PHENOTYPIC CHARACTERIZATION OF THE *Rvi15* (*Vr2*) APPLE SCAB RESISTANCE

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SUMMARY

Recently, a new apple scab resistance was found in the apple accession GMAL 2473 and named *Rvi15* (*Vr2*). The resistance reaction mediated by *Rvi15* (*Vr2*) appeared on the upper surface of young infected leaves 7 days post inoculation (dpi) as browning of epidermal cells. Starting from 11 dpi, browning of mesophyll cells was also observed suggesting a gradual involvement of the different leaf tissues. The resistance reaction resulted in pinpoint pits similar to those observed in *Malus* accessions carrying the *Rvi5* (*Vm*) or *Rvi4* (*Vh4*) resistance, which are considered to induce the classical hypersensitive response (symptoms appearing at both epidermal and mesophyll level 2-3 dpi). When the timing of the reaction is considered, *Rvi15* (*Vr2*) seems more similar to the *Rvi7* (*Vfh*) resistance found in *Malus floribunda* 821, which results in pinpoint pits 6 dpi. However, size of the pinpoint pits, number of cells involved and accumulation of fluorescent compounds at the site of pathogen penetration are very similar between all four resistance reactions.

Key *words*: *Malus*, resistance genes, apple scab resistance, *Rvi15*, pinpoint pits.

INTRODUCTION

Biotrophic pathogens colonize plants by avoiding or penetrating physical barriers and absorbing nutrients from the host without being detected by the host's constitutive surveillance machinery. To defend themselves against such pathogens, plants have developed inducible defense mechanisms mediated by disease resistance (*R*) genes. Theoretically, for resistance, a complementary pair of genes, one in the host (*R* gene) and the other in the pathogen (*Avr* gene) are required. Therefore, when corresponding *R* and *Avr* gene products are present in the host and in the pathogen, the result is disease resistance (incompatibility). When *R* or *Avr* genes are inactive or absent, the plant is susceptible (compatibility). This highly specific gene-for-gene mechanism of resistance proposed by Flor (1946) characterizes also the *Malus* spp. and *Venturia inaequalis* relationship (MacHardy, 1996). The observation of resistance reactions on the upper surface of young apple leaves after infection with the pathogen is a first approach to characterize resistances given by *R* genes. The phenotype can vary from complete resistance to complete susceptibility, depending on the type of resistance and on the length of the incubation period (Shay and Hough, 1952).

Up to now, sixteen major R genes against apple scab have been identified and mapped in different apple genotypes (Bus *et al*., 2009; Patocchi *et al*., 2009; Soriano *et al*., 2009). Most of them follow the gene-for-gene interaction model and were found to mediate different types of resistant phenotype (Gessler *et al*., 2006). Four of these genes [*Rvi5* (*Vm*), *Rvi4* (*Vh4*), *Rvi7* (*Vfh*) and *Rvi10* (*Va*), Bus *et al*., (2009)] induce an hypersensitive response (HR) characterized by a rapid response on the upper surface of young infected leaves leading to circular- to oval-shaped necrotic zones of dead epidermal and mesophyll cells under and around the pathogen penetration site (called pinpoint pit phenotype) (Goodman and Novacky, 1994). Well characterized examples of the involvement of an HR include resistances mediated by *Rvi5* (*Vm*), *Rvi4* (*Vh4*) and *Rvi7* (*Vfh*) genes derived from *Malus micromalus* 245-38, TSR33T239 (host 4) and *Malus floribunda* 821, respectively (Hernandez Castillo, 1990; Bus *et al*., 2005a; Benaouf and Parisi, 2000)[*]. However, the time elapsing between inoculation and the macroscopic appearance of a resistance reaction differs for these genes. *Rvi5* (*Vm*) and *Rvi4* (*Vh4*) require about two to three days before the symptoms become visible (Hernandez Castillo, 1990; Bus, 2006) while *Rvi7* (*Vfh*) requires six days (Shay and Hough, 1952).

