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Histopathological Study of the *Alternaria solani* Infection Process in Potato Cultivars with Different Levels of Early Blight Resistance

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Abstract

Histopathological analyses of the infection process of *Alternaria solani* were accomplished in three potato cultivars with different levels of early blight resistance. Leaflets of Aracy (resistant), Delta (moderately resistant) and Bintje (susceptible) cultivars were inoculated at the beginning of the flowering stage. In order to study the effect of leaf age, leaf samples were collected separately, in the lower, middle and upper third of the plants. Conidia germination, appressoria formation, penetration and number of penetration sites exhibiting hypersensitive response (HR) were qualitatively and quantitatively assessed at 6, 12, 24, 48 and 72 h after inoculation. Penetration occurred most frequently through the junctions of the epidermal cells. Penetrations through stomata were rarely observed. There was no association between the events and the resistance levels, except for the number of penetration sites showing HR that was highest in Aracy, intermediate in Delta and lowest in Bintje. Similarly, the number of sites with HR was the only event associated with the leaf age. Regardless of cultivar resistance levels, the number of the penetration sites with HR was higher in leaves in the upper part of the plant. These results suggest that HR may be one of the mechanisms associated with age-related and genetic resistance to early blight in potato.

Introduction

Potato early blight (EB), caused by the mitosporic fungus *Alternaria solani* Sor., is an important foliar disease that occurs in potato-growing regions worldwide (Christ, 1991; Rotem, 1994; Van der Waals et al., 2003). The disease may cause rapid plant defoliation, which may result in reduction of the vegetative cycle and yield losses. In Brazil, during the rainy season, EB is the most limiting disease for potato production (Reifschneider et al., 1984) and frequent

fungicide sprays are commonly used to prevent crop losses. Cultivar resistance is the most promising strategy to manage EB, but most commercially-grown potato cultivars in Brazil are susceptible to EB.

It has been repeatedly observed that resistance to EB is age-related: older leaves are more susceptible and susceptibility increases as plants grow older (Douglas and Pavek, 1972; Pelletier and Fry, 1989). In resistant cultivars as well as in young leaves, the incubation period is long and there are small numbers of lesions, suggesting that resistance mechanisms may act during the early stages of the infection process (Dita et al., 2006). However, to date, cytological and molecular studies to address the mechanisms involved in the resistance have not been conducted in the potato *A. solani* pathosystem.

Histopathological studies of the host–pathogen interaction can help identify events that occur during the pathogenesis, and ultimately lead to a better understanding of resistance mechanisms (Sillero and Rubiales, 2002; Vleeshouwers et al., 2000; Xi et al., 2000). Events associated with the pathogenesis of *Alternaria* spp. in different host species such as conidia germination, appressoria formation and penetration have been studied (Allen et al., 1983; Aveling et al., 1994; McRoberts and Lennard, 1996; Vandyke and Trigiano, 1987). However, most of these studies described the infection processes in susceptible cultivars. Histopathological studies comparing events in resistant and susceptible cultivars and in tissues with different ages are scarce. In studies, carried out with tomato, formation of few appressoria was associated with EB resistance (Araújo and Matsuoka, 2004) whereas the hypersensitive response (HR) to be related to non-host resistance to *Alternaria alternata* f.sp. *lycopersici* (Brandwagt et al., 2001). In the potato–*A. solani* interaction, however, no histopathological studies comparing genotypes with different levels of resistance to EB and tissues with different ages have been conducted.

Furthermore, there is no detailed description of the infection process in this pathosystem.

We have recently reported the effects of the leaf position on components of resistance to EB in potato cultivars with different levels of resistance (Dita et al., 2006). A step further would be to identify the cytological mechanisms associated with the resistance and to determine the time when they are deployed. Therefore, the purpose of this study was to accomplish a histopathological analysis of the infection process in three potato cultivars with distinct levels of EB resistance, aiming at describing the infection process as well as at identifying possible structural mechanisms related to the resistance levels of the cultivars including the age-related relationship.

