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Histological changes induced during the biotrophic phase of infection of three potato varieties by *Phytophthora infestans* (Mont.) de Bary

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Abstract

In this study defense responses in three potato varieties with different levels of reaction to the late blight disease caused by *Phytophthora infestans* were analyzed after inoculation with the pathogen. In the resistant cv. Pastusa Suprema, increased intensity of H_2O_2 and callose deposit accumulation was observed beginning at 24 hours after inoculation, followed by a hypersensitive response at the inoculation points. In the moderately resistant cv. Diacol-Monserrate, the same responses were observed as in the resistant variety, but with less intensity over time. For the susceptible cv. Diacol-Capiro, the responses observed occurred later than in the other two varieties, subsequent to the advance of the pathogen over extensive necrotic areas. These results suggest that early, intense peroxide and callose accumulation and a hypersensitive response are associated with the observed resistance of the cv. Pastusa Suprema and cv. Diacol-Monserrate to *P. infestans.*

Keywords: callose, hydrogen peroxide, hypersensitive response (HR), late blight disease, reactive oxygen species (ROS)

Introduction

As a foodstuff, potato (*Solanum tuberosum* L.) is surpassed only by rice and wheat at a global scale. Potato is widely cultivated around the world, is rich in carbohydrates, low in fat and rich in vitamins, primarily vitamin C (Camire *et al.* 2009). In Colombia, potato covered the third largest planted area during the past decade, with an annual average of 161,802.2 ha, and it had the second highest economic value, with production worth US$500 million/year (https://www.agronet.gov.co/estadistica/Paginas/home.aspx?cod=1, March 9th of 2019). Worldwide, the most limiting disease of this crop is late blight, caused by the oomycete microorganism *Phytophthora infestans* (Mont.) de Bary, which is also a limiting factor for other cultivated species of the botanical family Solanaceae, such as lulo, table and tree tomato, among others (Cardenas *et al.* 2011).

Widely cultivated varieties in Colombia including Diacol-Capiro, Parda Pastusa, and Criolla are highly susceptible to this pathogen. This disease is controlled almost exclusively by frequent spraying with chemical products, which leads to high production costs, as well as contamination of air, soil and water. They also impact wild flora and fauna and create risks to animal and human health (Andreu *et al.* 2006).

Plants have developed complex defense mechanisms to combat potential pathogens, which determine the resistance/susceptibility phenotypes. The first line of defense is known as molecular pattern-triggered immunity (PTI). It confers general resistance to a wide range of potential pathogens (McCann *et al.* 2012). The second line of defense is effector-triggered immunity (ETI) (Boller and Felix 2009; Bolton 2009;

Ge *et al.* 2013), which is activated when an avirulence protein is recognized by its cognate resistance (*R*) gene inducing a hypersensitive response, a form of programed cell death (Boyd *et al.* 2013; Michelmore *et al.* 2013). Processes of intracellular signaling and gene transcription are activated as a result of the induction of these forms of immunity. These are responsible for the protein synthesis associated with plant defense responses (Marone *et al.* 2013). Biochemical responses include cytoskeleton reorganization, relocation of peroxisomes, reinforcement of cell walls, callose deposits, synthesis of reactive oxygen species (ROS) and reactive nitrogen species (RNS), phytoalexins, and hormones for systemic signaling such as salicylic acid, jasmonic acid and ethylene, etc. (Buchanan *et al.* 2000).

One of the responses that is induced most quickly when a pathogen attempts to penetrate is the accumulation of ROS such as hydrogen peroxide (H_2O_2) (Jindřichová *et al.* 2011). This has a direct antimicrobial effect and mediates other defense responses such as callose deposits and programed cell death, or the hypersensitive response (HR). Accumulation of hydrogen peroxide stops the infection, thus preventing development of the disease (Zhang *et al.* 2001; Ge *et al.* 2013). Hypersensitive response develops through various cell changes, which are preceded by an oxidative explosion mediated by ROS and NRS (Vleeshouwers *et al.* 2000). The β(1-3) glucan polymer callose deposits (Luna *et al.* 2010) help reinforce the cell wall near the site of attempted invasion by the potential pathogen, thus blocking its penetration into the plant. Additionally, they can inhibit the secretion of effector proteins and the uptake of nutrients by the pathogen (Korgan *et al.* 2011; Ge *et al.* 2013).

In some pathosystems, it has been observed that resistant plants activate their defense responses earlier and with greater intensity than susceptible plants (Shimony and Friend 1975; Vleeshouwers *et al.* 2000; Torres *et al.* 2012; Ge *et al.* 2013; Rodríguez *et al.* 2016). It is generally accepted that the most efficient way of controlling plant diseases is through genetic resistance (De Vleesschauwer *et al.* 2012). In order to generate cultivars with stable and lasting resistance, it is essential to know the different genetic, biochemical and molecular mechanisms that determine the resistance/susceptibility spectrum in different genotypes (Marone *et al.* 2013). *Phytophthora infestans* exhibits a hemibiotrophic life cycle with an initial biotrophic phase followed by necrosis of plant tissues and sporulation. An understanding of the plant-pathogen communication that occurs in the initial stages of the interaction, primarily during the biotrophic phase, could contribute to an understanding of the mechanism by which some potato genotypes can stop *P. infestans* from attacking (Doke 2005).

In Colombia, there are native and cultivated genotypes of potatoes with different levels of susceptibility/resistance to the late blight disease (Thurston *et al.* 1962). These include cv. Pastusa Suprema (released in 2002), which has been reported as highly resistant to late blight; cv. Diacol-Monserrate (released in 1956), which has horizontal or polygenic resistance showing slow and restricted penetration, a limited cell tissue invasion rate and a decrease in the sporulation of the pathogen in the plant; and cv. Diacol-Capiro (released in 1961), which is highly susceptible (Ramos 2000; Segura *et al.* 2006; Ñústez 2011). Despite the long trajectory of Colombian plant breeding programs (Guzmán *et al.* 1960; Thurston *et al.* 1962; Guzmán 1964), very little is known about the resistance mechanisms of Colombian potato genotypes to late blight at histological, biochemical and molecular levels.

The objective of this study was to compare the expression of hydrogen peroxide, HR and callose in three potato varieties with contrasting resistance to late blight during the initial phases of interaction with *P. infestans*.

Materials and Methods

Pathogen isolation and inoculum preparation

Phytophthora infestans was isolated from a diseased leaf sample obtained from a commercial crop of cv. Diacol-Capiro of the *Solanum tuberosum* potato, located in Santa Elena in Medellín, Colombia, South America following the method reported by Gómez *et al.* 2019. A sample was put in a paper bag and immediately sent to the laboratory for further processing. Leaf tissue was rinsed thoroughly with tap water and carefully dried in a paper towel. Washed leaves were incubated in a humid chamber (Controlled growing cabinet Sanyo®), in darkness and at 16–18°C until profuse sporulation was observed. Then, sporangia were collected on a 10 μm filter with sterile distilled water. Sporangia of *P. infestans* were verified under light microscopy and the concentration was adjusted to 2×10^4 sporangia · ml⁻¹ with sterile distilled water. Two droplets of sporangia suspension of 20 μl each were placed and kept in potato slices and in healthy leaves of the susceptible cv. Careta (without *R* genes) incubated at 16°C with relative humidity >90%.