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[*] Please note that throughout the manuscript we will indicate both names of the apple scab resistances: the new name following the nomenclature system proposed by Bus *et al*. (2009), and the historical name between parentheses.

Gene-for-gene relationships have also been shown for other types of resistance not leading to the development of a classical HR. Examples are *Rvi2* (*Vh2*), *Rvi1* (*Vg*) and *Rvi6* (*Vf*) resistances. The *Rvi2* (*Vh2*) found in *Malus pumila* R12740-7A (host 2) conditions a stellate necrosis reaction 4-6 days after inoculation (Shay and Hough, 1952). The *Rvi1* (*Vg*) resistance discovered in cv. Golden delicious is expressed as a chlorotic and leaf necrotic reaction (Bénaouf and Parisi, 2000). The last example of resistance to apple scab not leading to an HR is the *Rvi6* (*Vf*) gene found in *M. floribunda* 821. This gene mediates different resistance phenotypes varying from no lesion to chlorotic and necrotic lesions with limited sporulation 12 dpi under optimal conditions for disease (Chevalier *et al.*, 1991). This large variation in symptom expression is attributed to minor genes or to modifiers of the *Rvi6* (*Vf*) gene that alter *Rvi6* (*Vf*) resistance expression (Gessler, 1989).

Recently, a new apple scab major resistance gene, denoted *Rvi15* (*Vr2*), has been found in the GMAL 2473 genotype (Patocchi *et al.,* 2004, 2009). We report a description of the resistance symptoms mediated by the *Rvi15* (*Vr2*) resistance gene to a local Swiss field-derived conidial suspension ("mixed" strains) of *V. inaequalis*. Furthermore, we compare the *Rvi15* (*Vr2*) resistance phenotype with those of *Rvi5* (*Vm*) present in cv. Murray.

MATERIALS AND METHODS

Two independent experiments were performed to assess the resistance phenotype of GMAL 2473 accession (PI 589835, from the Geneva National Germplasm Repository) carrying the *Rvi15* (*Vr2*) apple scab resistance. The first experiment was carried out in a greenhouse at the Swiss Federal Research Center of Wädenswil (ACW) inoculating five GMAL 2473 and five 'Gala' M9 grafted plants with a local field-derived conidial suspension ("mixed" strains) of *V. inaequalis* (10^5 conidia/ml) as described by Gianfranceschi *et al.* (1996). Symptoms were evaluated on young leaves 4, 7, 9, 11 and 15 dpi.

Inoculated leaf samples were cleared in 96% ethanol, 4% acetic acid at 75°C for 30 min, then stained at 75°C for 7 min in 70% ethanol, 29.9% water, 0.1% aniline blue. Next, leaf samples were rinsed three times in 70% ethanol at room temperature and left for at least 30 min in 50% ethanol and 50% lactic acid. Finally, leaves were mounted on a microscope slide with lactic acid. The samples were observed using a binocular loupe and a light microscope (Diaplan LEITX) both equipped with a LEICA DFC320 photocamera. Contrast and brightness of the pictures were adjusted in Photoshop.

The second experiment was performed in a greenhouse at the ETH of Zürich (Switzerland). Four plants of cv. Murray containing *Rvi5* (*Vm*), three of cv. Gala (susceptible) and three GMAL 2374 grafted on M9 rootstocks, were inoculated using the same procedure as the previous experiment. In addition to the samples prepared for light microscopy, young leaf sections 7 and 9 dpi, were examined for auto-fluorescence in the interference blue range (excitation filter 450-490 nm, dichroic mirror 505 nm, barrier filter 515 nm). Leaf samples were stained according to Bruzzese and Hasan (1983), and mounted in an arabic gum solution (Cunningham, 1972). The samples obtained from the second experiment were observed using a light microscope (Olympus BH-2) equipped with a UV lamp and digital images were taken with an Olympus E330 camera. Contrast and brightness of the pictures were adjusted in Photoshop.

