

# Effect of Photoperiod and Temperature on Resistance against *Phytophthora infestans* in Susceptible and Resistant Potato Cultivars: Effect on Deposition of Structural Phenolics on the Cell Wall and Resistance to Penetration

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## ABSTRACT

Deposition of phenolic compounds before and after inoculation with *Phytophthora infestans* was evaluated in two Mexican cultivars (Malinche and Tollocan) with major unknown R genes for resistance to potato late blight, two cultivars (393295.236 = CIP1 and 391046.22 = CIP2) without R genes from the International Potato Center (CIP) and the susceptible cultivar Atlantic. Before inoculation, plants were grown in growth chambers at two temperatures (16 or 24 C) and two photoperiods (PPD 12 or 16 h day length). Forty-eight hours after inoculation, the number of penetrations was recorded and depositions of phenolic compounds were classified according to detection and location in (a) the anticlinal cell wall, (b) the whole cell, (c) the stomatal cells, and (d) without detectable depositions of phenolic compounds. The concentration of phenolic compounds in the epidermal cells was slightly increased at 16 C and 16 h PPD and penetration frequency was lower at 16 C (12 h PPD). Concentration of phenolic compounds was not correlated with penetration frequency, but was correlated with the resistance level of the different potato cultivars. Atlantic had the highest number of penetrations followed by Tollocan, CIP1, CIP2, and Malinche. The cytological observations indicated that four types of deposition of phenolic compounds occurred in all five potato cultivars irrespective of their type and level of resistance. These results suggest that deposition of phe-

nolic compounds on epidermal cells is a general resistance mechanism in potato leaves that does not have a specific relation with resistance to the penetration of *P. infestans*. Phenolic depositions were intrinsically similar in potato cultivars with and without R genes, which stresses the difficulty in differentiating between horizontal and vertical resistance.

## RESUMEN

La deposición de compuestos fenólicos antes y después de la inoculación con *Phytophthora infestans* fue evaluada en dos cultivares mexicanos (Malinche y Tollocan) con genes mayores R desconocidos para resistencia al tizón tardío de la papa, dos cultivares (393295.236 = CIP1 y 391046.22 = CIP2) del Centro Internacional de la Papa (CIP), sin genes R y el cultivar Atlantic. Antes de la inoculación, las plantas estuvieron en cámaras de crecimiento a dos temperaturas (16 o 24 C) y dos foto períodos (FP 12 o 16 horas de longitud de día). Cuarenta y ocho horas después de la inoculación, se registró el número de penetraciones y se clasificó la deposición de compuestos fenólicos de acuerdo con la detección y localización en (a) pared celular anticlinal, (b) toda la célula, (c) células estomatales y (d) sin deposiciones detectables. La concentración de los compuestos fenólicos en las células epidérmicas se incrementó ligeramente a 16 C y 16 horas de FP y la frecuencia de penetración fue más baja a 16 C y 12 horas de FP. La concentración de compuestos fenólicos no estuvo correlacionada con la frecuencia de penetración, pero si con el nivel de resistencia de los diferentes cultivares de

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**papa. Atlantic tuvo en mayor número de penetraciones seguido por Tollocan, CIP1, CIP2 y Malinche. Las observaciones citológicas indicaron que se realizan cuatro tipos de deposición de compuestos fenólicos en los cinco cultivares, independientemente de su tipo y nivel de resistencia. Estos resultados sugieren que la deposición sobre las células epidérmicas es un mecanismo general de resistencia en las hojas de papa que no tiene relación específica con la resistencia a la penetración de *P. infestans*. Las deposiciones fenólicas fueron intrínsecamente similares en cultivares con y sin genes R, lo cual acentúa la dificultad de diferenciar entre resistencia vertical y horizontal.**

## INTRODUCTION

Potato late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, has long been recognized as a globally important disease. The efforts of many potato-breeding institutions have been focused on breeding cultivars with durable resistance and consequently there is great interest in elucidating the resistance mechanisms that operate in potato cultivars with and without major R genes. Successful breeding requires an understanding of changes that occur in the plants' defenses under different environmental conditions. Constitutive and induced deposition of phenolic compounds on the cell walls is thought to be a resistance mechanism against invasion by pathogens in many plant species (Ride 1978; Hammerschmidt and Nicholson 1999; Métraux and Raskin 1993). However, no information is available on the effect of temperature and photoperiod on the content and deposition of phenolic compounds in infected leaves of potato cultivars with horizontal and vertical resistance.

According to Ride (1978), lignin and its phenolic precursors may contribute to plant defenses by increasing cell wall resistance to mechanical penetration, decreasing the susceptibility to cell wall degrading enzymes, restricting the entrance of enzymes and toxins produced by the pathogen, preventing nutrient flow toward the pathogen, and as toxic products active against the pathogen. Métraux and Raskin (1993) suggested that certain phenolic compounds may be important internal signals to induce resistance pathways in the plant. These latter authors also made a distinction between the antimicrobial role of preformed phenolic products, like chlorogenic acid found in potato tissues, and those induced

after the infection, known as phytoalexins. Beckman (2000) gathered evidence to support the role of phenolic-storing cells in programmed cell death as a mechanism of resistance.

