Potato Research 44 (2001) 1 – 9

Production of phytoalexins, glycoalkaloids and phenolics in leaves and tubers of potato cultivars with different degrees of field resistance after infection with *Phytophthora infestans*

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Accepted for publication: 30 August 2000

Additional keywords: potato blight, pathogen defence, *Solanum tuberosum* L.

Summary

The kinetics of accumulation of phytoalexins, glycoalkaloids and phenolics was studied in two potato cultivars differing in their degrees of field resistance when infected with *Phytophthora infestans*. Tuber slices and leaves of cvs Pampeana INTA (high degree of field resistance, free of R genes) and Bintje (susceptible) were infected with race C (complex race 1, 3, 5, 7, 11) of *Phytophthora infestans*. Phytoalexins and phenolics accumulated in tuber and leaf tissues which had been inoculated. The levels of these compounds in the susceptible cv. Bintje were relatively low and similar to those found before inoculation. Leaves of cv. Pampeana INTA had a very high glycoalkaloid content, suggesting that glycoalkaloids may play a role in protection of leaves against the fungus. However, we could find no correlation between resistance and glycoalkaloid content of tubers. Our results suggest a major role of phytoalexins, phenolics and glycoalkaloids in the complex mechanisms of field resistance.

Introduction

Late blight (*Phytophthora infestans*) in Argentina is considered a very important problem. The ability of plant pathogens to evolve and produce many new and highly virulent races tempts us to conclude that stable control of diseases using resistance to some pathogens may not be feasible for some crops. Application of non-race specific resistance to control potato late blight is being advocated as the most effective method. This resistance, also called field resistance, is assumed to be multiple gene based and is effective against all races of the fungus (Umaerus, 1970). Also it is durable and would be commercially more attractive than race-specific resistance. It has been postulated that phytoalexins, phenolics and glycoalkaloids are involved in defence reactions against fungal infection (Friedman & McDonald, 1997, 1999; Kuc, 1995). In this paper we report on the kinetics of the accumulation of these molecules in two potato cultivars differing in their degrees of field resistance when infected with two races of *Phytophthora infestans*, in an attempt to determine whether these reactions contribute to this type of resistance.

Materials and methods

Biological material. P. infestans race C (1, 4, 7, 8, 10, 11) and race 0, mating type A2, were grown on V_8-agar medium and on potato tuber slices. Mycelia were harvested in sterile water and stimulated to release zoospores by incubation at 4 °C for 2–3 h. After filtration through muslin, each suspension was observed under light microscope for quantification of zoospores before use as inoculum.

Solanum tuberosum L. cv. Pampeana INTA (MP1 59.789/12 × Huinkul MAG) is a cultivar from the Argentine Breeding Programme (INTA-Balcarce).

Potato plants (*Solanum tuberosum* L. cvs Pampeana INTA and Bintje) were grown in pots containing a sterile mixture of soil:vermiculite (2:1 v/v) and maintained at 25 °C for 4 weeks with a 14 h photoperiod. Light was supplied by Osram L36W/20 cool white fluorescent tubes, which gave 120 µmol m^{-2} s^{-1} PAR measured 30 cm from the source. The plants were then transferred to 18 °C with the same photoperiod. Six week-old plants were used for inoculation with *P. infestans*; potato leaves were inoculated by spraying them with a suspension containing 2×10^4 sporangia ml^{-1} using a fine glass atomizer, while control leaves were sprayed with water. Plants were placed at 18 °C in a moist chamber. Leaves were harvested at different times post-inoculation.

Tubers were washed and sterilized by immersion in 5% (w/v) sodium hypochlorite for 20 min. Sterile disks of parenchyma (4–6 mm diameter, 10 mm thick) were prepared and inoculated with 1.25×10^3 spores or sterile water and incubated for different times at 18 °C in the dark.

