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RESEARCH PAPER

Revealing the importance of meristems and roots for the development of hypersensitive responses and full foliar resistance to *Phytophthora infestans* in the resistant potato cultivar Sarpo Mira

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Abstract

The defence responses of potato against *Phytophthora infestans* were studied using the highly resistant Sarpo Mira cultivar. The effects of plant integrity, meristems, and roots on the hypersensitive response (HR), plant resistance, and the regulation of *PR* genes were analysed. Sarpo Mira shoots and roots grafted with the susceptible Bintje cultivar as well as non-grafted different parts of Sarpo Mira plants were inoculated with *P. infestans.* The progress of the infection and the number of HR lesions were monitored, and the regulation of *PR* genes was compared in detached and attached leaves. Additionally, the antimicrobial activity of plant extracts was assessed. The presented data show that roots are needed to achieve full pathogen resistance, that the removal of meristems in detached leaves inhibits the formation of HR lesions, that *PR* genes are differentially regulated in detached leaves compared with leaves of whole plants, and that antimicrobial compounds accumulate in leaves and roots of Sarpo Mira plants challenged with *P. infestans*. While meristems are necessary for the formation of HR lesions, the roots of Sarpo Mira plants participate in the production of defence-associated compounds that increase systemic resistance. Based on the literature and on the presented results, a model is proposed for mechanisms involved in Sarpo Mira resistance that may apply to other resistant potato cultivars.

Key words: Hypersensitive response, late blight, meristem, *Phytophthora infestans*, plant signalling, potato, resistance, root, Sarpo Mira, *Solanum tuberosum*

Introduction

Potato (*Solanum tuberosum* L.) is the world's most important non-cereal food crop. Its high yield potential, high nutritional value, and its potential for industrial processing (e.g. bioethanol preparation) explain the ongoing global increase in potato

Abbreviations: AM, axillary meristem; *Avr*, avirulence factors; Cht, chitinase; DAB, 3,3'-diaminobenzidine; dai, days after inoculation; *Ef-1α*, elongation factor 1α (*Solanum tuberosum*); G418, geneticin; hai, hours after inoculation; HR, hypersensitive response; IAA, indole acetic acid; JA, jasmonic acid; MS, Murashige and Skoog medium; MS0, MS without added sucrose; QTL, quantitative trait locus; *Pitef*, *P. infestans Tef1* (translation elongation factor 1α); PR, pathogenesis related; R, resistance; RT-qPCR, quantitative RT-PCR; SA, salicylic acid; SAM, shoot apical meristem; SAR, systemic acquired resistance.

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production, which in 2005 exceeded 300 Mt (Haverkort *et al*., 2009).

Potato production is drastically reduced every year due to the late blight disease caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary. Late blight management strategies rely on frequent fungicide applications (Schepers, 2001; Haverkort *et al*., 2009) that both threaten food security and harm the environment.

Plant breeders have been trying to produce late blight-resistant potato cultivars for decades. The main breeding strategy used during the first half of the 20th century was based on the utilization of major dominant *R* genes that were discovered in the Mexican wild species *Solanum demissum* (Müller and Black, 1952; Umaerus and Umaerus, 1994). In many cases, a single *R* gene can provide complete resistance. Among 11 *R* genes derived from *S. demissum*, five of them (*R1*, *R2*, *R3*, *R4*, and *R10*) have been used for introgression in European breeding programmes (Vleeshouwers *et al*., 2011). The exploitation of new cultivars containing these *R* genes was initially successful; however, they have been overcome by new virulent races of the pathogen (Müller and Black, 1952; Wastie, 1991; Fry and Goodwin, 1997; McDonald and Linde, 2002). Novel *R* genes from diverse wild *Solanum* species have recently been discovered and have been widely studied (El-Kharbotly *et al*., 1996; Ruiz de Galarreta *et al*., 1998; Park *et al*., 2005; Smilde *et al*., 2005; Golas *et al*., 2010).

Phytophthora infestans has a remarkable capacity to adapt rapidly to resistant plants (McDonald and Linde, 2002; Fry, 2008; Haas *et al*., 2009). Disease effector genes and other virulence factors that seem to promote evolutionary plasticity and enhance genetic variation are localized in repeat-rich and gene-sparse regions of the genome of *P. infestans* (Raffaele *et al*., 2010). Furthermore, the existence of two mating types allows sexual reproduction that further contributes to the pathogen's adaptation to fungicides and host resistance (Turkensteen *et al*., 2000; Smart and Fry, 2001). At the early stages of infection, *P. infestans* penetrates the plant and translocates effectors into the host cells (Birch *et al*., 2006; Whisson *et al*., 2007). Some effectors can act as avirulence (*Avr*) factors and activate corresponding *R* genes as described in the gene-for-gene model (Flor, 1971), as well as in more recent models such as the Guard Hypothesis and the 'bait and switch' model (Dangl and Jones, 2001; Collier and Moffett, 2009). Upon recognition of the effector by the R protein, effector-triggered immunity is activated, often resulting in the hypersensitive response (HR) (Jones and Dangl, 2006). HR is frequently followed by systemic acquired resistance (SAR), although HR is not required for the initiation of SAR (Cameron *et al*., 1994; Mishina and Zeier, 2007). SAR, also called horizontal resistance, is an organism-wide state of enhanced defence characterized by an increased expression of a large number of pathogenesis-related (*PR*) genes both at the site of infection and systemically. SAR is race non-specific, and provides long-lasting systemic immunity to secondary infection against a broad spectrum of pathogens (Grant and Lamb, 2006). Despite many studies concerning the signal transduction events that lead to the establishment of HR and SAR, the signalling molecules and pathways involved are still not completely understood.

It has been suggested that the best strategy to improve resistance is to introgress different combinations of *R* genes and high levels of partial resistance, also known as field resistance, into each plant variety (Stewart *et al*., 2003). It has been widely implied that partial resistance is different from the resistance caused by the major *R* genes, but recent studies indicate that *R* genes do confer partial resistance against the late blight pathogen (Vleeshouwers *et al*., 2011). These *R* genes often segregate as quantitative trait loci (QTLs) in genetic mapping populations, and the molecular basis of partial *R* gene-mediated resistance is not fully understood (Vleeshouwers *et al*., 2011).