There is a wealth of evidence in the literature indicating that phenolic compounds play a role in resistance against *P. infestans* in potato leaves (Röthlisberger et al. 1984; Cuyppers and Hahlbrock 1988; Freytag et al. 1994), in potato tubers (Friend 1981), and against non-pathogenic fungi (Hammerschmidt 1984). However, there is not enough evidence to show the contribution of the structural phenolic compounds on resistance to penetration of *P. infestans* in potato leaves. It is important to distinguish the role of insoluble structural phenolics, like lignin, which strengthen the cell wall, and soluble phenolic compounds with antimicrobial, signaling, and toxic effects located mainly in the vacuoles. In this regard, based on cytological observations, Vleeshouwers et al. (2000) did not find any correlation between the deposition of phenolic globules on leaf cell walls and resistance levels of different *Solanum* species.

The phenolic content in leaves may be affected by environmental factors such as temperature and photoperiod. Recent studies on cabbage (Dan and Imada 2002) and on sweet potato (Islam et al. 2003) have shown that high temperatures decrease the concentration of phenolics in the leaves. Long photoperiods, such as 16 h, may increase the accumulation of phenolic compounds in tobacco leaves (Tso et al. 1970). Thus, assuming that the phenolic content of the leaves may be changed by environmental factors, the aim of this work was to manipulate temperature and photoperiod to induce changes in the phenolic content of the leaves of potato cultivars with and without R genes and to establish the relationship of those phenolic changes with resistance to the penetration of *P. infestans* into the leaves.

## MATERIALS AND METHODS

### *Potato Cultivars*

Two potato cultivars (Malinche and Tollocan) with unknown major R genes, two cultivars (391046.22 = CIP1 and 393295.236 = CIP2) without R genes and one susceptible cultivar (Atlantic; R1) were selected for this study. Malinche and Tollocan are Mexican cultivars bred by INIFAP (National Institute for Forestry, Agriculture and Livestock Research). These cultivars possess high and moderate resistance against late blight, respectively. Their resistance is based on a combination

of unknown major and minor genes derived from *Solanum demissum*. The cultivars CIP1 and CIP2 were bred in the CIP (International Potato Center) and form part of a group of cultivars identified by CIP as population B3 whose sources of horizontal resistance are genetic materials without R genes derived from *Solanum demissum* (Landeo 1997). Parents of both cultivars did not show the hypersensitivity reaction when they were inoculated with race 0 of *P. infestans*, indicating absence of R genes (J.A. Landeo, pers comm). The cultivars CIP1 and CIP2 have been tested in Toluca, Mexico, where they have shown medium and high resistance against late blight, respectively (C. Díaz and O. Rubio, unpublished data). Atlantic is a popular cultivar which is susceptible to late blight.

### **Plant Growth Conditions**

Details of the growth conditions for these experiments are reported in Rubio et al. (2005). Briefly, plantlets were grown for 3 weeks in sterile plastic magentas, then transferred into pots of peat moss and placed in climatic chambers. Ten plants (one per pot) of each cultivar were grown for 32 days in growth chambers under the four combinations of two constant temperatures (16 or 24 C) and two photoperiods (PPD, 12 or 16 h day length). Thirty-two days after planting, the fourth and fifth fully developed compound leaves (counted from the top) from each plant were detached. One leaf was used to evaluate the susceptibility to *P. infestans* and the other for determination of phenolic compounds.

### **Phytophthora infestans Isolate and Inoculation**

Details of the isolate of *P. infestans* and inoculation techniques for these experiments are reported in Rubio et al. (2005). Briefly, *P. infestans* isolate CO-42 (mating type A2; mefenoxam resistant; mt haplotype 1A; GPI 100/100; PEP 100/100) collected in Mexico and kindly provided by K. Deahl, USDA, was used in this study. The isolate was compatible with all five potato cultivars used in this study (Rubio et al. 2005). The virulence of the isolate was preserved by continuous propagation on potato leaves of the cultivar CIP2. Fresh sporangia collected on one or two leaves (8 days after inoculation) were harvested in a glass beaker with 50 mL of distilled water and incubated at 4 C. After 2 h, the concentration of zoospores was adjusted to  $3 \times 10^4$  mL<sup>-1</sup> for inoculation.

The primary and first pair of leaflets of the fourth leaf from each plant were detached, placed in a Petri dish with

water-saturated filter paper and a piece of plastic screen to avoid direct contact of the leaflets with the moistened paper and inoculated by pipetting a 10- $\mu$ L droplet on the abaxial side of each leaflet. The average diameter of the droplet was 4.1 mm, covering a leaf area of 12.7 mm<sup>2</sup>. The Petri dishes with the inoculated leaflets were incubated at 18 C in a Percival dew chamber with a photoperiod of 12 h light (RH > 95%).

