include the presence of: pathogenesis related proteins (PR) (PR-2: β-1,3-glucanases; PR-3: chitinases; PR-9: peroxidases), fungitoxic compounds (phytoalexins), reinforcement of the cell wall (phenols, callose and lignin) and others. The hydrolytic enzymes (β-1,3-glucanases and chitinases) posses a dual function in the resistance: they can exert a direct antimicrobial effect on the pathogen or they can accelerate and amplify the resistance response through the production of inducers (Tuzun 2001). On the other hand, peroxidases (POX) and polyphenol-oxidases (PPO) catalyze the formation of lignin and other oxidative phenols that contribute to the formation of defense barriers reinforcing the cell structure (Bashan *et al*. 1985). POX protects plant tissues from oxidative damage caused by reactive oxygen species that takes place during attack by the pathogen. The products of these anti-oxidative enzymes can have antimicrobial and antiviral activity, and they are

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also involved in the formation of precursors of substances that act as physical barriers (Avdiushko *et al*. 1993). PPO catalyzes the formation of highly reactive quinones (Mayer and Harel 1979). Quinones may act in several ways leading to protection of plants, for example, they can limit the development of diseases at the infected sites by accelerating the death of cells close to the infection site, by preventing the advance of infections and/or by generating a toxic environment, which will inhibit the growth of pathogens inside the cells (Bi and Felton 1995). Other enzymes such as phenylalanine ammonia-lyase (PAL) (Beaudoin-Eagan and Thorpe 1985; Dixon and Harrison 1991; Dalisay and Kuc 1995) are involved in phytoalexin or phenolic compound biosynthesis. Such enzymes have been related with defense against pathogens in several plants, including tobacco (Beaudoin-Eagan and Thorpe 1985; Goy *et al*. 1992), tomato (Bashan *et al*. 1985), cucumber (Rasmussen *et al*. 1991) and rice (Rajappan 1995).

The development of a system of biochemical markers, involved in the horizontal resistance to *P. infestans*, may help to implement a quick and economic technique to be used in the selection of resistant clones. The quick identification of resistant potato cultivars will contribute to the integrated management of the disease, with smaller economic, environmental and human costs.

The objectives of this work were: i) to quantify proteins related with the horizontal resistance to *P. infestans* in potato clones and cultivars of well-known resistance and ii) to select biochemical marker(s) that show a better correlation with the resistance response to Late Blight. The constitutive levels of the molecules related with the horizontal resistance may differ in resistant and susceptible potato clones to *P. infestans*. Therefore, the proteins profiles related with the horizontal resistance in potato can provide evidence of the metabolites that are responsible for the resistance and it may help to the selection of promising clones in breeding programs.

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 water embedded paper (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 and finally the concentration was adjusted to assays.

Potato cultivars of *Solanum tuberosum* L., 'Pampeana INTA' and 'Bintje' and *Solanum tarijense* clones, Oka 7494.3 and Oka 5874.33, were provided by the Balcarce Experimental Station of the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina. *Solanum tuberosum* L. cv. 'Pampeana INTA' (MP1 59.789/12 × 'Huinkul MAG') is a cultivar from the Argentine Breeding Program (INTA-Balcarce).

Evaluation of field resistance

Clones Oka 7494.3, Oka 5874.33 and cvs. 'Bintje' and 'Pampeana INTA' were planted in randomized complete blocks with two replicates. Four plants per experimental unit (clone) were planted in two contiguous rows. Two flanking rows were planted with susceptible 'Bintje' and with moderate resistant 'Pampeana INTA', both without major genes and used as reference cultivars. The plants were inoculated 40 days after planting with a sporangia suspension of *P. infestans*. The concentration was adjusted at 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. Four weekly readings were carried out beginning 7 days after the inoculation. For each clone it was determined the Area Under the Disease Progress Curve (AUDPC), as expression of the behaviour of the plant in relation to the pathogen (Shaner and Finney 1977).

$$AUDPC = \sum_{i=1}^{n} ((X_{i+1} + X_i)/2)\ (t_i+1 - t_i)$$

where Xi, is the tissue percentage affected in the observation i,

t, is the time in days of the observation i,

n, is the number of observations

The trials were carried out during the 2005/2006, 2006/2007 and 2007/2008 cycles. During the 2006/2007 cycle resistance results were not obtained in the field since the climatic conditions did not allow the development of the disease.