Materials and Methods

Plant growth and inoculum production

Three potato cultivars with different levels of EB resistance were selected: 'Aracy' was used as resistant, 'Delta' as moderately resistant and 'Bintje' as susceptible cultivar. Tubers of about 50 g were planted in 8 l-plastic pots filled with soil, sand and compost mixture in a 3:1:1 (v/v) ratio. All experiments were performed with a monosporic culture of *A. solani* (isolate ASA02), obtained from a naturally infected potato plant. Isolate was grown on Potato Dextrose Agar medium in Petri dishes kept on an incubator at 24°C, in the dark, until colonies reached 8 cm in diameter. Inoculum suspension was produced as described elsewhere (Dita et al., 2006).

Inoculation and evaluation of the infection process

Before inoculation, three sections: lower, middle and upper were properly labelled in each plant. The term plant position or leaf position, used along in the text, corresponds to these three sections. Although leaf age was not precisely determined, we assumed that leaves collected from lower, middle and upper sections, ranged from older to younger physiological stages, respectively.

Three consecutive series of experiments were performed. In each experiment 15 plants per cultivar were used. Ten plants were inoculated and five were used as control (not inoculated). Seven of the 10 inoculated plants were used for collecting samples. The remaining plants (three) were used to assess inoculation efficiency. A 10 μ l-drop of a 10^4 conidia per ml suspension supplemented with gelatin (Sigma® 1% w/v) was placed in an area of about 4.0 cm² previously marked in the adaxial surface of the leaflets. At least 30 leaflets were inoculated in each plant section. Aliquots (10 μ l) of gelatin without conidia were drop-placed on the leaflets of the non-inoculated control plants. After inoculation, plants were covered with polyethylene bags which had its interior part wetted with distilled water to form individual humid chamber and kept at 25°C for 24 h. After this incubation time, the plants were transferred to the greenhouse and the bags were removed. Three inoculated leaflets were randomly collected at 6, 12, 24, 36, 48 and 72 h after inoculation from the lower, middle and upper third sections of each plant. Leaflet

sections of about 0.5 cm² (1.0 cm \times 0.5 cm) were cut in the area previously marked on the leaflets and discolored in chloral-hydrated solution (2.5 g/ml). After 5 days, discolored leaflet sections were placed in microscopic slides and stained with trypan blue (0.05%). Three slides with four independent trypan-blue stained leaflet sections per treatment were examined by differential interference contrast microscopy (Olympus Vanox Microscope Platform, Olympus Japan Co., Ltd). The infection process was examined in all treatments and the following events were quantified:

Germination: Germinated conidia were considered as those in which germination tubes were present, regardless of either length or number of germ tubes per spore.

Appressoria: All globular structures with larger diameter than that of the germination tubes or hypha of origin were considered as appressoria and counted. No distinction between lateral and terminal appressoria was made.

Penetration: Penetration sites were recorded when fungal structures were observed beneath the epidermal cells. This event was recorded independently of the penetration place and host reaction.

Penetration sites exhibiting HR: This event was recorded when host epidermal cells had thickened cell wall, extensive cytoplasmic granulation and high levels of autofluorescence in response to pathogen penetration.

Scanning electron microscopy assay

In order to obtain a more accurate understanding of pre-penetration events of the infection process, scanning electron microscopy analyses were performed with infected tissues of 'Bintje' collected 24 h after inoculation. Plants were grown as indicated above and pre- and postinoculation conditions were also similar. Inoculation was accomplished by atomization of a 10^6 conidia per ml suspension onto all leaflets. This high inoculum concentration was used in order to increase the probability of finding sites with infection events. Leaflet sections of about 16 mm² (4 mm \times 4 mm) were cut and subjected to prefixation in glutaraldehyde (3%, buffered with sodium cacodylate pH 7, 0.05 M) for 2 h at room temperature. Sections were then washed six times in sodium cacodylate and fixation was carried out in osmium tetroxide (OsO₄ 1%) for 4 h, at 4°C. Leaflet sections were washed six times with sodium cacodylate buffer and dehydrated in alcoholic series (30, 50, 70, 80, 95 and 100%). After dehydration, leaflet sections were dried to the critical point using a 'Critical Point Dryer' (Balzers, model CPD020, BAL-TEC AG, Switzerland) then placed in a metal support and covered with gold by cathodic pulverization in 'Sputter Coater' coupled to a 'Freezing Drying Unit' (Balzers, FDU010, BAL-TEC AG, Switzerland). Samples were examined with a JEOL scanning electronic microscope (JSM-T2000, JEOL, Peabody, MA, USA) operating to 10 kV.