Late blight resistance could be improved by introgressing different combinations of *R* genes into each plant variety (Haverkort *et al*., 2009), since the pathogen would need to accumulate mutations in multiple *Avr* genes in order to overcome a combination of *R* genes. Importantly, *R* genes that have been only partially defeated remain useful for deployment in agriculture, especially when stacked with other *R* genes (Vleeshouwers *et al*., 2011).

Sarpo Mira is a potato cultivar selected at the breeding station SARPO KFT in Hungary for its high level of resistance to *P. infestans* and to the potato virus Y. The molecular mechanisms responsible for the late blight resistance of Sarpo Mira were unknown until recently and are still not fully understood. Recently, it was shown that at least five *R* genes are stacked in Sarpo Mira (Rietman, 2011) and that several *P. infestans*-responsive genes are differentially regulated in Sarpo Mira plants compared with the susceptible Bintje cultivar (Orłowska *et al*., 2012).

The progress of the late blight infection in detached leaves differed from that observed in the leaves of whole Sarpo Mira plants (Rietman, 2011; Orłowska *et al*., 2012). The present study elucidates the impact of plant integrity (whole intact plants compared with plants where tissues have been removed and plant parts) on the responses to the late blight pathogen. The roles of roots, shoots, and meristems in both the HR and the progress of infection were studied. *PR* gene responses to the infection in detached leaves were compared with those in attached leaves. Finally, a model for pathogen resistance based on the presented observations and previously published studies is proposed.

Materials and methods

Assessing the field resistance of Sarpo Mira and Bintje cultivars

Field late blight resistance of the potato cultivars Sarpo Mira (high resistance) and Bintje (low resistance) was assessed at the Danish Potato Breeding Foundation (LKF-Vandel, Denmark) during the past 7 years (2004–2008, 2010–2011). To identify the virulence of the *P. infestans* isolates used in the field trials, 11 Scottish potato differentials (Black differentials; Malcolmson and Black, 1966) carrying single *R* genes (*R1–R11*) were also scored annually for their late blight resistance. Both for Sarpo Mira and Bintje plants, as well as for the differentials, three plots of four plants were used for each cultivar.

The trial was inoculated on around 10 July by spraying infector rows of the varieties Bintje and Oleva with a solution containing a mix of *P. infestans* isolates collected in the previous season at a concentration of $\sim 5 \times 10^4$ sporangia ml^{-1}. The trial plots were irrigated with sprinklers to keep the humidity high and the sporangia from the infector rows were allowed to spread naturally to the test plots. The infection symptoms were recorded twice a week until the control variety Robijn exceeded 50% infection. Foliage late blight assessment was based on the percentage of leaf tissue diseased and was scored visually. The area under the disease progress curve (AUDPC) estimation, calculated by the trapezoidal method, was used to quantify disease intensity over

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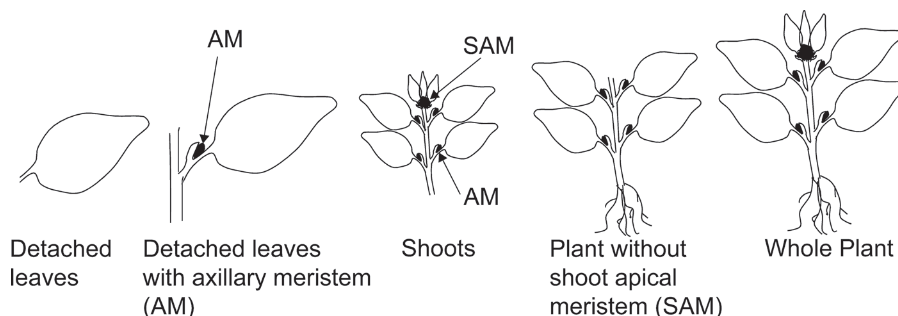

Fig. 1. Schematic overview of the potato plant physiological parts used for the experiments. The name used for each plant part is indicated. AM: axillary meristem, SAM: shoot apical meristem.

time (Madden *et al*., 2007). The AUDPC was calculated as a histogram: time interval since last estimation (3 d or 4 d) × percentage of diseased leaf area.

A score of 1–9, with 1 as the most susceptible and 9 as the most resistant, was calculated from the AUDPC value of each variety compared with the trial mean. The *Phytophthora* trials consisted of 800–1000 plots each year.

Growth and infection of potato plants under laboratory conditions

Phytophthora infestans strains were isolated from infected potato tubers provided by LKF-Vandel. To obtain sterile isolates, *P. infestans* mycelium from infected tubers was transferred to rye medium (Caten and Jinks, 1968) containing 50 μg ml^{-1} ampicillin, 10 μg ml^{-1} rifampicin, and 66 μg ml^{-1} carbendazim. The aggressiveness of the isolates was evaluated on *in vitro* grown potato plants with different levels of late blight resistance including Bintje, Sarpo Mira, and Danva (medium resistance) cultivars. None of the *P. infestans* isolates infected the Sarpo Mira cultivar but they were able to induce HR lesions. Several isolates had similar rates of disease progression, and an isolate from the potato cultivar Danva, *P. infestans* isolate API (A1 mating type, race 1.3.4.7.10.11) (Orłowska *et al.*, 2012), was used for this study. The isolate was grown under sterile conditions at 18 °C on rye agar supplemented with 2% sucrose as described by Caten and Jinks (1968).

Race specificity of the isolate used for the infection experiments was determined on the standard Black differentials (Malcolmson and Black, 1966). Mating type was determined as described by Fabritius *et al.* (2002) by co-cultivation with known isolates of mating type A1 and A2, kindly provided by Dr Sabine Ravnskov (Research Centre Flakkebjerg, Aarhus University, Denmark).