Evaluation of *in vitro* resistance

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 hours day-night cycle. Plants were irrigated with a sprinkler system when needed. Three experiments with a randomized complete design were performed during 2006 and 2007. Each experiment consisted of three replications each with 50 plants.

The foliage of plants after 30 days of emergence (period of maximum expression of resistance genes) 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^{-1}) under laboratory conditions ("detached-leaf" test) (Goth and Keane 1997). The assay was then placed in a growth chamber at 18-20°C and 80% relative humidity during the rest of the experiment. Disease development was recorded daily from 4 to 11 days after inoculation. Disease severity was estimated visually as the percentage foliage showing symptoms.

Extraction of β-1,3-glucanases and chitinases

Leaves without infection, from each clone and cultivar were homogenized with liquid nitrogen. The homogenized tissue was rinsed with sodium acetate 50 mM pH 5.2 (anhydrous, CH_2COONa, J.T. Baker), 0.1% sodium metabisulfite (granular, $Na_2S_2O_5$, Mallinkrodt Chemicals) and activated charcoal (Cat. C7606, Sigma-Aldrich) and filtered through a 0-20 mm nylon filter. For each gram of tissue 4 volumes were used. The tissue extract was centrifuged at 12.000 rpm for 10 min. The supernatant was dialyzed with 50 mM sodium acetate pH 5.2 (Cat. S7899, Sigma-Aldrich). All operations were carried out at 0-4°C. The supernatant was transferred to a 1.5 ml vial and stored at -80°C.

β-1,3-glucanase assay

β-1,3-glucanase activity was assessed by the rate of production of reducing sugar using laminarin (from *Laminarina digitata*, EC232.712-4, Cat. L9634, Sigma-Aldrich) as substrate. The reaction mixture consisted of 0.1 ml of 500 mM sodium acetate pH 5.2 containing 1% laminarin and 0.1 ml of enzyme extract. After 20 min incubation at 37°C the enzyme reaction was stopped by heating in boiling water for 2 min. Aliquots (0.1 ml) were taken and reducing sugar was determined as described by Ashwell (1957). Glucose (puriss Cat. G8270, Sigma-Aldrich) was used as a standard. Activity is expressed in units per gram of fresh weight, where 1 U = 1 µmol glucose released/h/ml enzyme.

Chitinase assay

Chitinase activity was assessed by measuring the rate of N-acetylglucosamine production using chitin purified powder from Crab Shells (Poly (*N*-acetyl-1,4-β-D-glucopyranosamine Cat. C9752, Sigma-Aldrich) as the substrate. The reaction mixture consisted of 60 µl of enzyme extract, 100 µl of partially hydrolyzed chitin in 50 mM sodium acetate buffer pH 5.2 and 80 µl of 50 mM sodium acetate buffer pH 5.2. After 5 hrs of incubation at 37°C the enzyme reaction was stopped by centrifugation for 10 min at 12,000

× *g*. Aliquots (100 µl) of the supernatant were separated and 20 µl of 0.8 M borate pH 9.1 (Buffer solution, puriss p.a. Cat. 82576, Fluka AG, Switzerland) and 0.5 ml of the DMAB (4-dimethyl-aminobenz-aldehyde, $C_9H_{11}NO$, puriss p.a. Cat. 02560, Fluka) were added. The mixture was incubated at 37°C for 20 min and absorbance at 585 nm was measured. *N*-actylglucosamine (*N*-Acetylglucosamine 6-sulfate sodium salt, $H_{14}NO_9SNa$, Cat. 44001, Sigma-Aldrich) was used as a standard (Reissig *et al*. 1955). Activity is expressed in units per gram of fresh weight, where 1 U = 1 µg of *N*-acetylglucosamine/h/ml of enzyme extract.

Extraction of PAL, POX and PPO

Uninfected leaves from each clone and cultivar were homogenized with liquid nitrogen in a mortar using a 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 -80°C.