Statistical analysis

Previous analyses with samples collected at different time-points revealed that sampling at 36 h after inoculation allowed the best assessment of the infection events. Furthermore, there was no interaction between time-points and cultivars, thus the data from 36 h were used for the statistical analyses. Data from samples taken 36 h after inoculation from the repeated experiments were combined and subjected to analysis of variance. Germination of conidia was analyzed by comparing the means of germinated conidia per tissue-area (2 cm^2) per treatment. The ratio (proportion) of appressoria per germinated conidia was calculated and analyzed. The number of penetrations per appressoria was estimated, as well as, the number of sites with HR relative to the number of penetration sites.

Because there was no significant interaction between resistance levels and plant sections, the effect of leaf position upon all pathogenesis events was analyzed using the data from each plant section (lower, middle and upper) pooled from the three cultivars. Thus, in each event the data from lower section of the plants of all cultivars were combined and compared with the data from the middle and upper plant sections. Mean values were compared using the Fisher LSD ($\alpha = 0.05$) test. All analyses were accomplished using the Statistical Analysis System version 8.0 (SAS Institute Inc., Cary, NC, USA).

Results

Infection process of *A. solani* in potato

For all cultivars, the first germinated conidia of *A. solani* were observed at 6 h after inoculation, increased at 12 h, reaching the maximum at 36 h after inoculation. All conidia formed at least one germ tube, but on average each conidium formed $3.21 (\pm \text{s.d. } 0.093)$ germ tubes that grew not oriented over the leaf surface. Germ tubes were not necessarily oriented towards stomata and often grew over them without forming appressorium (Fig. 1a). Germ tubes frequently branched and extended over various epidermal cells until forming appressorium and penetrating, predominately at the cell junctions. Conidia with short germ tubes, with an appressorium, also resulted in penetration (Fig. 1b). Appressorium formation increased significantly at 24 h after inoculation. At this time, appressoria were characterized by the hyaline dilations, prominent at the end of the germ tubes (Fig. 1c) or sidelong the hypha (Fig. 1d). Some non-penetrating appressoria originated new germ tubes, from which new appressoria could be differentiated and penetrate, or form a hyphae network. Most appressoria were formed at the cell wall junctions of the epidermal cells (Fig. 2a, b). On the appressorium surface, germ tubes and hypha, an extracellular substance was frequently observed (Fig. 2).

Penetration of tissues by *A. solani* began 24 h after inoculation increasing at 36 h and apparently only occurred after the formation of appressoria. The pathogen penetrated the leaf surface directly through the epidermis, usually at the site of cell wall junctions

(Figs 1d, 2a and 3c). Penetrations through the stomata were rarely observed. Once inside the tissue, the hyphae became thickened and abundant internal mycelium was formed (Fig. 1e). The internal mycelium branched and colonized the tissues (Fig. 1f). Tissue necrosis followed pathogen colonization and at 48 h after inoculation, macroscopic lesions in the discolored tissues were observed. The necrosis caused by the pathogen colonization prevented assessments in samples collected 72 h after inoculation.

Regardless of the penetration mode, host cell wall morphology at the point of infection was visibly modified in some cells (Fig. 3). These modifications were characterized by aggregated cytoplasm with abundant granulations and apparent cell wall thickening (Fig. 3e and f). These cells when observed under fluorescence microscopy presented high levels of autofluorescence (data not shown). This type of reaction which was observed with higher frequency in penetration sites near the stomatal complex was considered as HR (Araújo and Matsuoka, 2004; Vleeshouwers et al., 2000). The pathogen was visibly restricted in the infection sites with HR. However, in the susceptible genotype, successful colonization was occasionally observed despite HR. Similar to pathogen penetration, cells with HR began to be observed at 24 h after inoculation and reached the maximum number at 36 h after inoculation.