The potato cultivars Sarpo Mira (highly resistant to *P. infestans*) and Bintje (susceptible to *P. infestans*) were used in the experiments. Plants obtained from internode cuttings were grown in Murashige and Skoog (MS)–agar medium [Duchefa Biochemie BV, Haarlem, The Netherlands; 2% (w/v) sucrose, 8% Bacto™ agar, pH 5.7] in mini-greenhouses under sterile conditions, with a 16 h light period and temperatures of 20 °C during the day and 17 °C at night.

For the infection assays, whole *in vitro* grown potato plants and the fully expanded leaves with petiole but without axillary meristems from the third to sixth position (counting from the top) were used as detached leaves. Plants and plant parts were inoculated with 0.05–0.15 ml of a freshly isolated suspension of sporangia and zoospores with ~2.5×10^5 sporangia ml^{-1}, and mock-inoculated plants (control plants) were sprayed with 0.15 ml of cold water (4 °C). To avoid contamination of the media with the pathogen, the sporangia mix was sprayed on the plant parts/leaves in a different Petri dish and they were then transferred to Petri dishes or mini-greenhouses with MS0–agar media (Duchefa Biochemie BV; 8% Bacto™ agar, pH 5.7, no added sucrose) or with water–agar (water with 15% agar). The petioles of the leaves were inserted into the media so that the leaves were not touching the media. Leaves that accidentally fell on the media were excluded from the analysis. Similar results were obtained when using MS0–agar or water–agar media. These set-ups were used to support the petioles and to keep them submerged in order to avoid desiccation of the leaves. Foam was also tested as an alternative to water–agar and MS0–agar; however, it was not possible to place the leaves repeatedly on the foam without causing wounds that could affect the responses. The experiment was independently repeated five times and 10–40 replicates of water- and *P. infestans*-sprayed plants and detached leaves were used in each experiment.

In experiments aimed at revealing the role of different plant tissues in the resistance to late blight, the plants were divided into different physiological parts: detached leaves, detached leaves that include the axillary meristem (detached leaves with AM), de-rooted plants (shoots), plants where the shoot apical meristem was removed (plants without SAM), and whole intact plants (Fig. 1). The detached leaves with the petiole and without stem and without axillary meristems were prepared and maintained as described above. Leaves from the same developmental stage were used for detached leaves with AM, but they included the petiole and the axillary meristem and a short fragment of the stem. The shoots used were 3–4 cm long and were taken from whole plants from which the roots were removed immediately before the initiation of the experiment. For the plants without apical meristem, the apical meristem was removed with a sterile scalpel just before starting the experiment. In addition, whole Sarpo Mira plants grown in mini-greenhouses were used for the experiments.

Plants and plant parts were inoculated with 0.05–0.15 ml of a freshly isolated suspension of sporangia and zoospores with ~2.5×10^5 sporangia ml^{-1}, and mock-inoculated plants were sprayed with cold water (4 °C) in the same way as described above. The experiment was independently repeated four times and 20–50 replicates of water- and *P. infestans*-sprayed plants/plant parts were used in each experiment.

Grafting

Sarpo Mira and Bintje plants were grown on sterile MS–agar medium [Duchefa Biochemie BV; 2% (w/v) sucrose, 8% Bacto™ agar, pH 5.7] for 5 weeks prior to grafting. After incubation in the dark for 24 h, plant segments were prepared by obliquely cutting the stem with a razor blade ~2–4 cm above the roots and below the lowest leaf. Potato plants were grafted in four different combinations: B/S, Bintje's shoot (scion) and Sarpo Mira's roots (rootstock); S/B, Sarpo Mira scion and Bintje rootstock; and B/B and S/S, scion and stock from the same cultivars as controls. The scion to be grafted was then placed on top of the rootstock and both parts were connected by a small (0.5–1 cm in length) polyvinyl tube that prevented the plant segments from separating. Grafted plants were kept in sealed Petri dishes in the dark inside a growth chamber with temperatures of 20 °C during the day and 17 °C at night for 5 d. Thereafter they were exposed to a 16 h light period. Roots growing from the scions were removed before reaching the medium. After 6 weeks, the plants were used for infection assays as previously described (Orłowska *et al.*, 2012). Twenty replicates of mock- and *P. infestans*-inoculated plants of

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each grafting combination were used in each experiment. The experiment was independently repeated three times.

Characterization of the infection in plants grown under laboratory conditions

The progress of the infection was observed daily with stereo and light microscopes within 10 d after *P. infestans* inoculation. The following types of leaf reaction were recognized: (i) successful infection with *P. infestans* producing water-soak lesions and visible mycelium carrying sporangiophores; (ii) infection with *P. infestans* but no visible sporangiophores; (iii) HR lesions; and (iiii) no visible symptoms.

Disease severity was assessed by evaluating the percentage of infected leaves. Total leaf area and HR lesion area were analysed using the Image Analysis Software for Plant Disease Quantification (The American Phytopathological Society).

HR-related H_2O_2 accumulation was visualized by 3,3'-diaminobenzidine (DAB) staining according to Thordal-Christensen *et al*. (1997). In brief, leaves were incubated in 1 mg ml^{-1} DAB-HCl (pH 3.8) for 8 h and then cleared in boiling 96% ethanol for 10 min. Samples were stored in 96% ethanol before examination. Reddish-brown coloration indicated the presence of H_2O_2 (HR lesions).

Infection development at early time points [1, 4, 17, and 23 hours after inoculation (hai)] in both detached leaves and leaves of whole plants was analysed using quantitative RT-PCR (RT-qPCR) of the *P. infestans Tef1* (*Pitef*) gene (described in the next section). The progress of the infection in grafted plants was quantified at later stages of the infection (starting at 44 hai) using macro- and microscopic observations and by counting the number of HR lesions, when these were present. It has been previously shown that the expression of the *Pitef* gene at early stages of the infection corresponded to infection symptoms at later stages of the infection (Orłowska *et al.*, 2012).

Quantitative gene expression analysis

RNA was isolated from infected and mock-inoculated detached leaves and leaves of whole plants at 1 and 17 hai, when *P. infestans* mycelium was not visible on the leaves or on the surrounding media. The RNA and cDNA preparation, as well as the conditions and primers for the RT-PCR have been previously described (Orłowska *et al.*, 2012). RT-qPCR was used to study the expression of the potato ascorbate oxidase, osmotin, peroxidase, actin, *ChtA*, patatin, *Ef-1α*, *PR-1*, and *ChtB* genes, and for the *Pitef1* gene.