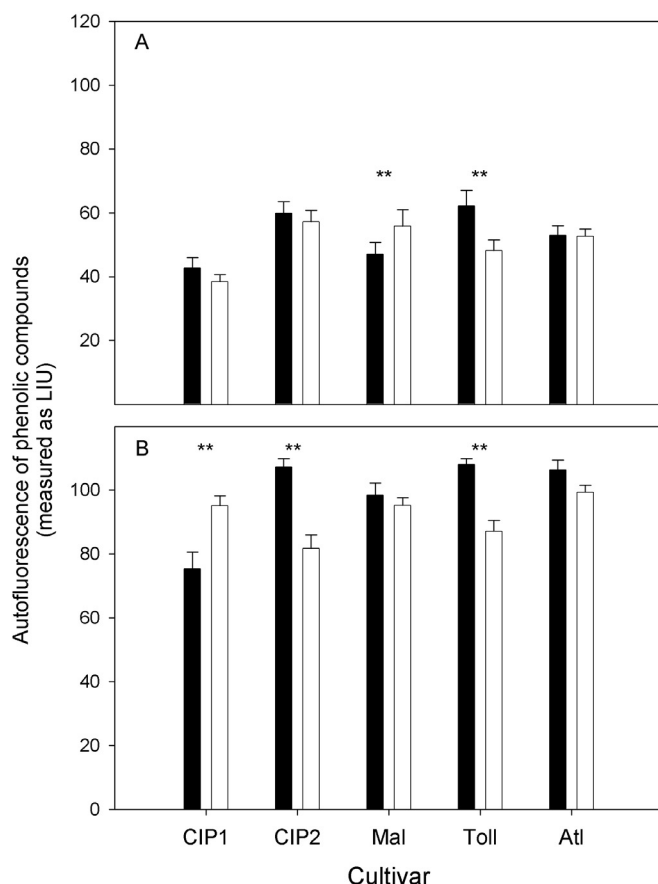
### **Staining of Leaves and Microscopic Examination**

Forty-eight hours after inoculation, a 6-mm-diameter disc was removed with a cork borer from the inoculated tissue of one of the three leaflets incubated in each Petri dish. The leaf discs were fixed in an ethanol:acetic acid solution (3:1). The fixing solution was changed two to three times during the first week until the chlorophyll and soluble phenols were completely removed and the leaf discs were discolored. The leaf discs were then re-hydrated in a series of solutions with progressively decreasing concentrations of the fixing solution and stained for 2 min in a solution containing 0.1% of toluidine blue in 0.1 M phosphate buffer at pH 6.5 (O'Brien et al. 1964), rinsed with distilled water twice, and mounted in glycerol (50%) for microscopic examination. The number of penetrations of *P. infestans* into the epidermal cells of the leaves were counted in the entire area of the inoculated leaf section (12.7 mm<sup>2</sup>) and the different reactions of the epidermal cells classified according to the phenolic compounds deposition (indicated by the blue-green color) in (a) the anticlinal cell wall, (b) the whole cell, (c) the stomata guard cells, and (d) cells without detectable depositions of phenolic compounds. In some penetration sites the phenolic depositions covered only part of the anticlinal cell wall near to the penetration site and were included in the category of phenolics on the anticlinal cell wall. The reaction classified as "phenolic compounds in the whole cell" included all the penetrated cells that showed depositions of phenolic compounds on more than 75% of the cell.

### **Analysis of Phenolic Compounds**

In our study the phenolic content was determined by quantifying the fluorescence intensity using image analysis. This technique is based on the fluorescence imaging system (FIS) described previously (Buschmann and Lichtenthaler 1997; Lichtenthaler and Mieh 1997). The primary and first pair of leaflets of the fifth leaf from each plant were detached and

a 6-mm-diameter disc was removed with a cork borer for the analysis of phenolic compounds. The leaf discs were cleared, as described above, then each disc was examined microscopically under UV light at 400x magnification. Three color pictures from different locations in each disc were taken, and the autofluorescence of phenolic compounds determined as the average light intensity by using image analysis software (Sigma Scan v.5; Jandel Scientific Software). This software has been previously used to quantify the average reflective intensity in late-blight-diseased potato tubers (Niemira et al. 1999). The autofluorescence was expressed in light intensity units on a scale where 0 corresponded to black and 255 to pure white.



**FIGURE 1.** The effect of photoperiod [(A) 12 and (B) 16 h] at two temperatures (16 and 24 C, ■ and □ bars, respectively) on mean concentration of phenolic compounds (measured as light intensity units [LIU] of autofluorescence under ultraviolet light; 0 = black, 255 = white) on leaf epidermal cells of five potato cultivars after inoculation with *P. infestans*. CIP1, CIP2, Mal = Malinche, Toll = Tollocan, and Atl = Atlantic). Data are means of two trials  $\pm$  SE (n = 20 leaf sections). The \*\* indicates a significant difference between temperature treatments at  $P = 0.05$ .

### Experimental Set-up

The 10 combinations of five cultivars (CIP1, CIP2, Malinche, Tollocan, Atlantic) and two levels of temperature (16 and 24 C) constituted the treatments of a factorial experiment; cultivar  $\times$  temperature. Two growth chambers were used for the experiments and each one had a capacity for 50 pots each containing one plant. There were 10 plants of each potato cultivar per chamber. Each combination of cultivar and temperature was repeated twice at 12 and 16 h PPD, representing two blocks (trials) per each photoperiod.

The effect of photoperiod could be included in the statistical analysis as a main factor to form a factorial design cultivar  $\times$  temperature  $\times$  photoperiod; however, there could be some differences in the experimental conditions among experiments that could not allow a valid statistical comparison of the effect of photoperiod. To avoid this risk, the variables were statistically analyzed as a factorial design cultivar  $\times$  temperature (Proc GLM — SAS/Stat, SAS Institute, Cary, NC, U.S.A.). This data analysis did not allow the estimation of the interactions of photoperiod with temperature and cultivars; however, by comparing the two photoperiods as two populations (n = 200) with a T-test, it was possible to define the single effect of photoperiod. In each experiment, Pearson's correlation coefficients between mean phenolic compounds and the mean number of penetrations were calculated.