RESULTS

Observations of the two infection studies gave similar results for resistance phenotype development on the GMAL 2473 young leaves inoculated with the conidial suspension, while there was no change in leaf appearance upon inoculation with water (water control). After four days of incubation, germinated conidia and appressoria were visible on the leaves of GMAL 2473 carrying *Rvi15* (*Vr2*) and on the susceptible Gala. However, up to this time no difference between behavior of the fungus and host reaction on susceptible and resistant tissue could be observed (Fig. 1, GM-4-Bl, GM-4-Lm, and Fig. 2, Ga-4-Lm). The first evidence of a resistance reaction in GMAL 2473 could be clearly observed seven dpi (Fig. 1, GM-7-Bl, GM-7-Lm). The response at the site of pathogen penetration was characterized by a necrotic zone composed of browned epidermal cells (5-20 cells in diameter). The shape and the number of cells affected were variable between the lesions. This was evident also on samples prepared for the auto-fluorescence study, where fluorescence was limited to a few (separated) epidermal cells (Fig. 1, GM-7-Bf/Bf'). Slightly fluorescent epidermal cells were also observed in some rare cases in the susceptible host at the penetration site of the pathogen or along the growing subcuticular mycelium (not shown). Subcuticular mycelium was observed in Gala but not in GMAL 2473 (Fig. 1, GM-7-Lm, and Fig. 2, Ga-7-Lm).

Nine dpi, the resistance phenotype of GMAL 2473 became macroscopically visible without the aid of an amplifying glass as a depression on the leaf surface characteristic of the pinpoint pit phenotype (not shown). Microscopic observations revealed that the necrotic zone around the pathogen penetration site reached a diameter ranging from 200 to 400 μm (approximately 20 to 40 browned epidermal cells across the diameter of the lesion) without further increase over time (Fig. 1, GM-9-Lm and GM-9-Bl). No additional formation of subcuticular mycelium was noted, while sporulation was