The potato elongation factor 1α (*Ef-1α*) was used as a reference gene (Nicot *et al.*, 2005; Orłowska *et al.*, 2012). The relative expression values of each gene in *P. infestans*-inoculated plants compared with mock-inoculated plants was calculated by the following method: first the expression values of each gene of interest in each sample are normalized to the expression values of *Ef-1α* in the same sample. This was performed both for pathogen-inoculated and for mock-inoculated plants. Then the average value of the three replicates at each time point is calculated. Finally, the averaged normalized values for each time point in the pathogen-inoculated samples are related to the averaged normalized values in the mock-inoculated samples.

The level of *P. infestans* infection was also quantified by RT-qPCR in the same samples used for the gene expression studies (detached leaves and leaves from whole plants). The analysis was done at four time points (1, 4, 17, and 23 hai). The transcript levels of the *Pitef1* gene were calculated relative to the expression levels of the potato *Ef-1α* gene as previously described (Orłowska *et al.*, 2012).

Three independent biological replicates of each sample and three technical replicates of each biological replicate were used for each analysis. All data were analysed using the Light Cycler Relative Quantification Software (Roche, Basel, Switzerland).

Phytophthora infestans *hyphal extension inhibition assay*

The antimicrobial activity of plant extracts was assayed using the modified hyphal extension-inhibition assay described by Roberts and Selitrennikoff (1986). *Phytophthora infestans* was grown on rye agar supplemented with 2% sucrose (Caten and Jinks, 1968) for 5 d before the experiments. A round (6 mm in diameter) rye media plug containing *P. infestans* mycelium was placed onto the centre of rye agar plates supplemented with 2% sucrose. Plant extracts were added on top of sterile paper discs (4 mm in diameter) placed at a distance of 2 cm around a central plug carrying the *P. infestans* mycelium. Extracts from both roots and leaves of mock- and *P. infestans*-inoculated (5 weeks after infection) Sarpo Mira plants as well as from non-infected Bintje plants were tested. Plant extracts were obtained by grinding 200 mg of tissue in liquid nitrogen. A 1 ml aliquot of the recovered liquid was diluted with 1 ml of distilled water and centrifuged at 8000 *g* for 15 min at 4 °C. The supernatant was recovered and the total protein content was quantified using the Bradford method (Bradford, 1976). All extracts were diluted to 60 mg of protein ml^{-1} and 15 µl of extract were added on top of each paper disc. Water dilutions (1:3, 1:6, and 1:12) of the extracts were also tested in the same way. Distilled water (15 µl) was used as a negative control and 15 µl of 5 µg ml^{-1} geneticin (G418) was used as a positive control for hyphal growth inhibition. Petri dishes were incubated at 18 °C to allow hyphae to grow. A crescent-shaped area of growth inhibition was observed around the discs containing antimicrobial compounds.

To assess if the inhibitory activity was heat labile, the plant extracts were boiled for 5 min or 10 min and then used for the extension-inhibition assay as described above. The antimicrobial activity of the plant extracts was also assessed after freezing the extracts at –80 °C for 5 weeks. Six replicates of each treatment were made and the experiment was repeated three times with *de novo* infected plants.

The inhibitory effect of plant extracts on the germination of zoospores/sporangia and initial hyphal growth was also studied. Sporangia were isolated from 7-day-old cultures of *P. infestans* and diluted to 4.4×10^4 sporangia ml^{-1}. A 20 µl aliquot of the of sporangia suspension was mixed with 80 µl of plant extracts (20 mg protein ml^{-1}), water, or G418, and incubated for 2 h or 5 h with gentle shaking at 19 °C in the dark. Then, the mix was diluted with 2900 µl of liquid rye medium and kept in small Petri dishes in the dark. The material was examined under a microscope within 48 h.

Statistical analysis

Data were analysed with the non-parametric Kruskal–Wallis test. The post-hoc analysis was done with a Mann–Whitney U-test. A non-parametric test was used for the data showing the percentage of the infected leaves because the infection of the leaves was measured using an arbitrary scale and in that case non-parametric tests are recommended. Furthermore, some other data did not meet the normal distribution that is an assumption of analysis of variance (ANOVA). The threshold for significant results was $P < 0.05$. Significant results are indicated by different letters above the bars on the figures.

Results

Characterization of the Sarpo Mira resistance in the field

Sarpo Mira plants were fully resistant to the late blight infection in field studies performed in Denmark with local isolates. The resistance of Sarpo Mira plants was not broken in any of the studied years between 2004 and 2011 (Table 1). In comparison, Bintje plants were susceptible to *P. infestans* in all the trials. The resistance to *P. infestans* of six of the 11 Scottish cultivars carrying single *R* genes (*R1–R11*) was broken in all the years studied (Table 1). Plants carrying the *R2* and *R6* genes were susceptible in all but one of the tested years, while the resistance of plants carrying the *R5* gene was broken in three out of the 7 years. Plants carrying either the *R8* or *R9* genes showed the highest

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Table 1 Late blight infection scores performed in field study at LKF-Vandel, Denmark. Results from three plots of four plants for each cultivar in every year are presented. Foliage late blight assessment was based on the percentage of leaf tissue diseased and was scored visually until the control variety Robijn exceeded 50% infection. The area under the disease progress curve (AUDPC) estimation was used to quantify disease intensity in time. AUDPC was calculated as a histogram: time interval since last estimation (3 d or 4 d) × percentage of diseased leaf area. A score of 1–9 with 9 as the most resistant was calculated from the AUDPC value of each variety compared with the trial mean.