Glycoalkaloid extraction from potato leaves. Accurately weighed portions (~1 g) of leaf tissue were stirred with 100 ml of 2% acetic acid for 2 h. The extract was concentrated to approximately 10–15 ml by evaporation on an air vacuum rotary evaporator. The pH was adjusted to 10–11 with concentrated ammonium hydroxide before the sample was placed into a water bath at 80 °C for 30 min. The solution was then refrigerated at 4 °C for at least 3 h prior to centrifugation. The precipitate was washed with 5 ml of 1% ammonium hydroxide and centrifuged, and the pellet was collected and air-dried. This was resuspended in 3 ml of a mixture of 50% ethanol and sulphuric acid (1:2, v/v). One ml of 1% formaldehyde was then added dropwise to the solution while the flask was swirled in an ice-water bath. The solution was allowed to stand at 23–25 °C for 90 min and the purple-red colour was measured at 562 nm using a Beckman spectrophotometer. A standard curve was established with recrystallized commercial solanine.

Phenol extraction from potato leaves and tubers. Tuber slices and leaves were homogenized with 80% aq. methanol (1 g frozen tissue per 10 ml solvent), allowed to stand with continuous stirring for 30 min and centrifuged. The supernatants were used for phenol quantification with the reagent of Folin-Ciocalteau (Bray & Thorpe, 1954).

Phytoalexin extraction and identification from potato leaves. Terpenoid phytoalexins from leaf tissue were extracted according to the method previously described

(Hammerschmidt & Kuc, 1979; Threlfall & Whitehead, 1992). Leaves (1 g) were cut and infiltrated under vacuum in 40% (v/v) aqueous ethanol for 10 min in a glass-stoppered conical flask fitted with a side arm to inflitrate solvent into leaf spaces. The flasks were shaked for 5 h and then the solvent was removed by filtration. The recovered solution was evaporated to dryness in a rotary evaporator at 40 °C (PF, phytoalexins fraction). For identification, the PF was dissolved in methanol and applied onto thin-layer chromatography plates of non-activated silica gel G 0.5 mm thick. The plates were developed twice in cyclohexane: ethyl acetate (1:1, v/v), according to Shih & Kuc (1973).

Determination of phytoalexins. Phytoalexins were determined according to the method described by Shih & Kuc (1973). The dried sample described above (PF) was dissolved in 1 ml of cyclohexane and 2 ml of concentrated sulphuric were added to the solution. The mixture was agitated and centrifuged at 1000 g for 3 min. The red colour of the lower sulphuric acid layer was measured at 500 nm, 20 min after the addition of the sulphuric acid. A standard curve was established using rhishitin or lubimin. Concentrated sulphuric acid and cyclohexane served as a blank.

Phytoalexin and glycoalkaloid extraction from potato tubers. Fresh tuber slices were blended in a mixture of chloroform, acetic acid and methanol (50:5:45, v/v) (ca 1:10 fresh w/v) using a Virtis homogenizer. The homogenate was allowed to stand overnight, filtered and evaporated to dryness. Equal volumes of chloroform and 0.2 M acetic acid were introduced into the container. The mixture was shaken and two layers were separated. The chloroform layer, which contained the phytoalexins, was evaporated to dryness and phytoalexins were quantificated as described above.

Glycoalkaloids were extracted and quantificated from the acetic acid layer as described for potato leaves.

Results

Accumulation of phytoalexins, glycoalkaloids and phenolic in tuber tissue. Results of accumulation of phytoalexins measured in tuber slices after inoculation with race C are shown in Fig. 1A. These showed that after five days of inoculation cv. Pampeana INTA tubers produced a higher accumulation of phytoalexins than cv. Bintje tubers.

Fig. 1C show that after inoculation with race C there was a higher level of phenolics in cv. Pampeana INTA than in cv. Bintje. Differences between the two cultivars become more pronounced three days after inoculation.

Glycoalkaloids were present at low levels in tubers of both cultivars, and no accumulation was observed after inoculation with race C (Fig. 1E).