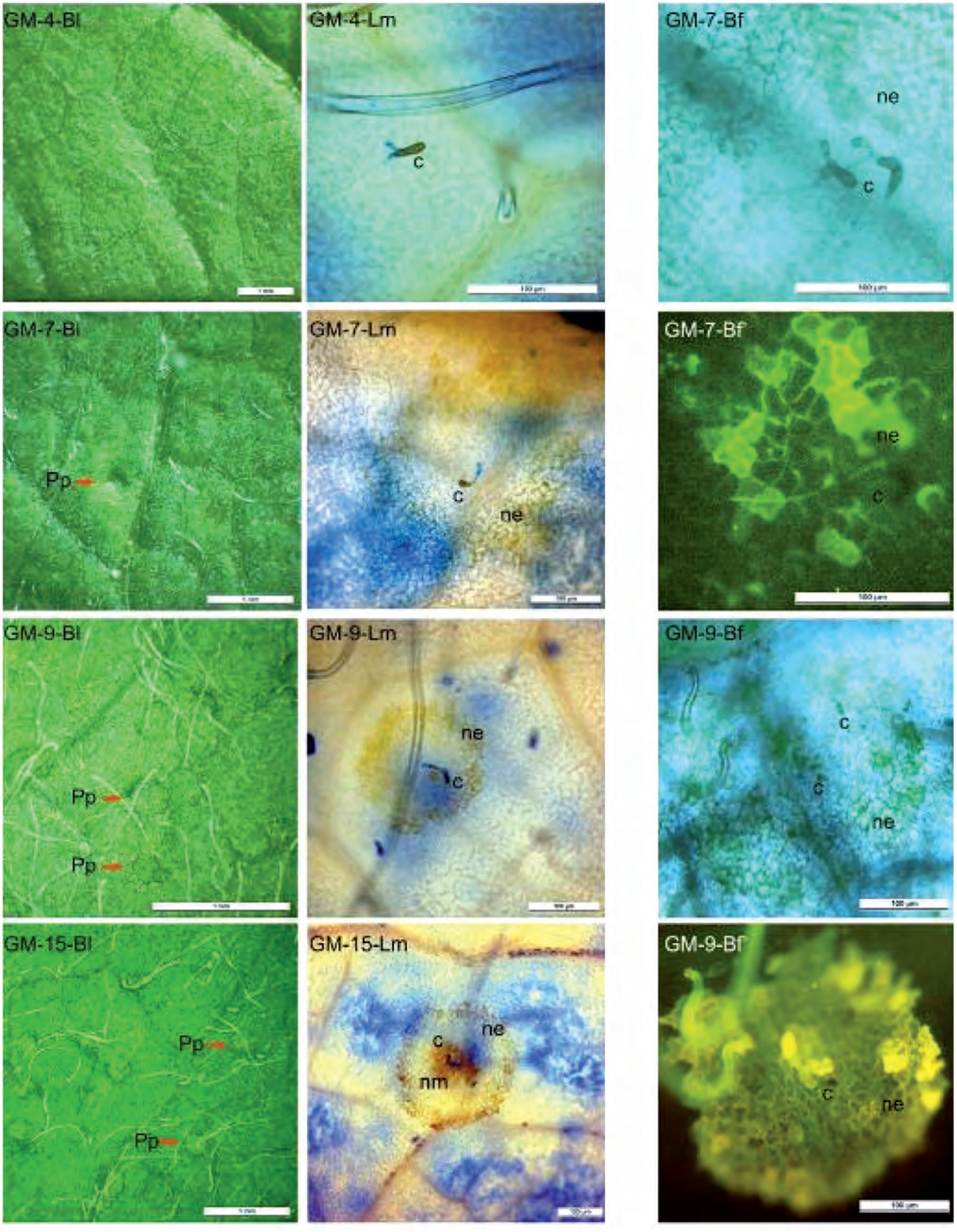

Fig. 1. Resistance reaction of GMAL 2473 leaves 4, 7, 9 and 15 days post inoculation with *V. inaequalis* conidia. Numbers indicate days post inoculation (dpi). GM = GMAL 2473; Bl = binocular loupe; Lm = light microscope; Bf (Bright field) and Bf' (interference blue auto-fluorescence) of the same lesion without moving the sample. Pp = pinpoint pit, c = conidium with appressorium, ne = necrotic epidermis cells, nm = necrotic mesophyll cells. Note: all the epidermal cells of the lesion at 9 and 15 dpi are brown. The brown ring is an artifact due to the concave form of the pinpoint pits.