Cultivar	*R* gene	2004	2005	2006	2007	2008	2010	2011	Mean
Sarpo Mira	*R3a*, *R3b*, *R4*, *Rpi-Smira1*, *Rpi-Smira2*[a]	9	9	9	9	9	9	9	9.0
Bintje	No *R* genes	1	2	1	2	3	2	3	2.0
Craigs Snow White	*R1*	2	3	3	4	3	5		3.3
1512c(16)	*R2*	3	6	2	4	3	9	2	4.1
Pentland Ace	*R3*	1		1	2	1	1	3	1.5
1563c(14)	*R4*	1	3	3	4	4	7	2	3.4
3053-18	*R5*	2	5	9	8	5	8	9	6.6
XD2-21	*R6*	4	7	5	5	4	9		5.7
2182ef(7)	*R7*	1	2	1	3	2	1	2	1.7
2424a(5)	*R8*	9	9	9	9	9	9	9	9.0
2573(2)	R9	9	9	9	9	9		9	9.0
3681ad(1)	*R10*	5	4	5	6	5	7	7	5.6
5008ab(6)	*R11*	1	3	2	3	3	3	1	2.3

[a] Rietman (2011).

resistance rates (grade 9) in all years (Table 1).

Progress of the late blight infection in Sarpo Mira whole plants and plant parts

Sarpo Mira is a potato cultivar that has a high level of resistance to *P. infestans*. Preliminary assays showed that the progress of the late blight infection was different in whole plants compared with detached leaves. Therefore, the aim of this study was to reveal the role of different plant tissues in the resistance against the late blight pathogen and in the induction of the HR.

Detached leaves without meristems, detached leaves with the axillary meristem and a short fragment of the stalk (detached leaves with AM), de-rooted plants (shoots), plants where the shoot apical meristem was removed (plants without SAM), and whole intact plants were used for the infection assays (Fig. 1). The assays showed reduced resistance to *P. infestans* after the removal of the roots or meristems. The infection progressed faster in detached leaves than in any other tissue (Figs 2–4). At 3 days after inoculation (dai), *P. infestans* mycelia and necrotic lesions were observed in most of the detached leaves (80%) (Fig. 3). All these leaves were completely covered by mycelia at 6 dai (Fig. 2B, 3A). On detached leaves with AM, the infection severity seemed to be slightly lower at 3 dai, but the difference was not statistically significant (Fig. 3A). All the detached leaves became totally infected at 6 dai. When shoots were inoculated with *P. infestans*, a significantly lower infection rate was observed. An average of 50% of the leaves were infected both at 3 and 6 dai (Fig. 3A). Later, these plants rooted and produced new healthy leaves free of *P. infestans* infection symptoms.

Phytophthora infestans mycelia were not observed on the leaves of whole plants or of plants without SAM at 3 dai. However, at 6 dai, mycelia were present in ~10% of these leaves but no sporangiophores were visible (Fig. 3A). At 8 dai, some of the leaves of whole plants and leaves of plants without SAM fell from the plants but the stems and stalks were still green and looked healthy. At 10 dai, these plants were still healthy and growing even though the entire medium was completely covered by *P. infestans* mycelia (Fig. 2G). Plants without SAM and whole plants started re-growing and new tissues were darker green or red (Fig. 7A), and always free of visible mycelia (Fig. 2G).

Interestingly, no HR lesions were observed on detached leaves without meristems (Fig. 3B). The number of HR lesions and the timing of their appearance differed between the inoculated plant parts (Fig. 3B). At 44 hai, HR lesions (Fig. 2C–D) were observed on the leaves of whole plants. HR lesions appeared at 48 hai on plants without SAM, at 55 hai on shoots, and at 62 hai on detached leaves with AM. The percentage of leaves with HR lesions was evaluated at 65 hai (Fig. 3B). At this time, HR lesions covered 5–10% of the leaf surface. Accumulation of H_2O_2, a marker for HR, was confirmed in infected leaves using DAB staining (Fig. 2E). The highest number of leaves with HR was observed in whole plants (Fig. 3B). On average, 45% of the leaves had HR lesions, and each leaf had between 28 and 54 lesions. Among the leaves showing HR lesions, the lowest frequency of lesions was observed in detached leaves with AM (Fig. 3B). They had 0–5 lesions per leaf and only 3% of the infected leaves developed HR lesions. During the following days, some larger and less defined necrotic areas appeared on these leaves. There were no significant differences between the plants without SAM and shoots; ~30% of the leaves had HR lesions in both cases (Fig. 3B).

Quantification of P. infestans *gene activity and gene responses in detached leaves and in leaves of whole Sarpo Mira plants*

To quantify the infection progress in leaves, the expression of the *Pitef1* gene was analysed as previously described (Orłowska *et al.*, 2012). *Pitef1* gene expression was low in all the samples and did not reach 2-fold of relative transcript accumulation until 23 hai

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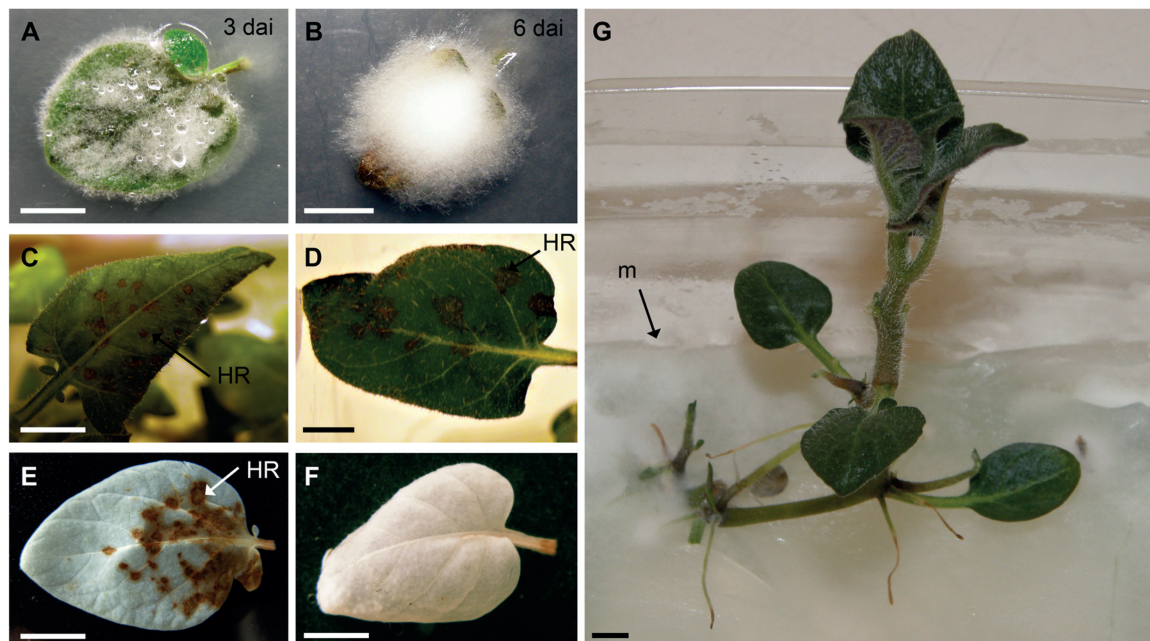