Depending on the sporulation intensity, the isolate was transferred to a rye-agar semi-selective media culture or sporangia were collected again and the process was repeated until enough sporangia were obtained to be transferred to semi-selective media. For long term storage, sporangia from isolates were frozen in liquid nitrogen. To obtain the pathogen inocula solution, the

inoculated potato slices were washed with distilled water and incubated for 7 days under conditions already described. Afterwards, the suspension of sporangia and mycelia was filtered through sterile gauze and sporangia were retained in a micro filter (10 µM). Sporangia of *P. infestans* were verified under light microscopy. Hematocytometry was used to adjust the resulting suspension to a concentration of 5×10^4 sporangia · ml^{-1} (Avrova *et al*. 2008).

Plant material

The following potato cultivars were selected for the experiments: Pastusa Suprema ([*S. stoloniferum × S. phureja* CCC 81 (Yema de huevo)] × Parda Pastusa, UNC), released in 2002 and classified as resistant to late blight; Diacol-Monserrate (Branca cascuda (CCC 334 ssp. *tuberosum*) × Pana blanca (ssp. *andigena*), ICA), released in 1956 and classified as having field resistance to late blight (Estrada *et al*. 1959); and Diacol-Capiro (CCC 751 (ssp. *tuberosum*) × Tuquerreña (CCC 61 ssp. *andigena*), ICA), released in 1961 and currently classified as highly susceptible to late blight (Segura *et al*. 2006; Ñùstez 2011).

Tubers of cv. Pastusa Suprema have a dun color, are round-flat in shape, with medium eye depth and creamy flesh. They have high androsterility with light-green foliage, uniform, first quality tubers and grow well between 2,500 and 3,200 meters above sea level (masl). Their potential yield is more than 45 t · ha^{-1}. Tubers exhibit 2 months dormancy at 15°C and 75% relative humidity. They have high culinary quality and frying aptitude in a short postharvest time period. They are susceptible to *Potato yellow vein virus* (PYVV) and to *Spongospora subterranea*, but show high resistance to *P. infestans* (Ñùstez 2011).

Cultivar Diacol Monserrate has creamy tubers, are round or oval in shape, uniform in size, with superficial eye depth and creamy flesh. Its potential yield is more than 52 t · ha^{-1} with uniform high quality tubers. Tubers exhibit between 3 to 5 months of dormancy at 14°C. There is high acceptance of its culinary quality. Several genes are involved in its tolerancet to *P. infestans* and is susceptible to viruses and insects (Estrada *et al*. 1959).

Cultivar Diacol Capiro has red tubers, with a round, slightly flattened shape, superficial eye depth and creamy flesh. Its foliage is dark green, and it has an adaptability from 1,800 to 3,200 masl with potential yield above 40 t · ha^{-1}. Tubers exhibit 3 months of dormancy at 15°C and 75% relative humidity. It is the main cultivar for industrial processing in Colombia because of its good frying aptitude and as a fresh product it is highly acceptable for its culinary properties. It is highly susceptible to *P. infestans*, *S. subterranea* and PYVV (Ñùstez 2011).

Certified seeds of these three potato cultivars (FEDEPAPA-Colombia) were planted in 15 cm diameter plastic pots with commercial substrate composed of organic matter, rice hulls and soil free of pathogens (DELPINO©-Medellín, Colombia). Potato plants were kept under greenhouse conditions at 2,600 masl, at an average temperature of 12°C, with 12 h of light on average. Irrigation was applied in order to maintain the substrate at field capacity and a relative moisture higher than 90%. Plants were fertilized with 10 g of NPK (15–15–15) at the time of planting.

Inoculation

Nine weeks after planting, 10 fully expanded and healthy leaflets of each variety were taken from the middle third of the plants. These were placed in trays on paper moistened with sterile distilled water. Between eight and ten drops of the sporangia suspension at a concentration of 5×10^4 sporangia · ml^{-1} were deposited on the abaxial side of each leaflet, on either side of the midrib. Following this inoculation, the plant material was incubated at 18°C, with relative humidity >90%. As a negative control, the same number of plant segments was inoculated with sterile distilled water, using the method described above. For cytological analyses, three inoculated leaflets and controls from each variety were collected at 12, 24, and 48 hours after inoculation (hai), corresponding to the early biotrophic phase of the life cycle.

Hydrogen peroxide accumulation

The presence of hydrogen peroxide (H_2O_2) was detected by staining with 3,3' diaminobenzidine (DAB) (Alfa Aesar™), using a modified version of the technique reported by Hao *et al*. (2011) and Yakimova *et al*. (2009). The leaflets were infiltrated under vacuum (100 bars) with the DAB solution for 2 min at a concentration of 1 mg · ml^{-1}. They were then preserved at 4°C for 2 h. Afterwards, they were bleached in a 3 : 1 ethanol-acetic acid solution for 24 h; the bleaching solution was changed two times. Finally, the leaflets were placed in object holders with 50% glycerol in order to be observed under a light microscope (Nikon Eclipse N*i*™ Microscope).

Trypan blue staining

Hypersensitive response was detected using a modified version of the protocol described by Weigel and Glazebrook (2002). Leaflets were bleached in a 3 : 1.1 ethanol (96%) – acetic acid (99%) solution. The solution was changed every 12 h, with two changes. The bleached leaflets were stained with lactophenol-trypan blue [2 ml of 96% ethanol, 2.5 mg of trypan

blue (Merck™), 1.0 ml of lactic acid, 1.0 g of phenol (Merck™)], infiltrated under vacuum (100 bars) for 2 min and incubated at 4°C for 2 h. Finally, the leaflets were taken out of the solution and mounted on object holders with 50% glycerol in order to be observed under a light microscope (Nikon Eclipse N*i*™ Microscope).

Aniline blue staining

The inoculated leaflets were bleached in a 3 : 1 ethanol-acetic acid solution for 24 h; the solution was changed two times during this period. Callose staining was performed using a modified version of the method described by Hao *et al.* (2011). Once the leaflets were bleached, they were placed in a solution of 100 mg of aniline blue in 20 ml of phosphate buffer (pH 7.0); they were gently infiltrated under vacuum for 2 min and preserved in this solution for 2 h at 4°C. Afterwards, the leaflets were placed in object holders with 50% glycerol in order to be observed under a fluorescence microscope (Olympus™ Microscope).

Data analysis

For each variety and time, the percentage of cells with the presence of hydrogen peroxide, callose and HR was calculated relative to a total number of 300 cells counted in an observation field using a Nikon Eclipse N*i*™ microscope. A total of 30 fields were quantified for each time and each variety evaluated. These were distributed randomly to either side of the leaflet's midrib. All experiments were repeated three times. In order to identify statistical differences, analysis of variance with separation of means were performed using the LSD method ($p < 0.05$), implemented in the software STATGRAPHICS Centurion XVI version 16.1.15 (32-bits).