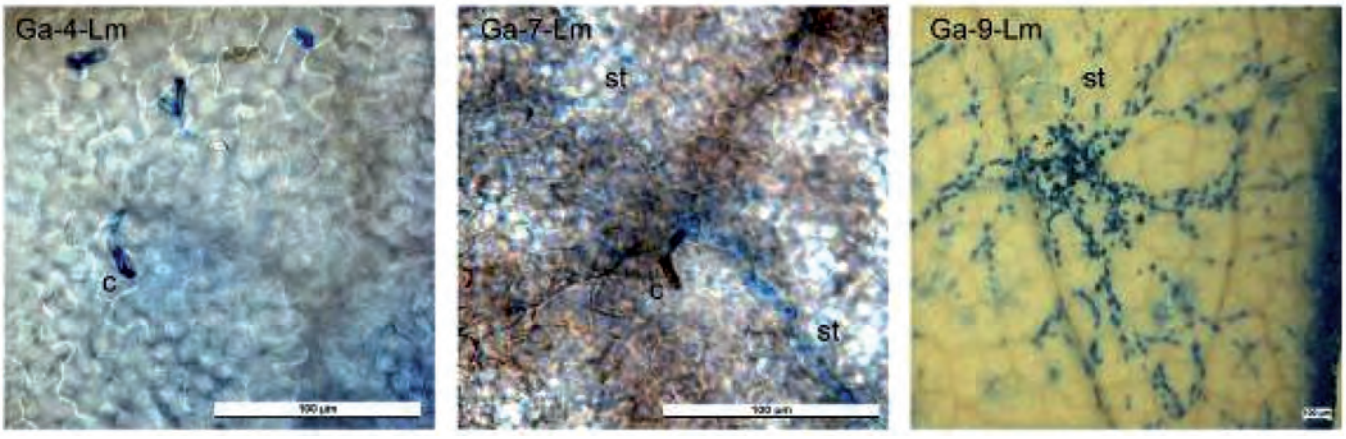

Fig. 2. Light microscope (Lm) observations of lesions on cv. Gala 4, 7 and 9 dpi with *V. inaequalis* conidia. Ga = Gala, c= conidium, st=stroma.

observed on the susceptible host (Fig. 2, Ga-9-Lm). Samples prepared for the auto-fluorescence study revealed a delimitation of the fluorescence to the browned epidermal cells of GMAL 2473 and fluorescence appeared mainly in the cell walls (Fig. 1, GM-9-Bf/Bf'). After 11 days of incubation, some pinpoint pits of GMAL 2473 showed browning of mesophyll cells under the pathogen penetration site (not shown). General browning of mesophyll cells below the pathogen penetration point was always observed 15 dpi (Fig. 1, GM-15-Lm). Browned mesophyll cells formed a zone of 50-150 µm in size (corresponding to a diameter of 5-15 epidermal cells) at the center of the browned epidermal cell zone. The reaction resulted in a complete block of fungal growth. Formation of subcuticular mycelium and sporulation on GMAL 2473 was never observed.

The Murray plants showed macro- and microscopically observable reactions 4 dpi, characterized by browning of epidermal and mesophyll cells below and around the pathogen appressorium (Fig. 3, Mu-4-Bl and Mu-4-Lm). Seven dpi pinpoint pits presented a round to oval zone involving browned epidermal cells with a darker zone of browned mesophyll cells at the centre (Fig. 3, Mu-7-Bl and Mu-7-Lm).

Size and number of cells involved in the resistance reaction were variable. The number of epidermal cells ranged from 15 to 40 (approximately 150-400 µm), while the zone of browned mesophyll cells had a diameter between 100 and 200 µm (corresponding to 10-20 epidermal cells in diameter). Nine dpi, pinpoint pits were more pronounced than 7 dpi (Fig. 3, Mu-9-Bl and Mu-9-Lm). The number of mesophyll cells involved in the resistance reaction seems higher than that observed at 7 dpi; however, variability between the different lesions on the same leaf was observed. Auto-fluorescence was delimited to the browned epidermal and mesophyll cells and was brightest in the mesophyll cells (Fig. 3, Mu-7-Bf/Bf' and Mu-9-Bf/Bf'). Formation of subcuticular mycelium and sporulation on Murray was never observed.

DISCUSSION

The *Rvi15* (*Vr2*) apple scab resistance found in GMAL 2473 leads to the formation of pinpoint pits on the upper surface of young infected leaves. These pits appeared gradually after inoculation with a local field-derived conidial suspension ("mixed" strains) of *V. inaequalis* under optimal conditions. A mixed conidial suspension was preferred to a monoconidial inoculum to reduce the risk of describing the phenotype of other *R* genes than *Rvi15* (i.e. ephemeral *R* genes). Nevertheless, the use of a mixed inoculum could be the cause of the observed variability of the pinpoint pit size in the resistant hosts (due to different aggressiveness of the different scab genotypes). However, we tend to exclude this hypothesis as difference in sizes of the pinpoint pits was observed also after inoculation with monoconidial suspension by Win *et al.* (2003).