## RESULTS

### Concentration of Phenolic Compounds (PC) in the Leaves before Inoculation

Temperature changes affected the PC concentration differently in the five potato cultivars. The main effect of temperature on PC was significant at 16 h PPD, but not at 12 h PPD. On average for all cultivars, the PC concentration at 16 C (16 h PPD) was 8.1% higher than at 24 C (16 h PPD). The effect of cultivar and the interaction temperature  $\times$  cultivar was significant at both photoperiods (Table 1).

In growth chambers set at 12 h PPD, Malinche had higher mean PC concentration at 24 C than at 16 C and Tollocan had the opposite temperature effect (Figure 1). At 16 h PPD, CIP2 and Tollocan had greater mean PC at 16 C, but CIP2 had greater mean PC at 24 C. Tollocan was the most responsive cultivar to temperature because the difference in mean phenolic compounds at the different temperatures was greater than the differences in all the other cultivars at 12 h

TABLE 1—Summary of ANOVA analysis. Effect of trials, temperature (Temp), and cultivar (CV) on the concentration of phenolic compounds (PC) in the leaves, total number of penetrations by *P. infestans* into the epidermal cells, and proportion (%) of penetration sites with different types of PC depositions. Each trial was a combination of two temperatures and five cultivars and was repeated twice at (A) 12 and (B) 16 h photoperiod in different experiments. The numbers are residual mean squares.

| Source              | d.f. | Conc. of PC | Total penetrations | PC on anticlinal cell wall | PC on whole cell | PC on stomatal cells | Without PC |
|---------------------|------|-------------|--------------------|----------------------------|------------------|----------------------|------------|
| A) 12 h photoperiod |      |             |                    |                            |                  |                      |            |
| Trials              | 1    | 31034**     | 24429**            | 477**                      | 93**             | 1092**               | 2670**     |
| Temp                | 1    | 301 n.s.    | 25541**            | 303 n.s.                   | 103**            | 2069**               | 4456**     |
| CV                  | 4    | 1830**      | 8570**             | 3664**                     | 175**            | 195 n.s.             | 2188**     |
| Temp*CV             | 4    | 663**       | 3850**             | 1391**                     | 67**             | 324**                | 2540**     |
| Error               | 188  | 96          | 486                | 286                        | 24               | 132                  | 190        |
| B) 16 h photoperiod |      |             |                    |                            |                  |                      |            |
| Trials              | 1    | 10938**     | 23932**            | 9963**                     | 1.6 n.s.         | 9175**               | 7.7 n.s.   |
| Temp                | 1    | 2709**      | 135095**           | 19363**                    | 4747**           | 5200**               | 3.5 n.s.   |
| CV                  | 4    | 1662**      | 7725**             | 12376**                    | 1142**           | 88 n.s.              | 247**      |
| Temp*CV             | 4    | 3171**      | 6900**             | 907**                      | 1157**           | 242**                | 46.5n.s.   |
| Error               | 188  | 168         | 1891               | 281                        | 187              | 87                   | 105        |

n.s. = not significantly different at  $P = 0.05$

\*\* = significantly different at  $P < 0.05$

PPD, and at 16 h PPD Tollocan and CIP2 were the most responsive cultivars (Figure 1). On average of the two temperatures, CIP1 had the lowest concentrations of phenolic compounds at both photoperiods; CIP2 and Tollocan had higher PC concentration than the rest of cultivars at PPD 12; and Atlantic was in the group of higher concentration of phenolic compounds at 16 h PPD (Table 3).

All cultivars had higher concentrations of phenolic compounds at 16 h than at 12 h PPD (Figure 1). On average, the PC concentration at 16 h PPD was 85% higher than at 12 h PPD (Table 2).

### Penetration of the Epidermal Cells

The penetrations into the epidermal cells generally occurred on or near the anticlinal cell walls. It was very common to find cells with more than one penetration site. The main effect of temperature and the interaction temperature  $\times$  cultivar in the factorial design were significant at 12 h PPD as well as at 16 h PPD (Table 1). The numbers of penetrations in all cultivars generally were higher at 24 C than at 16 C (Figure 2). Differences between CIP1, CIP2, Tollocan, and Atlantic were significant at 12 h PPD, and differences between CIP1, CIP2, Malinche, and Tollocan were significant at 16 h PPD (Figure 2). On average the number of penetrations increased

115% at the higher temperature at 12 h PPD and 138% at 16 h PPD across all cultivars.

Generally, the leaves of the more susceptible cultivars experienced a higher number of penetrations than the resistant ones. Over the two temperatures at 12 h PPD, Atlantic had the highest number of penetrations followed by Tollocan, CIP1, CIP2, and Malinche, and at 16 h PPD the order was Atlantic, CIP2, CIP1, Tollocan, and Malinche (Table 3).

There was a marked effect of photoperiod on the number of penetrations of *P. infestans* on the epidermal cells of the leaves. On average, the number of penetrations at 16 h PPD was twice that at 12 h PPD (Table 2).