When we tested the accumulation of each compound mentioned above in tuber tissue after inoculation with race 0 we found that the growth of the fungus was completely inhibited on the tuber slices and therefore, no induction was detected (results not shown).

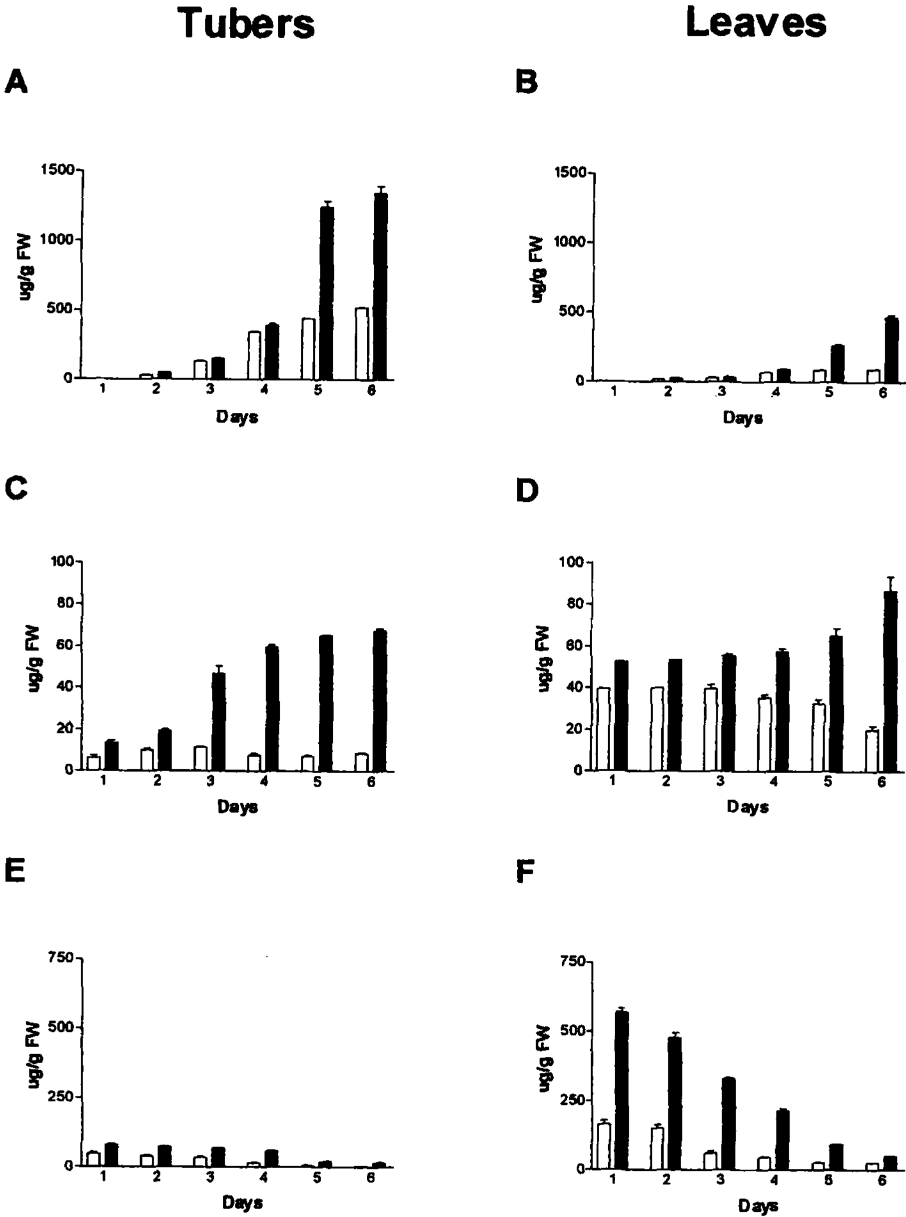

Fig. 1. Phytoalexin, phenolic and glycoalkaloid accumulation in potato tubers and leaves following infection with *P. infestans*. A and B, phytoalexins; C and D, phenolics; E and F, glycoalkaloids. Black bars, cv. Pampeana INTA; white bars, cv. Bintje.