Quantification of the infection process events

Germination

Germinated conidia of *A. solani* were observed in all cultivars, regardless of resistance levels. The highest numbers of germinated conidia occurred on the susceptible cultivar Bintje, in all plant sections. Germination rates on 'Bintje' differed from Delta in the lower and the upper and from 'Aracy' in the middle and the upper plant sections, respectively (Table 1). There was no relationship between the number of germinated conidia and leaf position (Table 2).

Appressoria

There was no consistent association of number of appressoria formed by germinated conidia (appressoria/germinated conidia) and cultivar resistance level (Table 1). The lowest values were recorded in the susceptible cultivar Bintje. This event was not influenced by the leaf position (Table 2).

Penetration

In all three cultivars, penetration of *A. solani* occurred predominantly by direct mode and there was no association of this event and resistance level (Table 1). In addition, there was no effect of leaf position on the number of penetration sites (Table 2).

Penetration sites with HR

Penetrations sites exhibiting HR were observed in all cultivars. In the three plant sections, highest numbers of penetration sites with HR were recorded in the

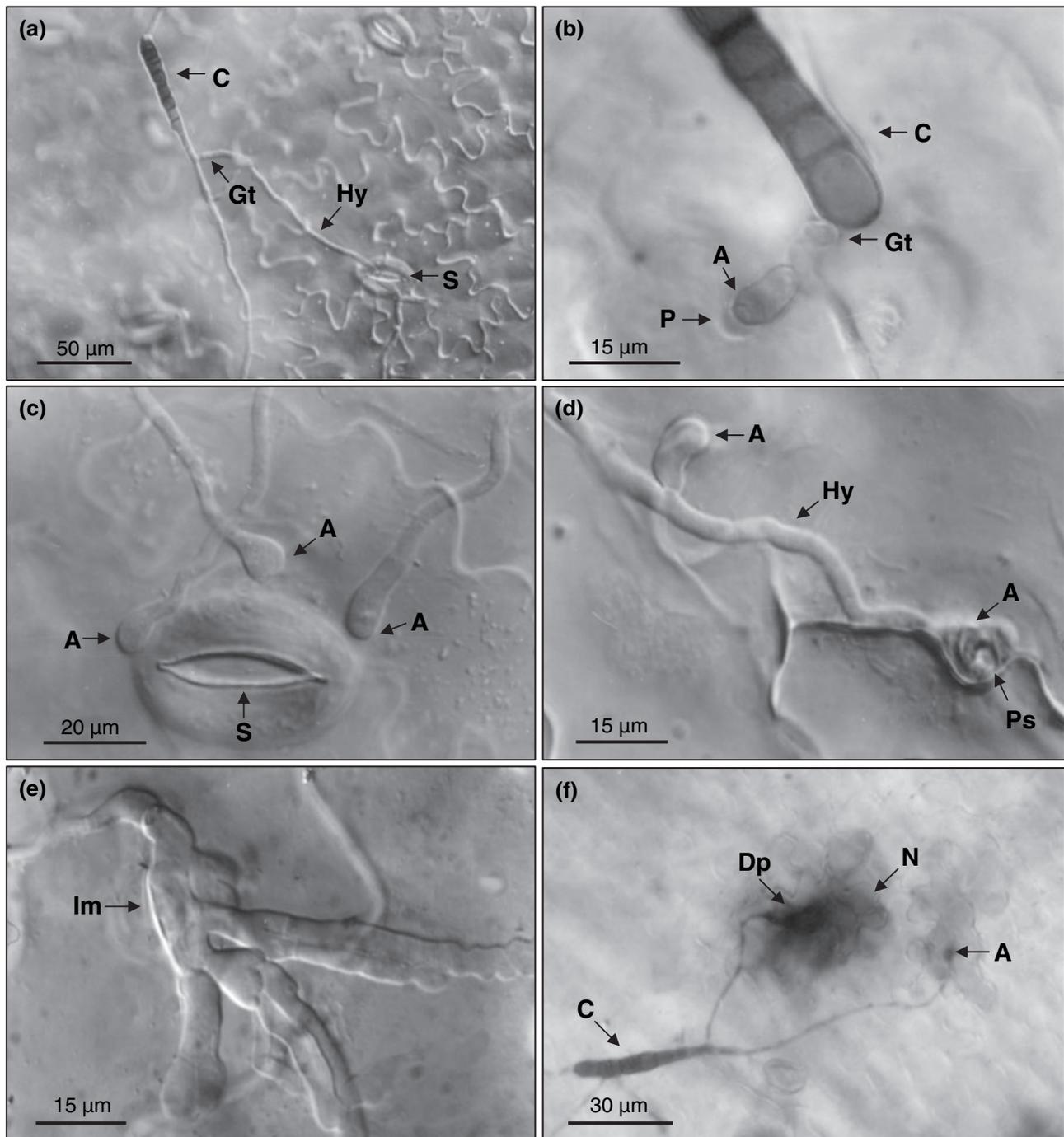


Fig. 1 Light microscopy micrographs of *Alternaria solani* pre- and post-penetration events in potato cv. Bintje, 24 h after inoculation. (a) Conidium (C) with two germ tubes (Gt) and a long hypha (Hy) crossing by a stoma (S) with no appressorium formation. (b) Conidium (C) with short germ tube (Gt) with appressorium (A) and semicircular papillae (P) at the attempted penetration site. (c) Three terminal appressoria (A) formed near of stomata (S). (d) Formation of two lateral appressoria (A) by a