A reaction on young leaves could be observed microscopically 7 dpi and was characterized by browning of epidermal cells below and around the penetration site of the pathogen. The zone of browned cells was characterized by auto-fluorescence after excitation with UV light. Two days later, the resistance phenotype became macroscopically visible as a depression on the leaf surface characteristic of pinpoint pits. The involvement of mesophyll cells (browning) in the *Rvi15* (*Vr2*) resistance response could be observed in some lesions at 11 dpi and became evident in all lesions 15 dpi. Browning of epidermal and mesophyll cells surrounding the infection site is associated with the collapse and death of epidermal and mesophyll tissue characteristic of an HR (Chevalier and Lespinasse, 1994).

Auto-fluorescence under ultraviolet light has been observed in many other pathosystems such as the cotton/*Xanthomonas campestris* pv. *malvacearum* or lettuce/*Bremia lactucae* interactions (Essemberg *et al.*, 1992; Bennett *et al.*, 2000) and is thought to arise from the production of a wide range of compounds possessing

a conjugation system and rigid structure, such as aromatic amino acids, flavonoids and phenolic acids (Davidson, 1996). The auto-fluorescent materials at the site of the resistance reaction have not been identified, although a strong correlation between deposition of auto-fluorescent materials and production of phytoalexins in cotton leaves (Essemberg *et al*., 1992) and phenolic compounds in lettuce (Bennett *et al*., 2000) has been demonstrated.

Classical examples of apple scab resistances leading to an HR and to the pinpoint pit phenotype characterized by browning and auto-fluorescence of epidermal