Accumulation of phytoalexins, glycoalkaloids and phenolics in potato leaves. Five days after inoculation with race C, leaves of cv. Pampeana INTA produced a higher accumulation of phytoalexins than cv. Bintje (Fig. 1B). This result was similar to those obtained in tuber tissue. However, the accumulation was three times lower in leaves as compared with tuber slices (Figs 1A and 1B).

In infected leaves of both cultivars, four sesquiterpenoid phytoalexins could be identified. Phytuberin (Rf = 0.75), rishitin (Rf = 0.21), lubimin (Rf = 0.31) and solavetivone (Rf = 0.63) were identified by comparing their Rfs with the Rfs of authentic compounds (Table 1). In samples of cv. Pampeana INTA, high amounts of the phytoalexins phytuberin, rishitin and lubimin had accumulated. Solavetivone was always detected at lower concentrations. In the susceptible cultivar cv. Bintje, the main constituent identified was phytuberin; all three phytoalexins were found at lower concentrations (Table 1).

High levels of phenolics were observed in both cultivars up to four days post-inoculation with race C. However, five days after inoculation, phenolics in leaves of cv. Pampeana INTA accumulated at similar levels to those observed in tuber slices (Figs 1C and 1D). In cv. Bintje phenolics decreased to 20% of the levels found in the resistant cultivar six days post-inoculation.

Levels of glycoalkaloids in leaves of cv. Pampeana INTA were five times higher than in leaves of cv. Bintje and in tuber slices of both cultivars (Figs 1E and 1F). After inoculation, the glycoalkaloid content decreased significantly in leaves of cv. Pampeana INTA, and was undetectable six days post-inoculation.

The results obtained after inoculation with race 0 were qualitatively similar to those described above; however the response was always quantitatively lower (results not shown).

Table 1. Phytoalexin concentration in potato leaves infected with *Phytophthora infestans*.

	μg phytoalexin g^{-1} fresh wt			
Cultivar	Phytuberin	Rishitin	Lubimin	Solavetivone
Pampeana INTA	147.56 ± 15.8	64.68 ± 9.65	126.32 ± 19.73	61.48 ± 9.63
Bintje	32.54 ± 2.66	15.52 ± 2	16.14 ± 0.94	14.16 ± 2.46

Discussion

Decrease in the mycelial growth of race 0 on potato tubers from both cultivars was an unexpected result. How tuber tissue delays or inhibits the growth of race 0 of *P. infestans* is not apparent from the data presented. It is possible that race C may be more aggressive on potato tubers than race 0. The lower accumulation of phytoalexins, phenolics and glycoalkaloids in leaves infected with race 0 when compared with race C, could be also due to a lower infection ability of race 0. In a

future study both races will be compared using two components of fitness that affect aggressiveness and therefore pathogenicity, infection frequency and sporulation on potato tubers and leaves (Day & Shattock, 1997). These data would explain results obtained with race 0 on tuber and leaf tissues.

Potato tubers of cv. Pampeana INTA infected with race C showed a strong increase in phytoalexin and phenolic production when compared with tubers from cv. Bintje. However, glycoalcaloid levels remained at low level in both cultivars (Figs 1A, 1C and 1E). In leaves of cv. Bintje no significant differences were observed in accumulation of phytoalexins, phenolics and glycoalkaloids before and after inoculation. By contrast, in cv. Pampeana INTA, a differential accumulation of phytoalexins and phenolics was observed (Figs 1B and 1D) and a high level of glycoalcaloids was also detected (Fig. 1F).