PAL assay

PAL activity was determined using Beaudoin-Eagan and Thorpe methodology (1985). One hundred µl of crude extract was mixed with 900 µM L-phenylalanine ($C_6H_5CH_2CH(NH_2)CO_2H$, P2126, Sigma-Aldrich) and 0.5 M Tris-HCl solution (Tris (Base) (Hydroxymethyl) Aminomethane) $NH_2C(CH_2OH)_2$, Sigma-Aldrich), (HCl, pro analysis, Merck, Argentina). The mixture was incubated at 37°C for 70 min. The amount of *trans*-cinnamic acid formed from L-phenylalanine was measured spectrophotometrically at 290 nm (UV). PAL (from *Rhodoturala glutinis*, Sigma-Aldrich) was used as the standard. Activity is expressed in µg of *trans*-cinnamic acid/g fresh weight/h.

POX assay

POX activity (Chan and Tian 2006) was analyzed using guaiacol as substrate (Yao and Tian 2005). The reaction mixture consisting of 0.5 ml of crude extract, 2 ml of guaiacol ($2\text{-}(CH_3O)C_6H_4OH$, Cat. G5502, Sigma-Aldrich) substrate (100 mM sodium phosphate, pH 6.4 (monobasic, monohydrate, granular, $NaH_2PO_4H_2O$, Millinckrodt Chemicals) and 8 mM guaiacol) incubated for 5 min at 30°C. The increase in absorbance at 460 nm was measured spectrophotometrically after the addition of 1 ml H_2O_2 (24 mM) (hydrogen peroxide solution, Cat. H3410, Sigma-Aldrich). 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 assays

PPO (cathecol oxidase) activity was determined using Chen *et al*. (2000). Two hundred µl of the leaf extract was 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. The results were expressed as units per gram of fresh weight, where 1U= 1 Abs/min/ml of enzyme extract.

Statistical analysis

Data were evaluated by analysis of variance procedures and by linear regression procedures using the routines included in SAS/SAT (SAS Inst. 1990). For comparisons of averages Tukey's test was used at P = 5%.

RESULTS

Evaluation of field resistance

A significant correlation (R^2 = 0.67) was found between both AUDPC seasons. **Table 1** shows that for both seasons *S. tarijense* clones Oka 7494.3 and Oka 5874.33 had a lower AUDPC than 'Bintje' (susceptible) and 'Pampeana INTA' (moderately resistant) cultivars used as standards. In particular, clone Oka 5874.33 showed the best performance.

Table 1 Relative Area Under the Disease Progress Curves (AUDPC), in potato clones of *S. tarijense* and in potato cultivars. Seasons 2005/2006 and 2007/2008.

Cultivar or Clone	AUDPC 2005/6*	AUDPC 2007/8**
Bintje	189.83	1853.5
Pampeana INTA	94.25	1343.4
S. tarijense Oka 7494.3	38.38	1496.7
S. tarijense Oka 5874.33	36.25	1380.6

* Season with low disease incidence
** Season with high disease incidence

"*In vitro*" evaluation of resistance

Potato clones Oka 7494.3 and Oka 5874.33 were more resistant than 'Bintje' and 'Pampeana INTA', as observed in the field. **Fig. 1** shows a significant reduction of the infected area in the *S. tarijense* clones with regard to 'Bintje' and 'Pampeana INTA'. The infection area in the clones were between fifteen and twenty times less than 'Bintje' and four times less than 'Pampeana INTA'.