Results

Foliar symptoms

The first visible foliar stains were observed as dark brown necrotic lesions, beginning at 48 hai for each of the varieties. At this time 72% of the necrotic area was restricted to the site of inoculation in the cv. Pastusa Suprema, whereas 20% of mild necrotic area with a clear restriction of pathogen colonization was observed in cv. Diacol-Monserrate. In marked contrast, at the same time cv. Diacol-Capiro presented a 12% necrotic area in the form of lesions that expanded outside the inoculation sites. The percentage of necrotic areas on the inoculated leaves can be observed in Figure 1.

A necrotic area was observed outside the inoculation site in the susceptible cv. Diacol-Capiro, while a necrotic area was observed in the tolerant cv. Diacol-

Monserrate, with pathogen colonization of the tissue restricted to the inoculation site. In the resistant cv. Pastusa Suprema, necrotic areas were seen as small points, with necrotic lesions restricted to the inoculation site. Overall, the disease known as late blight appeared in the cv. Diacol-Capiro. In the cv. Pastusa Suprema, a typical hypersensitive response was observed and in the cv. Diacol-Monserrate the lesion progressed very slowly compared to cv. Diacol-Capiro (Fig. 1).

Hydrogen peroxide accumulation

At 12 hai, no changes were observable with the naked eye or light microscope observation in the leaflets stained with DAB. However, at 24 hai coffee-brown spots could be seen with the naked eye at inoculation sites on leaflets of the cv. Pastusa Suprema. These were consistent with the spots associated with the expression of ROS. In the cv. Diacol-Monserrate, light spots of similar coloring were seen at the sites of inoculation. In contrast, small, barely visible spots in the cv. Diacol-Capiro, were observed (Fig. 2). At this time, the percentage of cells with peroxide accumulation was 56% in the cv. Pastusa Suprema, 19% in the cv. Diacol-Monserrrate, and 7% in the cv. Diacol-Capiro (Fig. 2).

At 48 hai, intense macroscopic spots localized at the inoculation sites were observed in the cv. Pastusa Suprema. This corresponded to an average of 66% of cells with peroxide accumulation. Microscopic observation confirmed that the localization of peroxides was restricted to the inoculation site, which stopped pathogen advance in this variety. In the cv. Diacol-Monserrate, a lower incidence of peroxide accumulation was seen at this time which was restricted to the inoculation site, represented by 30% of cells. In cv. Diacol-Capiro, peroxides were detected in large areas beyond the inoculation site, with a pattern of delayed appearance relative to colonization of plant cells by pathogen hyphae. At this time, lower intensity of peroxide accumulation was observed in 47% of cells in this variety, while the pathogen advanced through the leaflet tissue until sporulation (Fig. 3).

Hydrogen peroxide accumulation, observed in cells in the cv. Pastusa Suprema, was restricted to the inoculation sites. In contrast, in cv. Diacol-Capiro, hydrogen peroxide accumulation and necrosis were observed following the colonization and sporulation of the pathogen.

Hypersensitive response (HR)