Phytoalexins and phenolics seem to be important features in the resistance of cv. Pampeana INTA. The experiments reported in this paper show that these materials accumulated in tuber and leaf tissues which had been inoculated with race C. The levels of these compounds in the susceptible cultivar (cv. Bintje) are relatively low and similar to those found before inoculation.

Since 1970 (Metlitsky et al., 1970), this is the first report of substantial amounts of phytoalexins accumulating in leaf tissue of potato plants and phytuberin, lubimin, rishitin and solavetivone were detected in potato leaves inoculated with *P. infestans*. Phytoalexins in tubers from various potato cultivars with or without defined R genes to *P. infestans* have been extensively studied (Hildenbrand et al., 1989; Hildenbrand & Ninnemann, 1994; Price et al., 1976; Tomiyama et al., 1968). However, there are relatively few reports on the biochemical responses of potato leaves, although leaves are the primary infection site in the field (Govers et al., 1997; Thurston & Schultz, 1981). To our knowledge, the only reported observation of *P. infestans* inducing sesquiterpenoid phytoalexins in potato leaves (Metlitsky et al., 1970) has not been confirmed by independent experiments. However, rishitin was detected in cell suspension cultures originating from potato leaves (Rohwer et al., 1987; Threlfall & Whitehead, 1988) and antifungal activity was detected in diffusate of sporangial suspensions of an incompatible race placed on leaves (Metlitsky et al., 1970). The accumulation of sesquiterpenoid phytoalexins in non-tuber tissue of potato plants was also demonstrated; they were detected in appreciable amounts in stems, roots and stolons infected with *Erwinia carotovora* ssp. *atroseptica* (Abenthun et al., 1995).

Leaves of cv. Pampeana INTA (resistant) have a very high glycoalkaloid content and this suggests that glycoalkaloids could play a major role in protection of leaves against the fungus. However, we could find no correlation between resistance and glycoalcaloid content of tubers. If glycoalkaloids do contribute to host-plant resistance, it is possible that: 1, since growing potato tubers are not exposed to many of the organisms that harm leaves, their glycoalkaloid content is much lower (potato leaves are the primary site of infection by *P. infestans*) (Govers et al., 1997), or 2, resistance in foliage and in tubers are independent (Bonde et al., 1940; Gallegly, 1968; Inglis et al., 1996; Kadish et al., 1990; Roer & Toxopeus, 1961; Turkensteen, 1993); this means that a different degree of field resistance could be found in leaves and tubers of the same cultivar.

Our results show that the resistant reaction, where the damage was contained (cv. Pampeana INTA), resulted in a decrease in glycoalkaloid and an increase in phytoalexin levels in leaves. There have been several studies showing that *P. infestans* reduces glycoalkaloid production and induces production of the terpenoid phytoalexins (Holland & Taylor, 1979; Horikawa et al., 1976; Ishizaka & Tomiyama, 1972; Kuc, 1984; Kumar et al., 1991; Shih & Kuc, 1973). It seems probable that in cv. Pampeana INTA, infected potato leaves produce secondary metabolites such as the phytoalexins that might potentiate the biological action of glycoalkaloids. It is also possible that phytoalexins replace glycoalkaloids as resistance factors at late stages of the infection process. However, we can not exclude that enzymes of *P. infestans* race C can detoxify potato glycoalkaloids.

Our results suggest a major role of phytoalexins, phenolics and glycoalkaloids in the complex mechanisms of field resistance. As the molecular and genetic basis underlying field resistance is mostly unknown (Pieterse et al., 1992; Turkensteen, 1993) knowledge of plant responses, including the relative contribution of various synthesized compounds to the resistance reaction and their timing, is necessary to understand the whole resistance mechanism for further application in breeding programmes.

Acknowledgements

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), from Comisión de Investigaciones Científicas (CIC) and Universidad Nacional de Mar del Plata. G.D. is an established researcher of CIC.

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