Fig. 2. Progress of infection in Sarpo Mira plant parts. (A and B) Detached leaves without axillary meristem (AM) at 3 (A) and 6 (B) days after inoculation (dai). (C and D) HR lesions in Sarpo Mira leaves of whole plants at 3 (C) and 6 dai (D). (E) DAB-stained leaf of an infected Sarpo Mira plant showing HR lesions. (F) Sarpo Mira leaf of a mock-inoculated plant stained with DAB. (G) Infected Sarpo Mira plant at 20 dai, with new shoots; *P. infestans* mycelia covered the growth medium (m). Scale bar=1 cm. The experiment was independently repeated four times; representative results from one experiment using 20–50 biological replicates are shown.

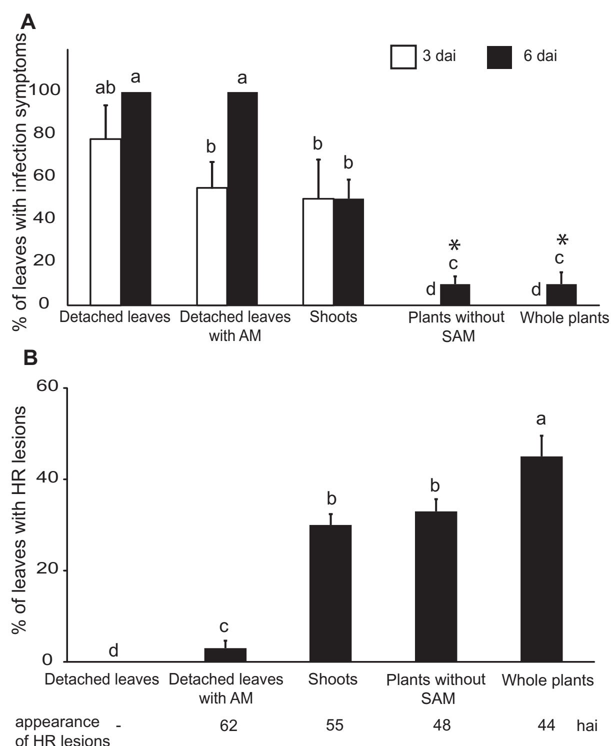

Fig. 3. Quantification of infection and the hypersensitive response (HR) in Sarpo Mira plant parts. (A) Percentage of leaves with infection symptoms at 3 and 6 days after inoculation (dai). (B) Percentage of leaves with visible HR lesions at 65 hours after inoculation (hai) with *P. infestans*. Appearance of HR lesions: the values below the *x*-axis show when (in hai) the HR lesions appeared. The experiment was repeated four times yielding similar results, and the data of one of the experiments is shown. The values are the mean ±SD of 20–50 biological replicates. Different letters indicate significant differences between plant parts and between days ($P < 0.05$; non-parametric Kruskal–Wallis test and Mann–Whitney U-test); *no sporangiophores observed.

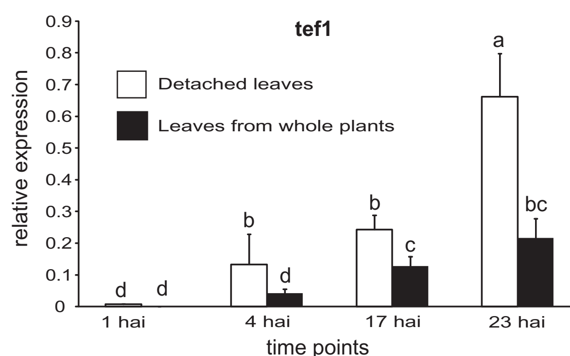

Fig. 4. Expression analysis of the *Phytophthora infestans Tef1* gene (*Pitef1*) in Sarpo Mira detached leaves and leaves of whole plants at 1, 4, 17, and 23 hours after inoculation (hai). The values are normalized to the potato *Ef-1α* gene. Hai are given on the *x*-axis and the levels of relative transcript accumulation on the *y*-axis (logarithmic scale); values are the means ±SD of three biological replicates. Different letters indicate significant differences between plant parts and between days ($P < 0.05$; non-parametric Kruskal–Wallis test and Mann–Whitney U-test).

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(Fig. 4). However, significantly higher gene activity was observed in detached leaves compared with that in leaves of whole plants at 4, 17, and 23 hai (Fig. 4). At these early time points no visible *P. infestans* mycelia were observed on the leaves or in the supporting media.

The differences in disease development observed between detached leaves and whole plants led to the investigation of whether these differences were linked to early gene responses to pathogen infection. For this analysis, genes that were induced early by the late blight infection in Sarpo Mira plants were selected (Orłowska *et al.*, 2012). The expression of *PR-1*, chitinase A (*ChtA*), chitinase B (*ChtB*), osmotin, peroxidase, patatin, ascorbate oxidase, and actin genes was studied at 1 and 17 hai. To analyse whether detaching leaves resulted in the induction of these genes, their expression was studied simultaneously in mock-inoculated detached leaves and in leaves from mock-inoculated whole plants. Of the selected genes, only the patatin-like gene showed differential expression in the mock-inoculated samples. This gene was induced 2.5- to 3-fold in detached leaves compared with leaves of whole plants. The levels of expression in detached leaves, relative to that of *Ef-1α*, were 0.24 and 0.28 at 1 and 17 hai, respectively. In comparison, the patatin gene expression levels in leaves of whole plants, relative to that of *Ef-1α*, were 0.08 and 0.11 at 1 and 17 hai, respectively.