Trypan blue accumulation was used as an indicator of a hypersensitive response (Bhadauria *et al.* 2010). At 12 hai, no cells were observed with the characteristic blue coloring in any of the three varieties evaluated, indicating that HR did not occur at this time.
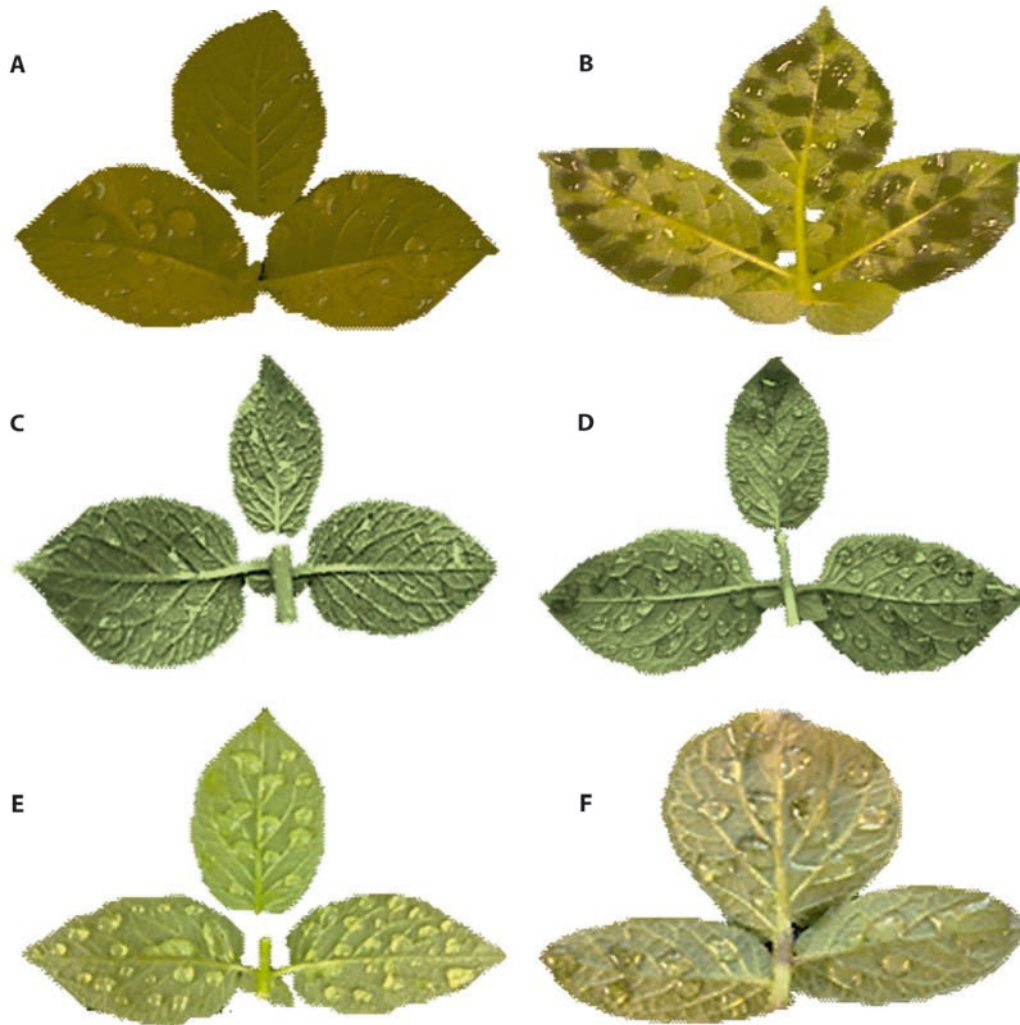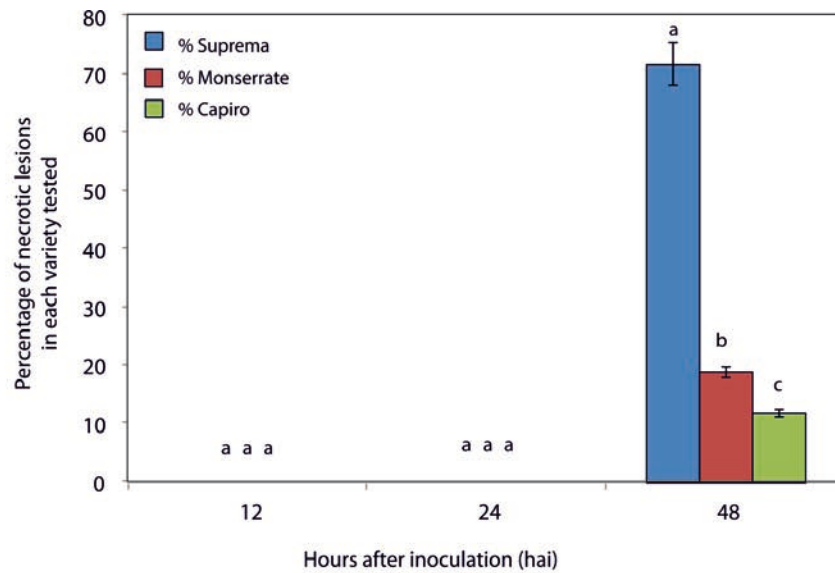
Fig. 1. Percentage of necrotic lesions observed in leaflets of cv. Pastusa Suprema, Diacol-Monserrate y Diacol-Capiro 48 hours after inoculation with *Phytophthora infestans*. Average percentage of necrotic lesions were calculated using data obtained from observations of 10 microscopic fields per each of three replicates in each of three independent repetitions. Different letters represent significant differences identified by the LSD statistical test ($p < 0.05$). Error bars represent the standard deviation. A – cv. Pastusa Suprema water mock-inoculated control; B – cv. Pastusa Suprema inoculated with *P. infestans*; C – cv. Diacol-Monserrate water mock-inoculated; D – cv. Diacol-Monserrate inoculated with *P. infestans;* E – cv. Diacol-Capiro water mock-inoculated; F – cv. Diacol-Capiro inoculated with *P. infestans*. Photographs are representative images from three independent repetitions
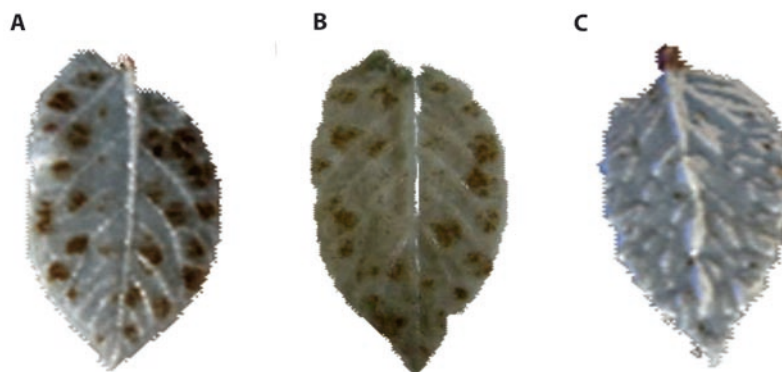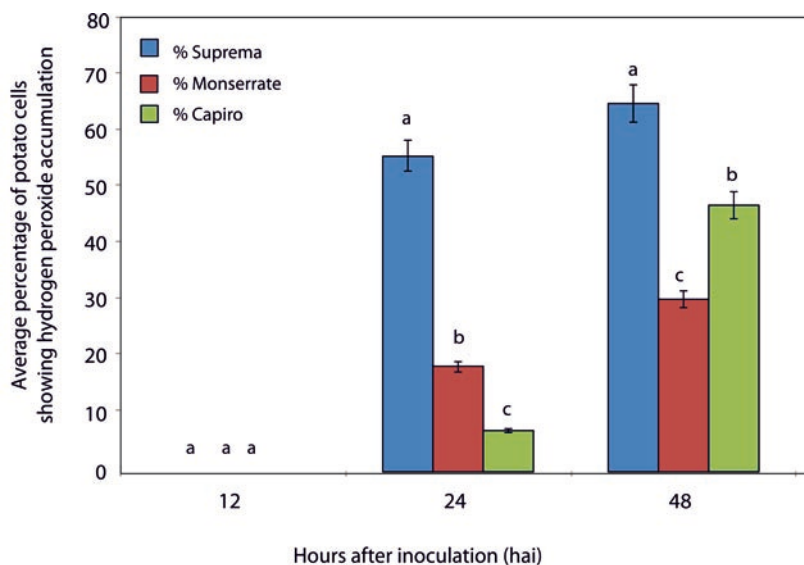
Fig. 2. Hydrogen peroxide accumulation on potato leaflets of the cultivars tested 24 hours after inoculation with *Phytophthora infestans*. Average percentage of potato cells showing hydrogen peroxide accumulation in the three cultivars tested. A – cv. Pastusa Suprema; B – cv. Diacol-Monserrate; C – cv. Diacol-Capiro. Photographs are representative images from three independent repetitions. Leaflets were stained with 3,3′ diaminobenzidine (DAB). Each average percentage was calculated using data obtained from observations of 10 microscopic fields in each replicate of the three independent repetitions. Different letters represent significant differences for the same time identified by the LSD statistical test ($p < 0.05$). Error bars represent the standard deviation. Cells were stained with 3,3′-diaminobenzidine (DAB)

At 24 hai, characteristic HR was observed in 7 and 6% of the stomatal cells in the cv. Pastusa Suprema and in the cv. Diacol-Monserrate, respectively (Fig. 4). Meanwhile, in the cv. Diacol-Capiro, no coloration indicative of HR was observed at this same time.

At 48 hai, 63% of cells in the cv. Pastusa Suprema were found to have dye accumulation indicative of HR; these were localized at the *P. infestans* inoculation sites. Meanwhile, 24% accumulation of HR indicator dye was observed in the cv. Diacol-Monserrate, and 7% of cells were observed to have HR in the cv. Diacol-Capiro at this same time (Fig. 4). At 48 hai, profuse sporulation was observed in the leaflets of the susceptible cv. Diacol-Capiro, indicating the termination of one cycle in the pathogen's asexual phase and the compatibility reaction of the pathogen with this variety.

In the cytological analysis performed at 48 hai, the cells that accumulated trypan blue dye in the

cv. Pastusa Suprema presented granular cytoplasm and condensed nuclei. These are changes characteristic of HR that correspond to the induction of programed cell death (Fig. 5). In the Diacol-Monserrate and Diacol-Capiro varieties, no significant changes in the cytological composition were observed at this time.