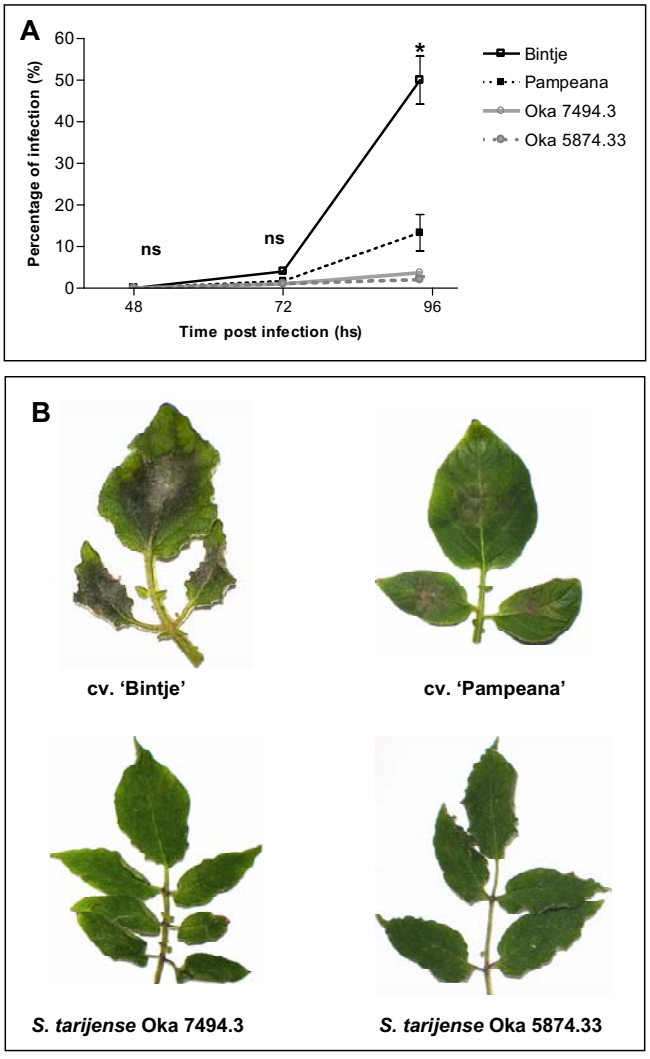

Fig. 1 "*In vitro*" evaluation of resistance. Detached leaves of plants after 30 days of emergence were inoculated with 5 µl of a suspension of 9.0 × 10^3 sporangia/ml of *P. infestans* under laboratory conditions. (**A**) Progress of the area of foliage injured after 24, 48 and 96 h post-infection. (*) Statistically different at 5% according to Tukey's test; (ns) not significant. (**B**) 96 h post-infection.