### Correlation between Phenolic Compounds and Penetrations

The correlations between the mean concentration of phenolic compounds (Figure 1) and the mean total number of penetrations (Figure 2) were not significant at either photoperiod treatment.

### Deposition of Phenolic Compounds after Inoculation

The ANOVA analysis (Table 1) shows that with the exceptions of PC depositions on the anticlinal cell wall at 12 h PPD

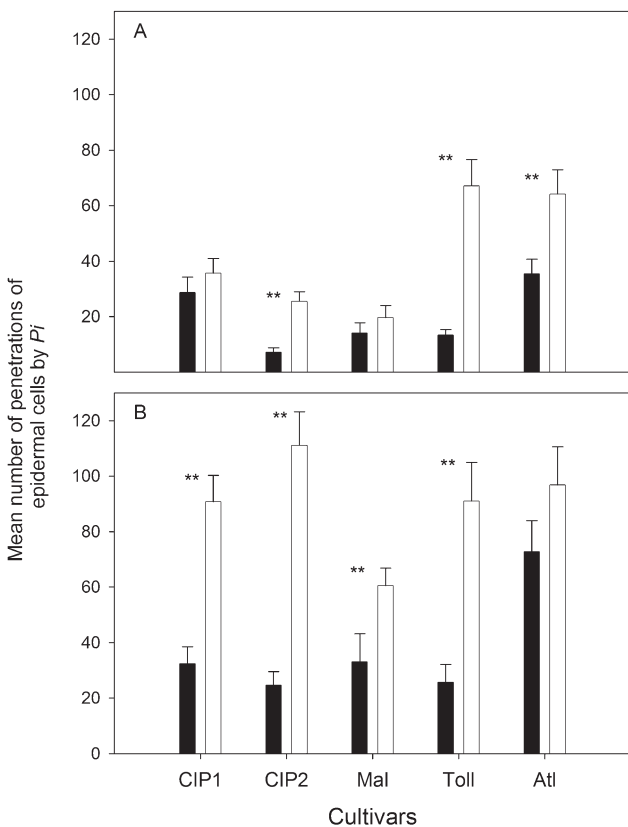
and PC depositions on the whole cell at 16 h PPD, the main effect of temperature was significant in the rest of the different kinds of phenolic depositions. Cultivars also differed in their phenolic deposition reactions and the interaction temperature  $\times$  cultivar was significant in most of the cases except in the proportion of penetration sites that did not shown PC depositions at 16 h PPD.

All potato cultivars had each of the four types of phenolic depositions at the two temperatures and at both photoperiods (Figures 3 and 4). Depositions of phenolic compounds in the stomatal cell guard complex occurred in one or two of the cells of the complex, indicating which cells were penetrated, and it was very common to observe that the auxiliary cells showed thickened cell walls but no deposition of phenolic compounds. The reactions classified as non-deposition of phe-

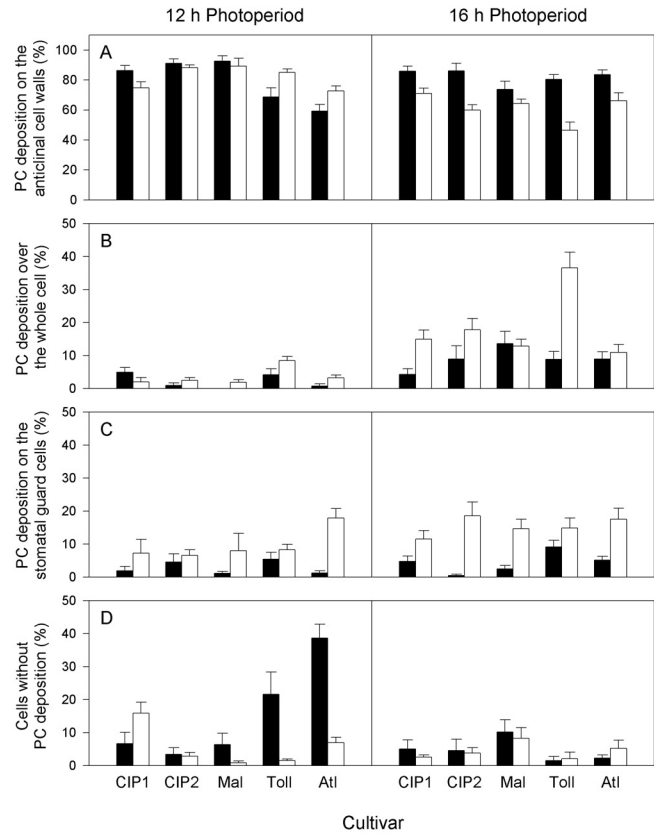
nolic compounds generally were characterized by thickening of the cell walls of the cells surrounding the penetration site.

Most of the penetrated cells in the five cultivars showed accumulation of phenolic compounds on the anticlinal cell wall (Figure 3). Under 12 h PPD, a change in temperature from 16 C to 24 C increased the proportion of PC depositions on the anticlinal cell walls of the cultivars Tollocan and Atlantic; however, in CIP1 the opposite effect was observed. Under 16 h PPD, all five cultivars showed a tendency to decrease their proportion of PC depositions on the anticlinal wall with increasing temperature.

PC depositions in the whole cell, PC depositions in the stomatal guard cells and penetration sites without PC depositions constituted less than 30% of the total reactions (Figure 3).