Callose

At 12 hai, fluorescence was not observed in any of the three varieties evaluated, suggesting that callose was not present at this time. At 24 hai, fluorescence consistent with callose deposits was identified in 33% of all the fields evaluated in the cv. Pastusa Suprema (Figs. 6 and 7). At this time, sites with sporangia germination were observed near the areas with fluorescence, indicating a rapid local response (Figs. 7A and B). In the cv. Diacol-Monserrate, the callose deposits were
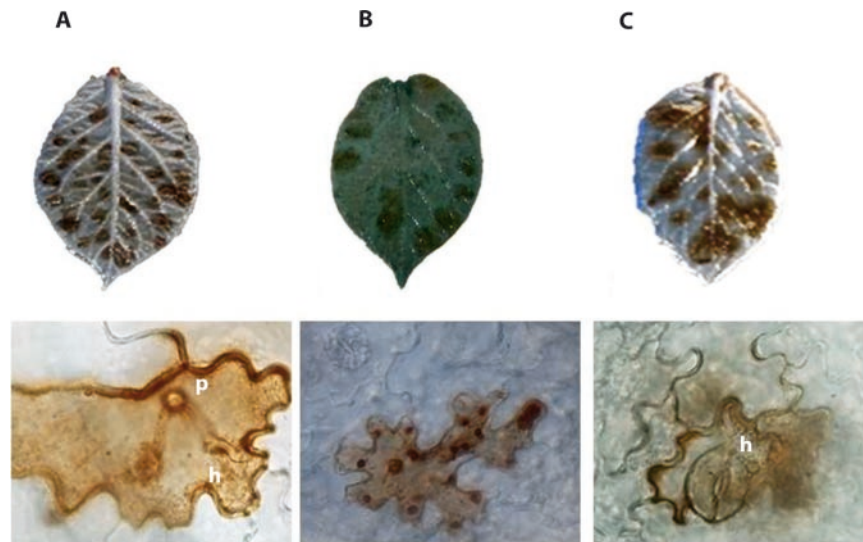
Fig. 3. Hydrogen peroxide accumulation in potato leaflets and cells from three cultivars with different resistance phenotypes to the late blight disease 48 hours after inoculation with *Phytophthora infestans*. A – cv. Pastusa Suprema; B – cv. Diacol-Monserrate; C – cv. Diacol-Capiro: p – pathogen penetration point, h – hyphae. Photographs are representative of images from three independent repetitions. Each photograph represents the cell response obtained from observations of 10 microscopic fields per replicate (400×). Leaflets and cells were stained with 3,3'-diaminobenzidine (DAB)
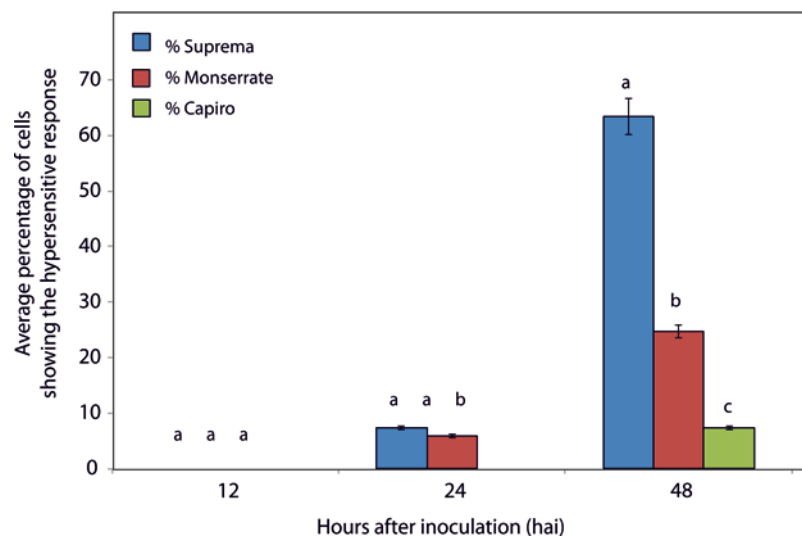

Fig. 4. Average percentage of cells showing the hypersensitive response (HR) in three potato cultivars with different late blight resistance phenotypes. Each average percentage was calculated using data obtained from observations of 10 microscopic fields per replicate of three independent repetitions. Different letters represent significant differences at each time tested identified by the statistical LSD test ($p < 0.05$). Error bars represent the standard deviation

observed in a lower proportion than in the cv. Pastusa Suprema (Figs. 7B and D). In the cv. Diacol-Capiro, in contrast to the resistant variety, no callose deposits were identified in these same observation fields (Fig. 7F).