single hypha (Hy) and direct penetration site (Ps) between cell wall junctions. (e) Host cell penetrated with internal mycelia (Im) larger than the penetration structure. (f) Germinated conidia (C) with appressoria (A) penetrating directly (Dp) in two different sites. Necrosis (N) of the colonized tissue is observed

resistant cultivar Aracy (Table 1). There was a significant association between number of sites with HR and cultivar resistance level when assessing this variable in the middle and upper third sections (Table 1). This event was also influenced by the position of leaves and higher number of sites with HR recorded in the upper than in middle or lower thirds of the plants (Table 2).

Discussion

As far as we are aware, this is the first histopathological study of the *A. solani*–potato interaction that describes and compares events of the infection process in cultivars with different levels of resistance and its relation with tissue-age. Similarly, to what has been reported in other *Alternaria* pathosystems, germination of conidia of *A. solani* on potato was neither associ-

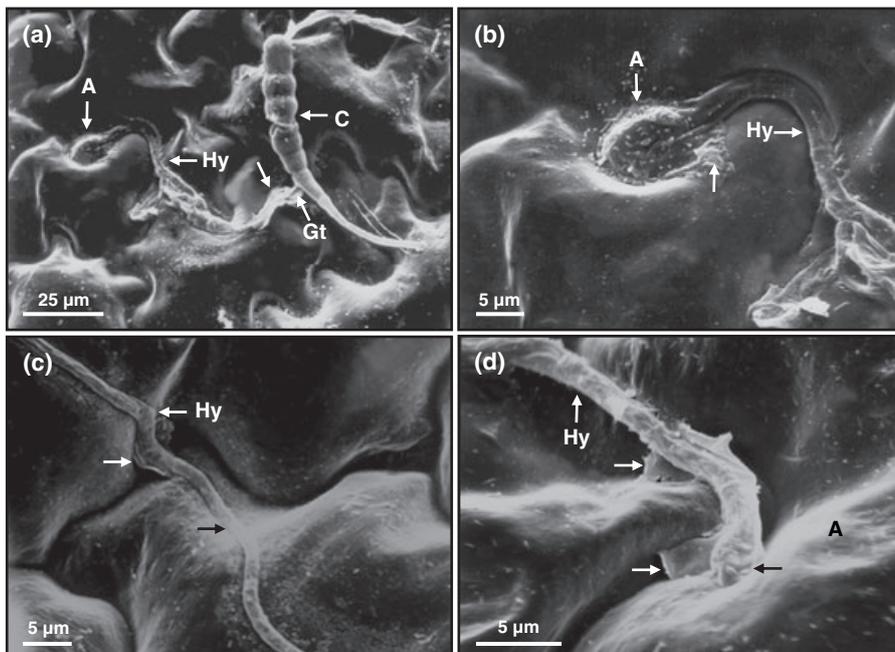


Fig. 2 Scanning electron micrograph of the pre-penetration and penetration structures formed by germinated conidia of *Alternaria solani* in the susceptible potato cv. Bintje, 24 h after inoculation. (a) Conidium (C) with germinating tube (Gt), hypha (Hy), and appressorium (A) at the cell wall junctions of the epidermal cells. (b) Detailed view of section (a). (c) Hypha (Hy) adhered in the host surface suggesting the presence of adhesive extracellular material. (d) Hypha (Hy) with appressorium (A) at initial stage in the cell wall junctions of the epidermal cells. Arrows indicate points with substance like extracellular material and apparent adhesion of fungal structure to the host surface