**FIGURE 2.** The effect of photoperiod [(A) 12 and (B) 16 h] at two temperatures (16 and 24 C, ■ and □ bars, respectively) on mean number of penetrations by *P. infestans* (*Pi*) in the epidermal cells of the inoculated leaf area (12.7 mm<sup>2</sup>). CIP1, CIP2, Mal = Malinche, Toll = Tollocan, and Atl = Atlantic). Data are means of two trials  $\pm$  SE (n = 20 leaf sections). The \*\* indicates a significant difference between temperature treatments at  $P = 0.05$ .



**FIGURE 3.** The effect of photoperiod (12 and 16 h) at two temperatures (16 and 24 C, ■ and □ bars, respectively) on the mean percentage of penetration sites with depositions of phenolic compounds on (A) anticlinal cell walls, (B) whole cell, (C) stomata guard cells, and (D) without detectable phenolic compounds on the leaf epidermal cells of five potato cultivars after inoculation with *P. infestans*. CIP1, CIP2, Mal = Malinche, Toll = Tollocan, and Atl = Atlantic. Data are means of two trials  $\pm$  SE (n = 20 leaf sections).

In the cultivars CIP1, CIP2, and Tollocan, the proportion of depositions on the whole cell markedly increased under 16 h PPD at the highest temperature (Figure 3). Generally, the proportion of depositions of phenolic compounds on the cell walls of the stomatal guard cells increased with temperature at 16 h PPD, and the penetration sites that did not show depositions of phenolic compounds increased with low temperature only in Tollocan and Atlantic at 12 h PPD (Figure 3).

There was no clear relationship between the kind of deposition of phenolic compounds and the different resistance levels of the five potato cultivars (Figure 3 and Table 3). Atlantic, the most susceptible cultivar, had the lowest proportion of PC depositions on the anticlinal cell wall at 12 h PPD; however, at 16 h PPD, this cultivar had the same proportion as CIP1 and CIP2. Tollocan, a moderately resistant cultivar, had the highest proportion of deposition of phenolic compounds on the whole cell at both photoperiods. The cultivars with the greatest proportion of reactions without PC depositions were Atlantic and Malinche at 12 h and 16 h PPD, respectively.

## DISCUSSION

In the present work, it was observed that the content of phenolic compounds in the leaves of different potato cultivars may be affected differently by changes in temperature. The potato cultivars exposed to the lower temperature (16 C, 16 h PPD) showed a slight increase in phenolic compounds content in the leaves. This finding is in agreement with earlier studies on cabbage (Dan and Imada 2002) and on sweet potato (Islam et al. 2003). Thus, it appears that the effect of temperature on production of phenolic compounds may be widespread in plants.

Our results indicate that potato plants grown at 16 C had

fewer penetrations in the epidermal cells regardless of cultivar. In an extension of this study, a negative effect of the higher temperature (24 C) on the total leaf area infected by *P. infestans* in four potato cultivars was noted (Rubio et al. 2005). Our results are in agreement with the results of Jenns and Leonard (1985), who observed that different maize lines diminished in resistance to *Bipolaris maydis* with increasing temperature, and also with the findings of Harder et al. (1979), who demonstrated that increasing temperatures inhibited the expression

TABLE 2—Effect of two photoperiods (PPD) after inoculation with *P. infestans* on the mean concentration of phenolic compounds (PC) in the leaves, total number of penetrations into the epidermal cells, and proportion (%) of penetration sites with different kind of PC depositions.

| PPD  | Concentration of PC <sup>a</sup> | Total number of penetrations | % of penetrations with PC depositions on: |            |                |            |
|------|----------------------------------|------------------------------|---|------------|----------------|------------|
|      |                                  |                              | anticlinal cell wall                      | whole cell | stomatal cells | without PC |
| 12 h | 51.4 a <sup>b</sup>              | 31.1 a                       | 80.6 a                                    | 3.0 a      | 6.38 a         | 10.4 a     |
| 16 h | 95.4 b                           | 64.1 b                       | 71.5 b                                    | 13.9 b     | 10.25 b        | 4.4 b      |

<sup>a</sup>Concentration of phenolic compounds was measured as light intensity units (LIU) of autofluorescence under ultraviolet light; LIU 0 = black, 255 = white

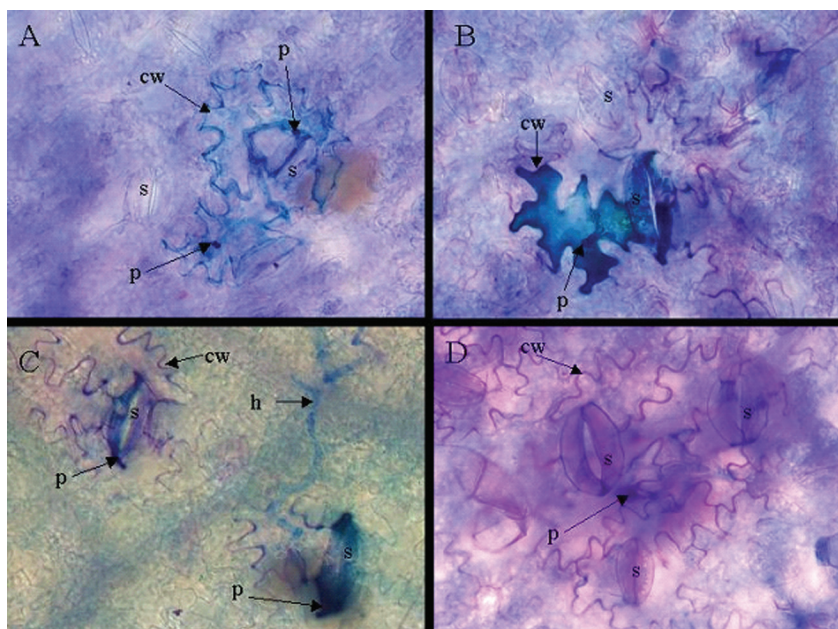
<sup>b</sup>The comparisons of the means (n = 200) are by a T-test and values followed by the same letter are not statistically different at  $P < 0.05$