After 48 hai, 51% of the fields were found to have callose deposits in the cv. Pastusa Suprema, 32% in the cv. Diacol-Monserrate, and 11% in the cv. Diacol-Capiro at this same time. From 24 hai until the end of the evaluation, cell wall thickening associated with callose deposits and hydrogen peroxide accumulation at the inoculum germination sites and attempted penetration of the plant cells by the pathogen in Pastusa Suprema and Diacol-Monserrate varieties were observed (Fig. 7). In contrast, in the cv. Diacol-Capiro no reinforcement of the cell wall or callose deposits associated with the pathogen structures were observed (Fig. 7).
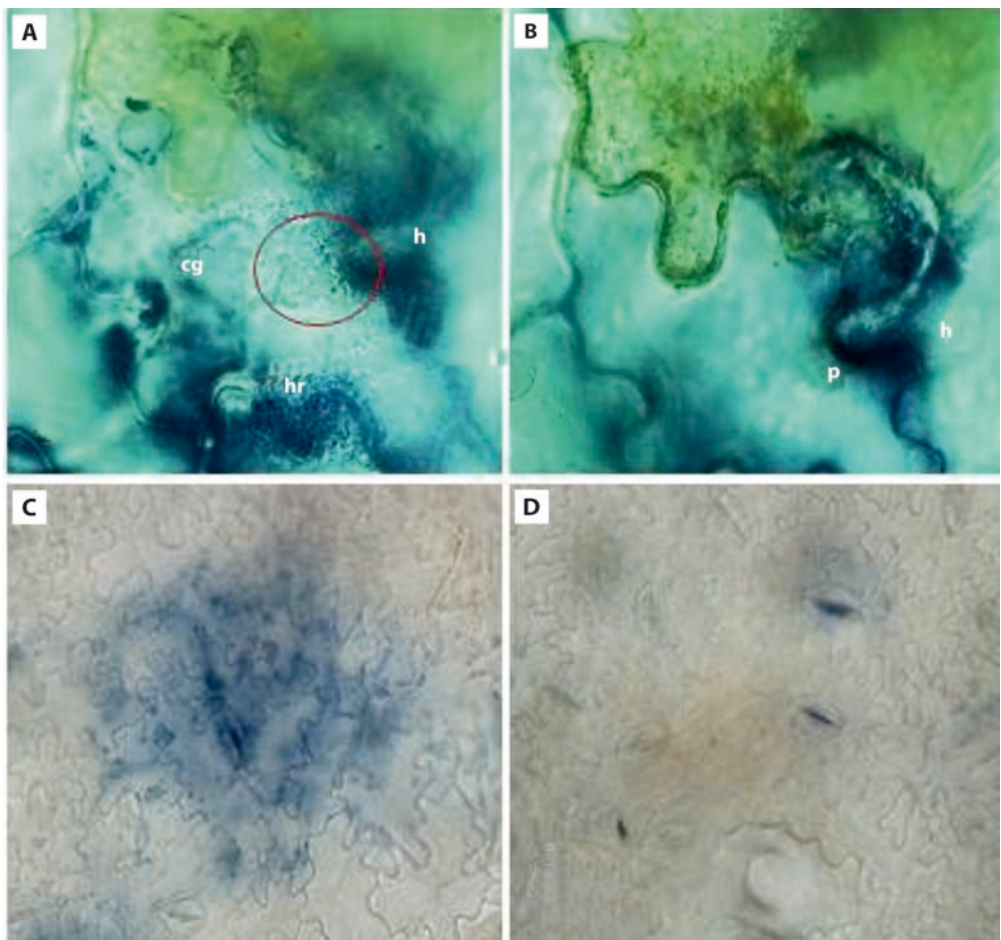
Fig. 5. Epidermal cells 48 hours after inoculation with *Phytophthora infestans*. A and B – cv. Pastusa Suprema; C – cv. Diacol-Monserrate; D – cv. Diacol-Capiro: cg – granular cytoplasm, h – hyphae, NC – condensed nuclei, hr – hypersensitive response, p – hypha penetration point. Photographs are representative of images obtained from observations of 10 microscopic fields per replicate of three independent repetitions. Cells were stained with trypan blue
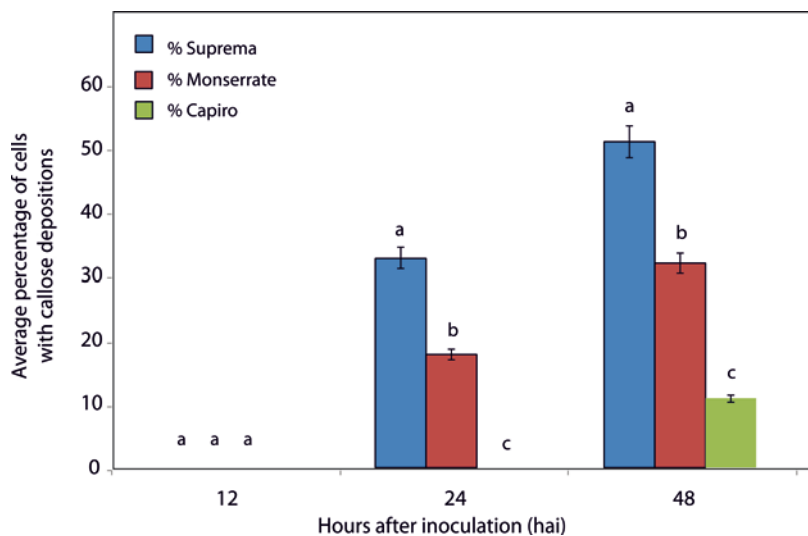

Fig. 6. Average percentage of cellular callose depositions on three potato cultivars after inoculation with *Phytophthora infestans*. Each average percentage was calculated using data obtained from observations of 10 microscopic fields per replicate of three independent repetitions. Different letters represent significant differences identified by the statistical LSD test ($p < 0.05$). Error bars represent the standard deviation
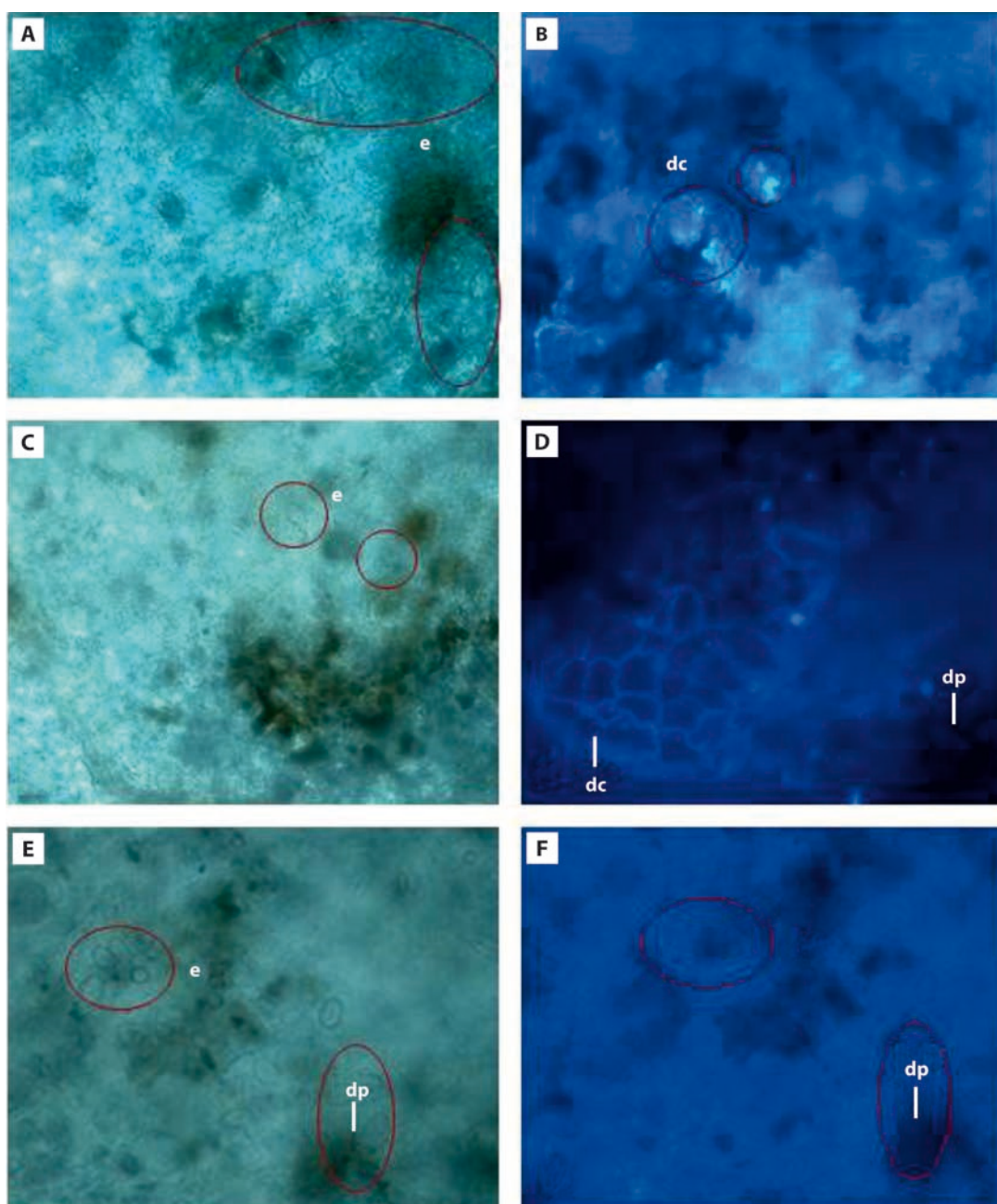
Fig. 7. Callose depositions on three potato cultivars 24 hours after inoculation with *Phytophthora infestans*. A and B – cv. Pastusa Suprema; C and D – cv. Diacol-Monserrate; E and F – cv. Diacol-Capiro: A, C and E – light microscopy showing sporangia on inoculated points; B, D and F – epidermal cells under fluorescence microscopy; dc – callose depositions, e – sporangia, dp – peroxide depositions. Cells were stained with aniline blue. Photographs are representative of images obtained from observations of 10 microscopic fields per replicate of three independent repetitions