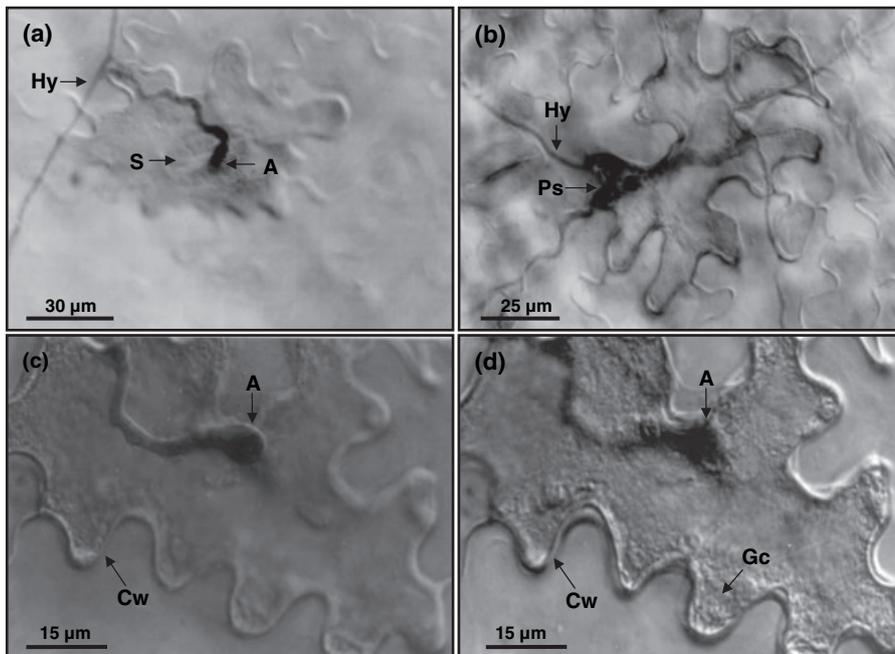


Fig. 3 Light microscopy micrographs of *Alternaria solani* hyper-sensitive response (HR) in resistant potato cv. Aracy, 36 h after inoculation. (a) Hypha (Hy) branched with appressorium (A) penetrating through the stoma (S) and host cell with HR (darker area). (b) Hypha (Hy) penetrating directly and host cell with HR at the penetration site (Ps). (c) Appressorium (A) at initial phase of direct penetration in a site with thickened cell wall (Cw) and HR. (d) Details of HR from section (c) (with different focal view) showing granular structure of the cytoplasm (Gc), and thickened cell wall (Cw)

ated with cultivar resistance levels (Araújo and Matsuoka, 2004) nor with the tissue-age (Green and Bailey, 2000). However, germination of conidia was slightly lower at all leaf positions in the resistant cultivar (Aracy) suggesting that this event could be affected by host resistance in this pathosystem. Detailed studies properly designed to address this issue should be conducted as inhibition of conidial germination has been already associated with the host resistance to fungal pathogens (Cook, 1980; McLean and Byth, 1981).

Multiple germination tubes growing in any direction over the leaf surface were also observed, in the *A. solani*-*Solanum lycopersicum* (Araújo and Matsuoka,

2004), *A. cirsinoxia*-*Cirsium arvense*, (Green et al., 2001), *Alternaria porri*-*Allium cepa* (Aveling et al., 1994) and *Alternaria cassiae*-*Vigna unguiculata* (Van den Berg et al., 2003) pathosystems. The fact that only germinated conidia were present on the examined leaf samples could be related to the presence of an extracellular substance observed in the germ tubes, appressorium surface and hypha. According to Gold and Mendgen (1984), in addition to protection of the germ tubes from drought or other stresses, this material might also be involved in the adherence to host surface. Thus, this substance could have facilitated the adhesion of these structures to the potato leaf surface