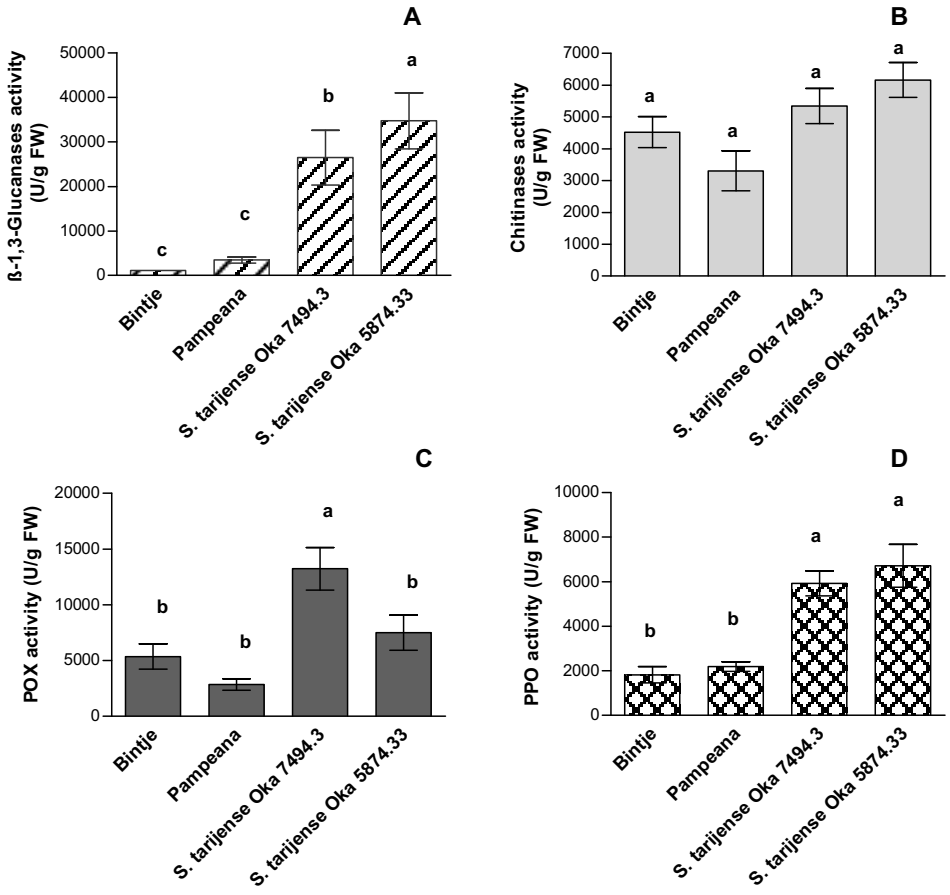

Fig. 2 Constitutive levels of PR-proteins activities in potato cultivars and *Solanum tarijense* clones. (**A**) β-1-3 Glucanases activity, (**B**) Chitinases activity, (**C**) Peroxidases activity, and (**D**) Polyphenoloxidases activity. Bars represent the mean values ± SD of three independent experiments, with five replicates per experiment. Columns with the same letter are not statistically different at 5% according to Tukey's test.

Constitutive levels of PR protein activities

S. tarijense clones showed higher constitutive levels of PR-proteins activity than 'Bintje' and 'Pampeana INTA', which were used as standards (**Fig. 2**). Significant differences were observed in glucanase, chitinases, POX and PPO activities among potato clones of *S. tarijense* and 'Bintje' and 'Pampeana INTA'. The enzymatic activities were 15, 1.5, 2, and 3 times higher, respectively for *S. tarijense* clones than for 'Bintje' and 'Pampeana INTA'.

A linear relationship was found between β-1,3-glucanase and PPO activity levels against AUDPC season 2007/2008 and *in vitro* resistance behaviour (**Table 2**).

Constitutive levels of PAL activity were not detected in any of the cultivars and clones examined in this study.

DISCUSSION

It has been reported that the expression of PR proteins, PPO, PAL, phytoalexins and phenols differs with the level of resistance of the different potato cultivars. For example, cultivars with high resistance level to *P. infestans* showed an early expression and larger accumulation of these compounds (Bi and Felton 1995; Dalisay and Kuc 1995; Tonón *et al*. 1998; Andreu *et al*. 2001; Conrath *et al*. 2001).

Table 2 Ordinate (a), slope (b) and coefficient of determination (R^2) of the linear regressions between β-1,3-glucanase, PPO, AUDPC and *in vitro* resistance.

	a	b	R^2
PPO vs AUDPC 2007/2008	1802.4	-0.0577	0.52
PPO vs *in vitro* resistance	50.4	-0.0074	0.56
β-1,3-glucanase vs AUDPC 2007/2008	1710.7	-0.0086	0.57
β-1,3-glucanase vs *in vitro* resistance	38.3	-0.0011	0.58

In addition, many authors have demonstrated that there is a strong correlation between the levels of horizontal resistance and the increase in the expression of genes that code for PR proteins, suggesting that their constitutive expression or their induction could contribute to the horizontal resistance against *P. infestans* in *Solanum* species (Vleeshouwers *et al* 2000; Tuzun 2001; Evers *et al*. 2003, 2004). Specifically, Vleeshouwers *et al*. (2000) reported a positive correlation between the constitutive levels of RNAm of: PR-1 (protein with antimicrobial activity), PR-2 (β-1,3-glucanases) and PR-5 (thaumatin like proteins) and the resistance of potato cultivars to *P. infestans*, suggesting that the RNAm could serve as molecular markers in the breeding programs. Recently, Ros *et al*. (2004) showed that the expression of PR-1, PR-2, PR-3 and PR-5 differed in susceptible and moderately resistant potato cultivars to *P. infestans*. On the other hand, the loci corresponding to genes related to PAL and other genes of the defense mechanism in plants were associated with the quantitative resistance to *P. infestans* (Trognitz *et al*. 2002). In other *Solanum* species, for example in tobacco, various resistant lines to *Peronospora tabacina*, obtained from wild species through breeding, showed the presence of high glucanase and chitinase levels before the attack of the pathogen, as likewise the early induction of the same enzymes after the attack (Robertson 1995; Tuzun *et al*. 1997). Also, resistant tomato clones to *Alternaria solani*, expressed high constitutive levels of β-1,3-glucanases and chitinases compared to the susceptible plants (Lawrence *et al*. 1996, 2000). Therefore, the constitutive expression of PR proteins in plants with high levels of natural resistance to the disease is of great importance, suggesting that these proteins would have a role as defense barriers (Edreva 2005). Also, it has been shown that chitinases, either alone or in combination with β-1,3-glucanases, can inhibit the *in vitro* growth of different fungi (Mauch *et al*.