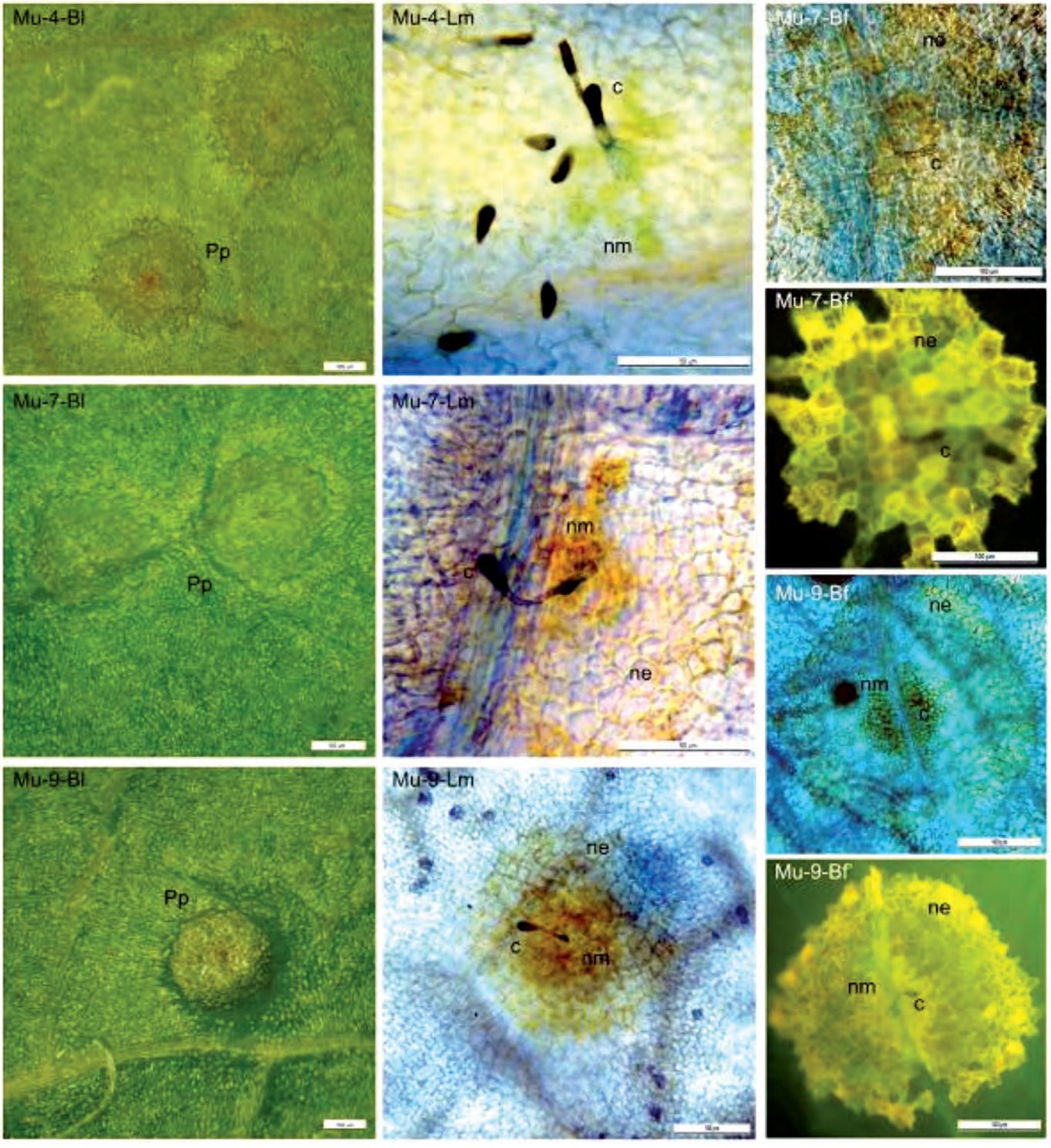

Fig. 3. Resistance reaction of CV. Murray leaves 4, 7 and 9 dpi with *V. inaequalis* conidia. Numbers indicate dpi. Mu = 'Murray'; Bl = binocular loupe; Lm = light microscope; Bf (Bright field) and Bf' (interference blue auto-fluorescence) of the same lesion without moving the sample. Pp = pinpoint pit, c = conidium with appressorium, ne = necrotic epidermis cells, nm = necrotic mesophyll cells.

and mesophyll cells are *Rvi5* (*Vm*) and *Rvi4* (*Vh4*) resistances, which are found in *M. micromalus* 245-38 and TSR33T239 (host 4), respectively (Hernandez Castillo, 1990; Bus *et al*., 2005a). The *Rvi5* (*Vm*) resistance was previously described micro- and macroscopically by Chevalier and Lespinasse (1994) observing different apple selections carrying *Rvi5* (*Vm*) (progeny plants of the cross cv. Golden Delicious x 9AR2T196) and by Win *et al*. (2003) on a progeny of the cross cv. McIntosh x ('Wolf River' x *M. micromalus* 245-38). The *Rvi4* (*Vh4*) resistance was described following inoculation of young leaves of TSR33T239 (host 4) with a conidial suspension of *V. inaequalis* race 4 (Bus, 2006). Both *Rvi5* (*Vm*) and *Rvi4* (*Vh4*) were found to induce a fast HR leading to macroscopic symptoms 2-3 dpi and to a restriction of the fungal growth at the site of pathogen penetration. These two resistances are characterized by auto-fluorescence after excitation with UV light and formation of a necrotic zone (200-500 µm) at the site of pathogen penetration with a darker zone in the centre, composed of necrotic mesophyll cells (150-50 µm). Similar results were observed in our *Rvi5* (*Vm*) phenotype analysis inoculating young leaves of cv. Murray with a local field-derived conidial suspension.

The descriptions of the *Rvi5* (*Vm*) and *Rvi4* (*Vh4*) resistance phenotypes are very similar to those reported in this study for *Rvi15* (*Vr2*) 15 dpi However, *Rvi15* (*Vr2*) differs clearly in the amount of time needed for the resistance phenotype to become macroscopically visible and completely develop compared to *Rvi5* (*Vm*) and *Rvi4* (*Vh4*).

When the timing of the development of the resistance response is considered, *Rvi15* (*Vr2*) resistance seems more similar to the *Rvi7* (*Vfh*) resistance identified in *M. floribunda* 821 than to *Rvi5* (*Vm*) and *Rvi4* (*Vh4*). *Rvi7* (*Vfh*) was distinguished from the *Rvi6* (*Vf*) resistance (also present in *M. floribunda* 821) by segregation analysis of the cross cv. Golden Delicious x *M. floribunda* 821 and by the different phenotypes that the two resistances confer [*Rvi7* (*Vfh*), pinpoint pit; *Vf* (*Rvi6*), chlorosis 7-10 dpi] (Benaouf and Parisi, 2000). The pinpoint pit phenotype on *Malus floribunda* 821 appeared after a minimum of 6 dpi (Shay and Hough, 1952).