Discussion

The response reactions of plants to attack by a micro-organism with pathogenic potential, determines the resistance or susceptibility phenotype that is observed during the interaction (Marone *et al.* 2013). Deposits of glucans, such as callose, and the synthesis of reactive oxygen species, such as hydrogen peroxide, are associated with a hypersensitive response and have been widely linked to defense responses in multiple pathosystems in both model and cultivated plants (Zhang *et al.* 2001; Ge *et al.* 2013). Comprehensive proteome analysis performed in the interaction between potato cv. Sarpo Mira and *P. infestans*, suggested that expression patterns of proteins during the early stages of infection are consistent with coordination and enrichment of cell wall-associated defense responses and that during the late stages of infection, they correspond to protein modification processes, membrane protein complex formation and cell death induction (Xiao *et al.* 2019).

In the present study, we found callose and hydrogen peroxide accumulation and a hypersensitive response to be probable response mechanisms of the Colombian potato varieties Pastusa Suprema, Diacol-Monserrate and Diacol-Capiro to infection by *P. infestans*. Although these defenses were identified in varieties that showed different levels of resistance/susceptibility (Hohl and Suter 1976; Ge *et al.* 2013), factors such as appearance time and response intensity seemed to be more decisive in determining disease development (Vleeshouwers *et al.* 2000; Chen and Halterman 2010). The cv. Pastusa Suprema has a phenotype of high resistance to late blight under field conditions (Segura *et al.* 2006; Ñústez 2011). In general, the responses studied appeared earlier and with greater intensity in this variety than in Diacol-Monserrate, in which they appeared later, or in the susceptible cv. Diacol-Capiro, in which they appeared even later.

Following the arrival of pathogen inoculums to different plant surfaces, very rapid metabolic and molecular changes occur which to a large extent determine the result of the interaction (Kamoun and Smart 2005; Balmer *et al.* 2013). *Phytophthora infestans* sporangia can germinate directly or liberate zoospores when the temperature and humidity conditions are favorable (Kamoun and Smart 2005). Zoospores are mobile and encyst when they recognize the surface of the host, after which they emit a germinative tube (Avrova *et al.* 2008). *Phytophthora infestans* uses its infection structures to attack all of the epidermal tissue, but increased appressoria formation and hyphae development has been observed when the zoospores establish themselves near the stomatal complex (Oyarzún *et al.* 2004). In fact, most germinative tubes penetrate the host through the stomata (Gees and Hohl 1988). A similar pattern was found in the present study, with rapid cell death occurring in the stomatal cells during the initial hours after inoculation in the cv. Pastusa Suprema. This programed cell death (PCD) has been widely described as the process by which the pathogen is confined or even destroyed, thus preventing its development in the host. This process, which is known as hypersensitive response (HR), is characterized by small necrotic lesions that prevent pathogen colonization and disease development in the plant tissue (Cavalcante *et al.* 2011). Hypersensitive response in plant stomata has been recognized for the resistance observed in some pathosystems, such as that of *Vitis inifera – Plasmopara vitícola* (Lambert *et al.* 2013), among others (Oyarzún *et al.* 2004; Cavalcante *et al.* 2011).

During the plant cell penetration phase, an appressorium is formed (Pristou and Gallegly 1956), followed by a penetration peg that dilates and penetrates the cuticle and epidermal cells. This forms an infection vesicle from which one or more secondary hyphae develop (Kamoun and Smart 2005). In susceptible potato varieties like Diacol-Capiro, the colonization of intercellular spaces of the apoplast was observed. The apoplast acts as the interface between the plant cells and the pathogen and is where the biotrophic phase of the interaction is established. This pattern was also observed in the cv. Diacol-Monserrate, but the biotrophic phase was more delayed and restricted than in the susceptible variety. Similar results have also been observed in other potato varieties (Coffey and Gees 1991; Avrova *et al.* 2008). In cv. Pastusa Suprema, penetration and limited development of the intercellular mycelium was observed; this finding was consistent with a variety that inhibits pathogen colonization (Vleeshouwers *et al.* 2000; Chen and Halterman 2010).

During the first 12 hai, no peroxides, hypersensitive response or callose deposits were observed in any of the three varieties. The detection of the pathogens or of self-damage by the plant occurs very early in the interaction (Rodríguez *et al.* 2016). This induces molecular signaling activity through the cell membrane receptors of the host and reprograming of the transcriptome and proteome for the activation of the defense responses (García-Brugger *et al.* 2006; Balmer *et al.* 2013). For this reason, it is possible that in the early phases the dyes evaluated will not be macroscopically observed, as has been reported by Vleeshouwers *et al.* (2000); Chen and Halterman (2010) and Rodríguez *et al.* (2016).

The biotrophic phase of the disease is established during the first 24 h of the interaction (Shimony and Friend 1975; Avrova *et al.* 2008; Chapman 2012). This is characterized by the colonization of the live tissue of the foliar mesophyll. The rapid accumulation of hydrogen peroxide during the pathogen's attempt to penetrate the plant cells has been reported as the most decisive response in the failure of plant colonization (Kotchoni and Gachomo 2006). Hydrogen peroxide is one of the molecules known as oxygen free radicals; these are involved in the hypersensitive response, which determines host and non-host resistance in numerous plant species and varieties (Zhang *et al.* 2001; Ge *et al.* 2013).

In the potato-*P. infestans* pathosystem, the expression of the oxidase glucose gene in transgenic potato plants increased H_2O_2 accumulation levels, supporting strong host resistance to this pathogen. This indicates a high sensitivity of *P. infestans* to this reactive oxygen species (Wu *et al.* 1995; Zhang *et al.* 2001). Five peroxidase proteins involved in ROS metabolism were observed up-regulated during the *Solanum tuberosum* (cv. Sarpo Mira) – *P. infestans* incompatible interaction (Xiao *et al.* 2019), suggesting the induction of the oxidative burst as was observed in the present work for the incompatible interaction between potato cv. Pastusa Suprema and *P. infestans*. In the sorghum-*Colletotrichum sublineolum* pathosystem, it has been found that

in the resistant variety, a large and early accumulation of peroxides occurs under the pathogen attack site, while in the susceptible variety the appearance of peroxides is more delayed (Basavaraju *et al.* 2009).