Significant differences were observed in the relative expression levels of all the studied genes in *P. infestans*-inoculated samples when detached leaves were compared with leaves of whole plants (Fig. 5). Already at 1 hai, *ChtA*, *ChtB*, *PR-1*, and the patatin-like gene were expressed at higher levels in leaves of whole plants (Fig. 5). *ChtA* and ascorbate oxidase were more highly expressed in the leaves of whole plants than in detached leaves at 17 hai (Fig. 5). In contrast, the expression of patatin, *ChtB*, osmotin, and actin was higher in detached leaves than in whole plants at 17 hai (Fig. 5).

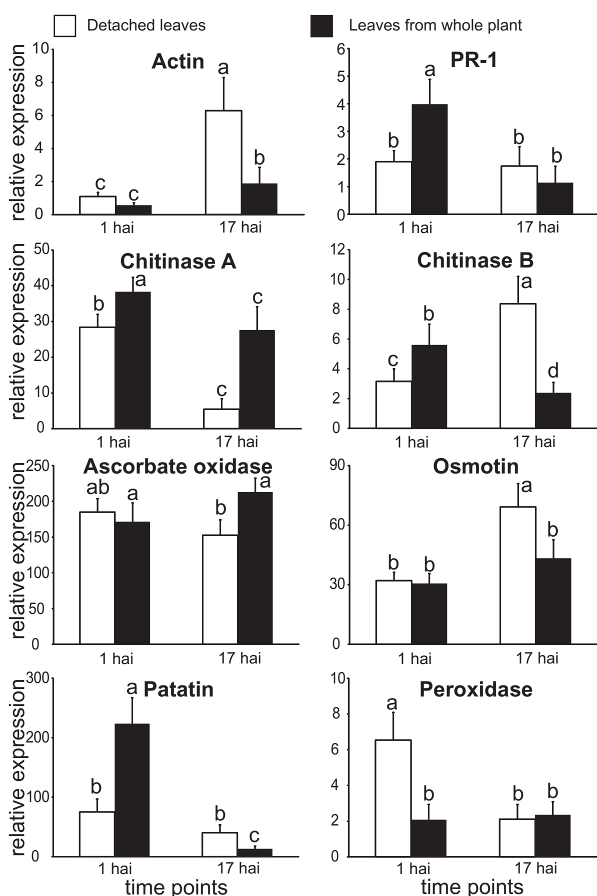

Fig. 5. Relative expression of pathogen-induced genes in Sarpo Mira detached leaves and in leaves of whole plants at 1 and 17 hours after inoculation (hai) with *Phytophthora infestans*. The expression of the genes was normalized to the expression of the *Solanum tuberosum Ef-1α* gene in each sample and to the expression in mock-inoculated samples from the same time point. The values are the mean ±SD of three biological replicates. Different letters indicate significant differences between plant parts and between days ($P < 0.05$; non-parametric Kruskal–Wallis test and Mann–Whitney U-test).

Symptoms of late blight infection in grafted plants

The differences in resistance observed between shoots and whole plants indicated that the roots might have an impact on disease resistance. To address whether the increase in resistance was a specific property caused by the Sarpo Mira roots, grafting experiments were performed in which roots from the susceptible Bintje cultivar were grafted on Sarpo Mira shoots (S/B) and shoots from Bintje plants on Sarpo Mira roots (B/S). A higher resistance to the pathogen infection was observed in B/S plants compared with Bintje/Bintje grafted plants (B/B) and Bintje non-grafted plants at early stages of infection. At 3 dai, ~40% of the leaves of B/S plants inoculated with *P. infestans*

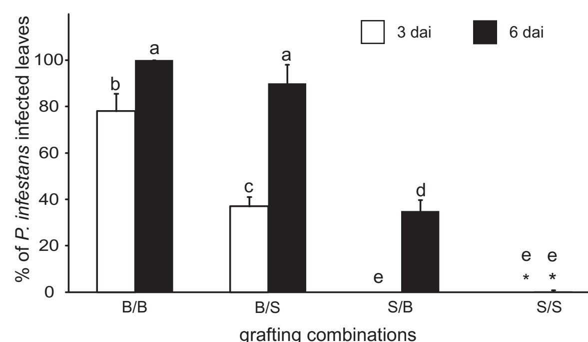

Fig. 6. Progress of the late blight infection in grafted Sarpo Mira and Bintje potato plants. Percentage of leaves with infection symptoms at 3 and 6 days after inoculation (dai) in grafted plants: B/B, shoot and root from susceptible Bintje cultivar; B/S, shoot from Bintje and roots from the Sarpo Mira cultivar; S/B, shoots from Sarpo Mira and roots from Bintje; S/S, shoot and root from Sarpo Mira. The experiment was repeated four times, yielding similar results. The values are the mean ±SD of 20 biological replicates from one experiment. Different letters indicate significant differences between grafted plants and between days ($P < 0.05$; non-parametric Kruskal–Wallis test and Mann–Whitney U-test); *no sporangiophores observed.

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showed infection symptoms typical for the susceptible Bintje cultivar and B/B plants (Fig. 6). At the same time point, 80% of the leaves of B/B plants showed infection symptoms (Fig. 6). These symptoms were characterized by expanded soaked lesions and *P. infestans* sporangiophores occurring on the leaves. These B/B plants died within 6 dai. At 6 dai, 25% of the B/S plants were still alive and 85% of their leaves were heavily infected and collapsed (Fig. 6). The upper parts of these plants were green and still growing. None of the B/S plants survived past 10 dai. These results indicated that the Sarpo Mira roots were able to provide a weak protection against *P. infestans* to the Bintje shoots, but finally this protection was overcome by the pathogen.