Chevalier *et al*. (1991) described macro- and microscopically six classes of resistance symptoms on plants obtained from a cross pollination between a susceptible cultivar and a cultivar or a clone carrying the *Rvi6* (*Vf*) gene. Among the six classes, they described the pinpoint pit phenotype (as class 1), which was never observed on other cultivars carrying the *Rvi6* (*Vf*) gene (Gessler, 1986; Gessler and Stumm, 1984; Valsangiacomo and Gessler, 1988). Therefore, it is possible that Chevalier *et al.* (1991) described in class 1 the resistance mediated by the *Rvi7* (*Vfh*) gene, which would lead to the expression of the resistance phenotype before *Rvi6* (*Vf*). Macroscopically, class 1 resistance reaction was described as appearing 4 to 6 dpi in the form of small pits whose size did not enlarge with time. Microscopically, the symptoms of *Rvi7* (*Vfh*) were described by Chevalier *et al.* (1991) as a typical HR where epidermal cells collapsed (100-500 µm) and the cytoplasm became necrotic. At the centre of the pit there was a conidium with a very restricted subcuticular stroma. This description is clearly similar in both timing and phenotype with the resistance of *Rvi15* (*Vr2*). However, Chevalier *et al.* (1991) reported that mesophyll cells did not undergo any important modification, but they did not specify the day at which they observed class 1 symptoms microscopically. Therefore, it could well be that these authors observed the symptoms before 11 dpi, without noticing the changes in mesophyll cells that we observed only 11-15 dpi.

MacHardy (1996) suggested that HR resulting in pinpoint pit phenotypes occurring after a minimum of 4-6 days incubation should be considered a different type of 'resistance system' compared to those with a faster response (2-3 dpi). The description of the two groups of resistances reflects the *Rvi5* (*Vm*)/ *Rvi4* (*Vh4*) and *Rvi7* (*Vfh*)/ *Rvi15* (*Vr2*) groups. The fact that the end-phenotypes of both resistances are highly similar at the macroscopic and microscopic level, suggests that they might involve the same defense process.

The resistance reaction can be considered as a three-step mechanism: pathogen recognition, signaling cascades and defense process. The defense process, which includes the production of several antibiotic radicals and compounds (such as reactive oxygen species, phenol oxidation products and phytoalexins), seems to be common to many defense reactions not necessarily leading to HR (Király *et al*., 2007). Therefore, any difference between the defense signaling cascades induced by the different *R* genes could generate a delay in expression of the resistance reaction. The delay in the resistance expression could also be ascribed to the different elicitors recognized by the *R* genes. Assuming that the Avr15 (AvrVr2) elicitor is generated at a later time-point during the infection process than Avr5 (AvrVm), a delayed resistance response would simply arise from the recognition of the elicitor released later in the infection process.

Win *et al*. (2003), observing the reaction of mesophyll cells at the sites of fungal penetration without direct interaction with the fungus, suggested the presence of a signalling between epidermal and mesophyll layers mediating the reaction in the mesophyll cells. In the case of *Rvi15* (*Vr2*), mesophyll cells seem to be involved in a later phase of the resistance reaction, since they show browning at least four days later than epidermal cells and no fluorescence of mesophyll cells could be observed at 7 and 9 dpi. A different pathway or differential regulation of the same signalling pathway or the absence of a signalling pathway (in this case the response in distant cells will be mediated by the diffusion

of an elicitor) between the tissues might regulate the involvement of the mesophyll cells in the *Rvi15* (*Vr2*) resistance reaction causing their death in a second phase of the reaction.

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