TABLE 3—The effect of photoperiod ([A] 12 and [B] 16 h) after inoculation with *P. infestans* on the mean concentration of phenolic compounds (PC) in the leaves, total number of penetrations by *P. infestans* into the epidermal cells, and proportion (%) of penetration sites with different kind of PC depositions in five cultivars grown under two photoperiods. Values are the means of two temperatures (16 and 24 C) over two trials.

| Cultivar            | Concentration of PC <sup>a</sup> | Total number of penetrations | % of penetrations with PC depositions on: |            |                |            |
|---------------------|----------------------------------|------------------------------|---|------------|----------------|------------|
|                     |                                  |                              | anticlinal cell wall                      | whole cell | stomatal cells | without PC |
| A) 12 h photoperiod |                                  |                              |   |            |                |            |
| CIP1                | 40.6 a <sup>b</sup>              | 32.4 b                       | 80.7 b                                    | 3.5 b      | 4.3 b          | 11.4 b     |
| CIP2                | 58.6 c                           | 16.6 c                       | 89.8 a                                    | 1.7 bc     | 5.4 ab         | 3.0 c      |
| Malinche            | 51.4 b                           | 17.2 c                       | 91.1 a                                    | 1.0 c      | 4.4 b          | 3.5 c      |
| Tollocan            | 55.1 bc                          | 40.2 b                       | 76.8 b                                    | 6.4 a      | 6.8 ab         | 11.7 b     |
| Atlantic            | 52.8 b                           | 49.8 a                       | 65.9 c                                    | 2.0 bc     | 9.6 a          | 22.9 a     |
| B) 16 h photoperiod |                                  |                              |   |            |                |            |
| CIP1                | 85.2 a                           | 61.6 bc                      | 78.3 a                                    | 9.7 b      | 8.1 b          | 3.8 b      |
| CIP2                | 94.5 b                           | 67.8 ab                      | 72.9 ab                                   | 13.3 b     | 9.5 ab         | 4.2 b      |
| Malinche            | 96.8 b                           | 46.8 c                       | 68.5 bc                                   | 13.3 b     | 8.7 ab         | 9.5 a      |
| Tollocan            | 97.6 bc                          | 58.6 bc                      | 63.3 c                                    | 22.7 a     | 12.0 a         | 1.9 b      |
| Atlantic            | 102.8 c                          | 84.8 a                       | 74.9 ab                                   | 10.0 b     | 11.3 ab        | 3.8 b      |

<sup>a</sup>Measured as light intensity units of autofluorescence under ultraviolet light; 0 = black, 255 = white

<sup>b</sup>The comparisons of the means (n = 40) are by a T-test and values followed by the same letter are not statistically different at  $P < 0.05$



**FIGURE 4.** Different sites of deposition of phenolic compounds after inoculation with *P. infestans*; (A) on the anticlinal cell wall, (B) on the whole cell, (C) on the stomatal cell guard, and (D) without PC depositions. Captions; cw = cell wall, h = hyphae, s = stomatal guard cell, p = penetration site. Cells are from the cultivar Atlantic.

of resistance genes against *Puccinia graminis* f. sp. *tritici* in wheat. Thus, it seems that resistance breaks down with increasing temperature.

The correlation between phenolic compounds and penetration frequency was not statistically significant at either photoperiod, 12 or 16 h. Atlantic, the most susceptible cultivar to *P. infestans*, had a high concentration of phenolic compounds in the epidermal cells; however, it was also the cultivar with the higher number of penetrations. This suggests that the concentration of structural phenolic compounds on the cell wall is not a factor that plays an important role in resistance to penetration of the epidermal cells by *P. infestans*. In previous work, Vleeshouwers et al. (2000) demonstrated that *P. infestans* is able to penetrate indiscriminately in leaves of susceptible and resistant potato cultivars as well as in non-host *Solanum* species. Thus, it seems that *P. infestans* has a great ability to penetrate the cells of leaves and that structural phenolic compounds on the cell walls are not an efficient mechanism to avoid the intrusion of the pathogen.

The reduction of penetrations at 16 C and 12 h photoperiod was observed in most of the tested cultivars, including

Atlantic. This suggests that the effect of temperature is independent of the resistance levels of the different potato cultivars used in this study. This also suggests that a structural or chemical modification occurred in the leaves of all the cultivars that caused a higher penetration rate. Wynn and Staples (1982) gathered information to conclude that chemotropism (chemical) and thigmotropism (contact) are the most common tropisms to orient the penetration structures of fungi. In this study and in previous works (Wilson and Coffey 1980; Gees and Hohl 1988; Vleeshouwers et al. 2000) it was observed that most of the penetrations occurred in epidermal cells adjacent to stomatal guard cells and preferentially close to the depressions formed by the junction of anticlinal cell walls. This suggests that thigmotropism based on the topography of the leaf surface may orient the penetration structures of *P. infestans*.