Table 1
Quantification of *Alternaria solani* infection events in leaves from the lower, middle, and upper plant sections of three potato cultivars, 36 h after inoculation

Cultivar ^a	Germination ^b	Appressoria ^c	Penetrations ^d	Penetration sites with HR ^d
Lower				
Aracy (r)	67.3 ab ^e	0.61 a	0.66 a	0.32 a
Delta (mr)	62.0 b	0.66 a	0.62 a	0.12 b
Bintje (s)	90.6 a	0.55 a	0.80 a	0.08 b
Middle				
Aracy (r)	71.6 a ^e	0.78 a	0.83 a	0.41 a
Delta (mr)	83.3 ab	0.87 a	0.76 a	0.18 b
Bintje (s)	99.3 b	0.72 a	0.80 a	0.04 c
Upper				
Aracy (r)	57.3 a ^e	0.54 ab	0.85 a	0.58 a
Delta (mr)	61.0 a	0.81 a	0.59 a	0.35 b
Bintje (s)	88.6 b	0.42 b	0.76 a	0.18 c

^aLetters in brackets indicate level of early blight resistance of the cultivars: r = resistant, mr = moderately resistant, s = susceptible.

^bAverage germination percentage. For each cultivar, the mean value was estimated from seven replicates.

^cAverage number of appressoria per germinated conidium.

^dProportion of penetration sites with or without HR in relation to the average number of appressoria formed per conidium.

^eValues in a column followed by different letters are significantly different at $\alpha = 0.05$ according to Fisher LSD test.

Table 2
Effect of leaf position of three potato cultivars on the *Alternaria solani* infection events, 36 h after inoculation. For each plant section, the mean value of each variable was estimated across three cultivars ('Bintje', 'Delta' and 'Aracy') and seven replicates

Plant sections	Germination ^a	Appressoria ^b	Penetrations ^c	Penetration sites with HR ^c
Upper	69.0 a ^d	0.58 a	0.73 a	0.36 a
Middle	84.5 a	0.79 a	0.80 a	0.21 b
Lower	73.3 a	0.60 a	0.70 a	0.17 b

^aAverage germination percentage.

^bAverage number of appressoria per germinated conidium.

^cProportion of penetration sites with or without HR in relation to the average number of appressoria formed per conidium.

^dValues in a column followed by different letters are significantly different at $\alpha = 0.05$ according to Fisher LSD test.

preventing them from being removed during the preparation of the sample. Extracellular substances produced by germinating conidia have already been described in other plant pathogenic fungi (Braun and Howard, 1994; Hau and Rush, 1982; Kuo and Hoch, 1995; Wheeler and Gantz, 1979), including *Alternaria* species such as *A. porri* (Aveling et al., 1994) and *A. cassiae* (Mims et al., 1997). However, to date, there was no report of the presence of extracellular substances associated with conidia germlings of *A. solani*.

Formation of appressoria and penetration occurred predominantly between cell wall junctions at the epidermis and very rarely at the stomata. On this regard, *A. solani* was similar to *A. porri* (Aveling et al., 1994) and *A. cirsinioxia* (Green et al., 2001), but different from *A. lternaria tenuis* that penetrates through the stomata to cause leaf spot in beans (Saad

and Hagedorn, 1969). Appressoria formation was a necessary step for successful infection of tomato by *A. solani*. In this pathosystem, there was a clear relationship between low levels of appressoria formation and low number of EB lesions in the resistant tomato genotype CNPH 417 (Araújo and Matsuoka, 2004). However, in some species, such as *A. cassiae*, the pathogen can penetrate the host tissue with or without forming appressoria (Van den Berg et al., 2003). Despite its importance for the infection process, no relationship between appressoria formation and cultivar resistance levels or leaf age was detected in the present study. This result suggested that resistance to EB in these cultivars is not associated with inhibition of appressoria formation.