1988). Tonon *et al*. (2002), suggest that β-1,3-glucanases may have a role in field resistance. Differential expression of β-1,3-glucanases was observed between 'Pampeana INTA' and 'Bintje'. In the moderately resistant cultivar ('Pampeana INTA'), β-1,3-glucanases induction (four-fold with respect to healthy tubers) occurred 14 hours after inoculation and remained over basal levels at 38 hours after inoculation with *P. infestans*. On the contrary, in susceptible 'Bintje', β-1,3-glucanases were induced at lower levels than those observed in 'Pampeana INTA'. Also, β-1,3-glucanases purified from 'Pampeana INTA' showed antifungal activity against *P. infestans*, by inhibiting the sporangia germination.

POX has been associated with resistance of potato to Late Blight (Umareus 1959). An increase of POX activity upon infection by *P. infestans* has been described and rates of accumulation may differ slightly between resistant and susceptible clones (Dowley *et al*. 1991). Evers *et al*. (2004) and Lozoya-Saldaña *et al*. (2007) detected an increase in POX activity in clones with horizontal resistance after infection by *P. infestans*. The increase was much more pronounced in the resistant clones as compared to the susceptible one. The relation of POX activity to resistance phenomena could be explained by its function as a generator of toxic free radicals and its involvement in the production of lignin and phenolic compounds (Reuveni 1998).

Recently, Li and Steffens (2002) have obtained direct evidence of such role for PPO in plants. These authors observed that transgenic plants of tomato overexpressing PPO had a higher oxidizing capacity and displayed increased resistance to *Pseudomonas syringae* pv. tomato.

Our results were in agreement with those obtained by the authors previously mentioned. The *S. tarijense* clones had higher resistance levels, than 'Bintje' (susceptible) and 'Pampeana INTA' (moderately resistant) cultivars (**Table 1, Fig. 1**), and showed higher constitutive levels of β-1,3-glucanases, POX and PPO activities than the reference cultivars (**Fig. 2**).

PAL regularly exhibits greater activity or the novo synthesis in the diseased tissues or in resistant genotypes and is a precursor of most of the phenolic compounds, including phytoalexins and lignin (Dixon and Harrison 1991). The synthesis of these substances is related with the level of genetic resistance of potato to the oomycete (Andreu *et al*. 2001). Our results showed that constitutive levels of PAL activity were not detected in none of the cultivars and clones tested. These results agree with those from Lozoya-Saldaña *et al*. (2007). However, these authors found that after four weeks of infection by *P. infestans*, their action increased suddenly in resistant cultivars and clones. The lowest activity was detected in the susceptible cultivar (Lozoya-Saldaña *et al*. 2007).

CONCLUSIONS

Solanum tarijense clones Oka 7494.3 and Oka 5874.33 showed high levels of resistance to Late Blight as likewise high levels of activity of constitutive proteins PR, such as, β-1,3-glucanases, POX and PPO.

These results and future studies on *S. tarijense* - *P. infestans* interaction, will allow us to introgress potato genes from *S. tarijense* into *S. tuberosum* in breeding programs as well as use some PR proteins as biochemical markers for the selection of new resistant clones.

The development of a system using biochemical markers will be a fast and economic technique for the selection of resistant clones. Finally, these results will contribute to the selection of promissory cultivars and their use in the breeding programs for integrated pest management.

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