The marked increase in the concentration of phenolic compounds on epidermal leaf cells exposed to the longer photoperiod in all five potato cultivars may corroborate similar results observed in tobacco leaves by Tso et al. (1970). Greater accumulation of phenolic compounds in potato may be expected under a longer photoperiod because the production of phenylalanine and tyrosine, precursors of phenolic compounds in tobacco plants, is enhanced by 16 h PPD. The works of Dan and Imada (2002) on cabbage and Islam et al. (2003) on sweet potato, with different shade percentages, also reflect the dependence of phenolic compounds production on photoperiod.

According to previous field tests in the Toluca Valley in Mexico, Malinche and CIP2 had high resistance to *P. infestans*, CIP1 and Tollocan had medium resistance, and Atlantic was susceptible (C. Díaz and O. Rubio, unpublished data). The same results were found in the study by Rubio et al. (2005). Here we found that the number of penetrations was associated with the resistance levels of the five cultivars. Our results were similar to those of Wilson and Coffey (1980), who compared the potato cultivars Pimpernel, Majestic, and Shamrock and concluded that the very low frequency of penetrations in Pimpernel was associated with its high resistance level. However,

Vleeshouwers et al. (2000) did not find a correlation between the penetration frequency of *P. infestans* in different *Solanum* species and their resistance to *P. infestans*. A similar conclusion was reported by Gees and Hohl (1988), who compared several potato cultivars with general and specific resistance genes. These different results suggest that resistance to penetration of epidermal cells may be a resistance component that is determined by the pool of genes of each cultivar. As the source of resistance to *P. infestans* in CIP1, CIP2, Malinche, and Tollocan is *S. demissum*, it is possible that these cultivars may have a common resistance mechanism to penetration expressed at different levels.

In addition to the quantification of the penetrations of *P. infestans* in the epidermal cells, we made observations of the different reactions that occurred in each penetration site. The occurrence of the four types of deposition of phenolic compounds irrespective of the level and type of resistance of the five potato cultivars is not a surprise. In previous work, Vleeshouwers et al. (2000) observed accumulation of phenolic compounds on the cell wall of a range of *Solanum* species, including resistant and susceptible commercial cultivars with and without R genes as well as non-host and wild *Solanum* species. Vleeshouwers et al. (2000) also observed that the hypersensitive reaction (HR) is a common resistance mechanism that occurred within 22 h after inoculation in fully resistant *Solanum* species and between 16 and 46 h in partially resistant cultivars. In the present study, observations were made in infected tissue fixed 48 h after inoculation, and it is assumed that all the cultivars, including the susceptible Atlantic, had enough time to react by depositing phenolic compounds in the cell wall and in the whole cell, which is an indication of an HR. It is important to point out that there was no correlation between the resistance of the different cultivars and the proportion of penetration sites that were hypersensitive with accumulations of phenolic compounds on the whole cell. Probably, timing of the induction of the HR is a more important factor in resistance than the HR itself (Vleeshouwers et al. 2000).

Based on auto fluorescence, cell wall browning, and presence of haustoria, Freytag et al. (1994) defined five different types of reactions in potato leaves infected with compatible and incompatible races of *P. infestans* and found the HR in 30% to 56% of the total infection sites irrespective of the type of interaction, compatible or incompatible. In this study, all the reactions indicated compatibility between the host and the

pathogen and the percentage of penetrations with depositions of phenolic compounds on the entire cell, associated with the HR, varied from 1.7% to 4.3% in plants that were grown with a 12 h PPD and 8.3% to 19.4% under a 16 h PPD. The substantial increase of the proportion of reactions with depositions of phenolic compounds on the whole cell with temperature of 24 C and photoperiod of 16 h cannot be fully explained. A partial explanation is that the higher accumulation of phenolic compounds under long photoperiod may be associated with higher availability of phenolic compounds and consequently higher accumulation of them in the penetration sites.

From the genetic point of view, the presence of the four types of phenolic depositions in each cultivar is a particularly remarkable observation because it suggests a similar response among the cultivars with horizontal resistance (CIP1 and CIP2), those with a combination of horizontal and vertical resistance (Malinche and Tollocan), and the susceptible cultivar (Atlantic). This finding implies that the deposition of structural phenolics on the cell wall should be considered as a nonspecific mechanism of resistance present in potato cultivars. This finding is in agreement with previous cytological studies that have demonstrated that the HR is a defense reaction associated with all forms of resistance (Wilson and Coffey 1980; Gees and Hohl 1988; Vleeshouwers et al. 2000).

In conclusion, in this study it was found that the concentrations of phenolic compounds on the epidermal cells of potato leaves are influenced by temperature and photoperiod and that resistance to penetration of *P. infestans* into the epidermal cells was correlated with the resistance level of the cultivars, but not with their phenolic content. It was also observed that all the cultivars had similar depositions of phenolic compounds after inoculation and that the different types of reaction were independent of the type and level of resistance.

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