The host reaction considered here as HR was characterized by high levels of autofluorescence, granular structure of cytoplasm and thickened cell wall. Host responses with similar characteristics were detected in both resistant and susceptible genotypes of potato when challenged with *Phytophthora infestans* (Ferris, 1955; Freytag et al., 1994; Schröder et al., 1992; Vleeshouwers et al., 2000) and *Potato virus Y* (Hinrichs-Berger et al., 1999) and in tomato inoculated with *A. solani* (Araújo and Matsuoka, 2004). In all cases, the response was considered as HR. However, due to the necrotrophic nature of *A. solani*, additional studies are necessary to determine whether the reaction observed in the present study is similar to the HR associated with programmed cell death. Thus, detailed studies with programmed cell death inhibitors, DNA laddering and other techniques are necessary to better characterize this HR response.

The high frequency of HR observed in areas near the stomatal complex may be related to differential accumulation of organ-specific substances involved with plant response to pathogens. Buchter et al. (1997) and Hoegen et al. (2002) observed higher accumulation of pathogenesis-related proteins in areas of the stomatal complex of potato tissues in response to *P. infestans* infection. It is possible that similar events can occur when potato leaves are penetrated by *A. solani* in this area.

Similarly, for germination of conidia and appressoria formation, penetration frequency did not show any relation with the resistance levels of cultivars or with tissue-age. Resistance to EB in these cultivars is likely to be determined by mechanisms acting after pathogen penetration. The capacity of *Alternaria* to penetrate cells of non-host plants (Brandwagt et al., 2001; McRoberts and Lennard, 1996) supports the hypothesis that major resistance responses may take place after pathogen penetration. One factor that seems to be involved in the post-penetration resistance to EB in potato is the number of penetration sites displaying HR. This event was associated with the resistance levels of cultivars and leaf age. The association between number of penetration sites exhibiting HR with levels of partial resistance has been demonstrated in other pathosystems (Gees and Hohl, 1988; Kowalska and

Nuiks, 1999; Vleeshouwers et al., 2000). However, as far as we are aware, this is the first report that suggests the possible involvement of HR with quantitative resistance of potato cultivars as well as with the age-related resistance to EB.

The frequency and/or the timing of HR occurrence might explain the resistance levels of genotypes (Gees and Hohl, 1988; Kowalska and Nuiks, 1999; Vleeshouwers et al., 2000). We have not found differences in the timing of HR occurrence, but it is possible that with shorter sampling intervals, these differences could be detected. On the contrary, HR frequency can contribute to resistance by reducing the number of lesions. Lesions formed in tissues of resistant cultivar and in young leaves had slower expansion rate than those in susceptible cultivars or in old leaves (Dita et al., 2006). Thus, other defense mechanisms that restrict the pathogen growth may contribute to the slower colonization observed in resistant cultivars.

The elicitation of HR by *A. solani* in potato is an interesting issue. Although this defense reaction is more commonly reported in interactions with biotrophic pathogen, HR has been observed in tomato leaves inoculated with *A. solani* (Araújo and Matsuoka, 2004); when challenged with elicitors obtained from the conidia and germlings of this pathogen (Lawrence et al., 2000) or by direct inoculation. Additionally, other species of *Alternaria* considered as necrotrophic are also able to elicit HR in their hosts (Brandwagt et al., 2001; Ortega et al., 2005). It seems that HR may also be part of a quantitative defense mechanism that is present in *A. solani*-solanaceous species interactions. Interestingly, Govrin and Levine (2000) showed that hypersensitive cell death did not protect plants against infection by the necrotrophic pathogens *Botrytis cinerea* and *Sclerotium sclerotiorum*, but instead, HR seems facilitating colonization of plants. In the *A. solani*-potato interaction the pathogen was apparently restricted to the infection sites in most penetrations sites exhibiting HR. Differences in the strategies employed by the pathogens to overcome host resistance could explain these contrasting observations.

In summary, this study describes the infection process of *A. solani* in potato, comparing cultivars with different level of resistance and tissues with different ages. Data based on the light microscopic observations suggested that HR may be one of the mechanism involved with age-related and genetic resistance to EB in potato. However, further studies are needed in order to characterize this response at the molecular level, and to determine whether the reaction observed in the present study is similar to the HR observed in plant-pathogen interactions that follows the gene-to-gene interaction model.

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