According to Vleeshouwers *et al.* (2000), *P. infestans* has an extraordinary ability to penetrate the epidermal cells and on occasion the mesophyll, of any plant, including non-Solanaceous species. In the present study, *P. infestans* passed the first epidermal barrier in all varieties; however, starting from the initial hours after inoculation (24 hai), quantitative and qualitative differences were identified in the responses of the three varieties. At the pathogen attack sites in cv. Pastusa Suprema, hydrogen peroxide was present in 56% of cells, HR was present in 7% of cells and callose deposits were present in 33% of cells. In the cv. Diacol-Monserrate, hydrogen peroxide was present in 19% of cells, 6% of cells presented HR, and callose deposits were present in 18% of cells. Meanwhile, in the cv. Diacol-Capiro, hydrogen peroxide accumulation was present in 7% of cells, and neither HR nor callose deposits appeared in any. This suggests early activation of the resistance mechanisms in the differentially resistant varieties. The early peroxide accumulation response associated with HR that occurred in cv. Pastusa Suprema and cv. Diacol-Monserrate suggests that at least some components of the resistance phenotype are due to the hypersensitive response (Segura *et al.* 2006; Ñústez 2011).

The cytological events that occur during programed cell death lead to a series of events that follow a chronological order: (i) migration of the nucleus to the pathogen penetration site and intense cytoplasmic activity; (ii) cessation of cytoplasmic activity, condensation of the nucleus, accumulation of granular cytoplasm in the periphery and shrinking of the protoplasm; and (iii) collapse of the cytoplasm and infected cell death (Morel and Dangl 1997). Accumulation of granular cytoplasm in the periphery and condensation of the nucleus were observed repeatedly in the present study. It is worth highlighting that these characteristics were also seen frequently in the cells that accumulated peroxides. Results indicate that peroxide is associated with the hypersensitive response observed, perhaps as a component of the oxidative burst induced by the reactive oxygen species.

After the first 24 hours of the interaction, a series of events occur in the *P. infestans*-potato interaction, which lead the biotrophic phase to move into the necrotrophic phase. This latter phase is characterized by being highly destructive and involving abundant sporulation, which causes cellular collapse (Hohl and Stössel 1976; Hohl and Suter 1976; Pieterse *et al.* 1994; Avrova *et al.* 2008). The lesions present in the necrotrophic phase lead to the liberation of thousands of sporangia, primarily in the susceptible varieties (Kamoun and Smart 2005). The development of the disease with the

characteristics described is determined by a compatible relationship in which the initial macroscopic lesion response is observed as a soaked area, similar to the reaction observed in cv. Diacol-Capiro, in which 10% of cell death was found at this time (Pieterse *et al.* 1994; Avrova *et al.* 2008).

Once the sporulation process has occurred in the susceptible variety, extensive colonization of the foliar tissue and stem occurs, followed by necrosis and mass cell death in the invaded tissue. According to Palavan-Unsal *et al.* (2005), two forms of cell death occur following the attack of a potential pathogen. The first of these is related to HR; this response involves the oxidative burst, among other responses, which causes irreversible damage in the plant cells with the objective of inhibiting the invasion and propagation of the pathogen. The second form is caused by the symptoms of the disease and results from the production of toxins generated by the invasion of the pathogen.

In an incompatible relationship, a rapid hypersensitive response causes the premature death of the infected tissue, provoking the death of the pathogen and the cells of its host (Hohl and Stössel 1976; Hohl and Suter 1976; Friend 1991). This was observed in 63% of cells in the cv. Pastusa Suprema at 48 hai. The percentages of cell death in each variety and the resistance/susceptibility phenotypes were consistent with the higher accumulation of hydrogen peroxide and callose deposits in the cv. Pastusa Suprema at 48 hai (65 and 51%, respectively), than in the susceptible cv. Diacol-Capiro (47% and 11%, respectively). This result coincides with the findings of Shimony and Friend (1975), who observed this response at the same time points in two potato varieties with strong and weak resistance, respectively. In the cv. Diacol-Monserrate, the percentages of HR obtained were 31 and 32%, respectively, which suggests an intermediate resistance phenotype.

Cells evaluated in the cv. Diacol-Capiro showed necrosis with extensive colonization of the foliar tissue, accompanied by peroxide accumulation, with fields exhibiting callose deposits which were formed near the sites where the pathogen structures were fixed (Buchanan *et al.* 2000; Voigt and Somerville 2009; Piršelová and Matušíková 2013). In marked contrast, the cv. Pastusa Suprema showed a hypersensitive response of cells which was associated with the accumulation of peroxides, and callose deposits in cells. These responses were clearly restricted to the inoculation sites. In the cv. Pastusa Suprema, neither colonization nor profuse sporulation was observed adjacent to these sites, as occurred in the cv. Diacol-Capiro. It is important to highlight that the accumulation of callose deposits was found to be strongly related to the level of resistance (Korgan *et al.* 2011), since after 24 hai a high percentage of this defense response was observed in the cv. Pastusa Suprema, while in the susceptible

variety, this occurred much later and in a very low percentage. In the cv. Diacol-Monserrate, these responses were maintained at an intermediate point between the two contrasting varieties, which confirms the presence of partial resistance, involving a restriction rate that limits pathogen penetration and invasion of the plant tissues (Ramos 2000).

In a similar work, Xiao *et al.* (2019) reported differential regulation of 70 proteins during the early phases of infection of the resistant potato cv. Sarpo Mira with *P. infestans*. Most proteins regulated indicated early activation of cell-wall defense-related responses and initiation of effector-triggered immunity (ETI) resistance activation consistent with a potato genotype that restricts pathogen tissue invasion.

Altogether, the results obtained suggest that plant responses in the form of callose deposits, peroxide accumulation and hypersensitive response are related to the level of resistance of the potato genotype tested with the late blight disease. Resistance responses in cv. Pastusa Suprema were characterized by appearing early, within the first 24 h, and with high intensity. Similar results have been obtained in numerous pathosystems, where the activation of genes involved in defense proteins occurs very quickly and with greater intensity in the resistant genotypes than in the susceptible genotypes (Wang *et al.* 2005; Torres *et al.* 2012). In the cv. Diacol-Monserrate, a similar early response behavior was observed, but with less intensity than in the resistant variety. Finally, in the susceptible cv. Diacol-Capiro, all responses evaluated were produced in a delayed manner, after the first 48 hai, and following the necrotrophic phase of the pathogen life cycle, accompanied by profuse sporulation. The failure to inhibit the growth of the pathogen enabled mass tissue colonization, abundant sporulation and clear symptoms of full disease development. Cell death in the form of a hypersensitive response effectively restricted colonization in the cv. Pastusa Suprema, preventing disease development.

In summary, significant differences in the speed and magnitude of expression of the defense responses were observed between the highly resistant variety and the highly susceptible variety; in the latter, the defense mechanisms were delayed and appeared with less intensity in the initial phases of the interaction with *P. infestans*. To the best of our knowledge, this result has not been previously reported in varieties cultivated in Colombia. The speed and intensity with which defense responses to the attack of a potential pathogen occur constitutes an important mechanism for identifying materials with resistance to disease in potato varieties. This can serve as a complementary resource in plant breeding programs, when searching for varieties with resistance to the late blight disease.

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