Control Sarpo Mira (S/S) grafted plants showed HR lesions typical of non-grafted Sarpo Mira plants. HR lesions were visible at 40 hai and the plants had a survival rate of 100% at 6 dai. At 3 dai, some leaves were shed, but the plants kept growing and produced new non-infected leaves. When Sarpo Mira shoots were grafted with Bintje roots (S/B), 70% of the plants survived the infection at 6 dai and they were still alive at 10 dai. The infection symptoms were similar to those of Sarpo Mira plants, but HR lesions appeared ~12 h later. *Phytophthora infestans* mycelium was observed in ~30% of the leaves at 6 dai (Fig. 6) and very rarely sporangiophores were present. These results indicate that the shoots of Sarpo Mira are determinant for the resistance and that the Sarpo Mira roots, to a greater degree than the Bintje roots, contribute to the *P. infestans* resistance.

Antimicrobial activity of plant extracts

Sarpo Mira leaves changed their colour after the late blight infection (Fig. 5A). No pathogen growth was observed on the plants even when a high pathogen pressure was present (Fig. 2G). These observations suggested that plants accumulated compounds that could have antimicrobial activity. To test this hypothesis, the antimicrobial activity of plant extracts was assayed using the modified hyphal extension-inhibition assay. Control discs containing water or extracts from the susceptible cultivar Bintje were quickly covered by *P. infestans* mycelia,

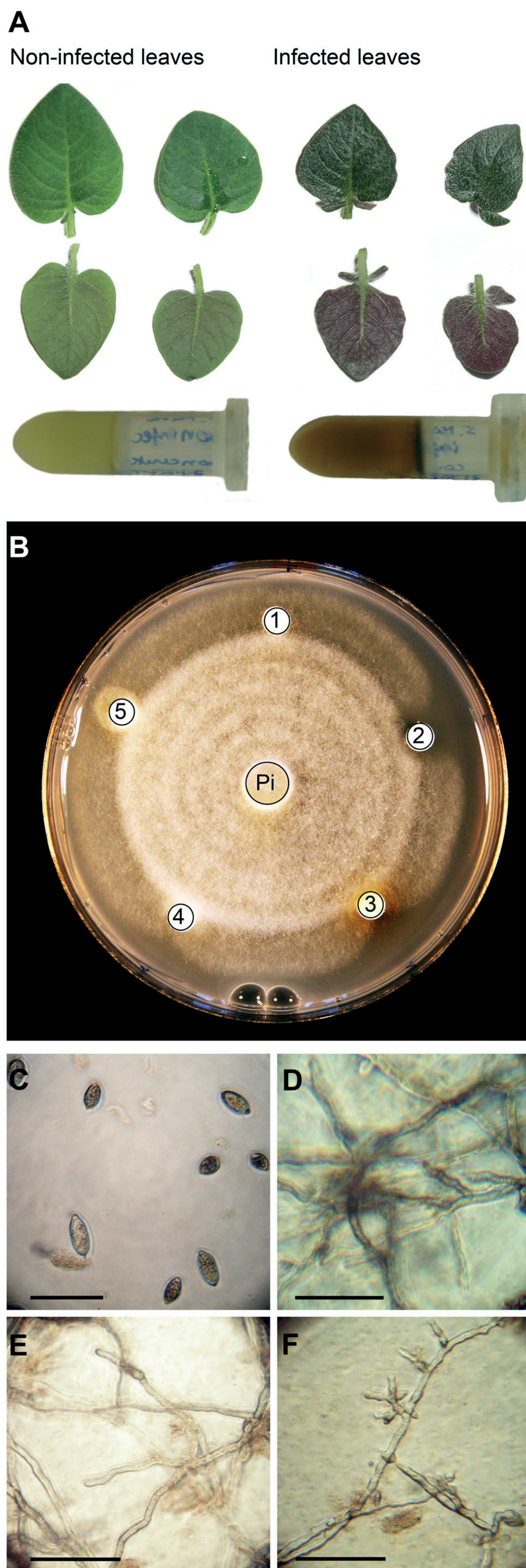

Fig. 7. Antimicrobial activity of plant extracts. (A) Leaves and extracts from leaves of Sarpo Mira plants mock-inoculated (left column) and infected with *P. infestans* (right column) at 5 weeks after inoculation. (B) Modified hyphal extension-inhibition assay showing the growth inhibition of *P. infestans* mycelia on rye media. Discs contained 15 μl of: water (disc 1), G418 (5 μg ml^{-1}) (disc 2), extract of Sarpo Mira leaves from *P. infestans*-infected plants (disc 3), extract of Sarpo Mira leaves from mock-inoculated plants (disc 4), and extract from leaves of mock-inoculated Bintje plants (disc 5). (C–F) *Phytophthora infestans* sporangia germination and hyphal growth after incubating the sporangia with G418 (C) and with different plant extracts (D–F). (D) Extract from leaves of mock-inoculated Bintje plants. (E) Extract from leaves of mock-inoculated Sarpo Mira plants. (F) Extract from leaves of *P. infestans*-infected Sarpo Mira plants. The experiment was repeated three times with *de novo* infected plants, yielding similar results. Representative results of six replicates are shown. Scale bar=50 μm.

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whereas a strong inhibition of *P. infestans* growth was observed in control discs containing the antibiotic G418. The concentrated extracts from infected Sarpo Mira leaves strongly inhibited the mycelial spread, and a slight inhibitory activity was present in concentrated extracts from mock-inoculated Sarpo Mira leaves (Fig. 7B). Growth inhibition was also observed when concentrated extract from roots of infected Sarpo Mira plants was used, and this inhibition was similar to that of extract from leaves of infected Sarpo Mira plants (not shown). The inhibitory effect was still visible when the extracts were diluted 3-fold with water, but no inhibitory effect was observed when dilutions of 1:6 or 1:12 were used (not shown). No inhibition of *P. infestans* growth was observed when root extract from mock-inoculated Sarpo Mira plants was tested.

Using extracts that had been frozen at –80 °C for 5 weeks yielded similar results to those obtained using fresh extracts, while boiling the extracts for 5 min or 10 min deactivated their antimicrobial activity (not shown).

Testing the extracts to inhibit the germination of zoospores and the growth of mycelium in liquid rye medium yielded similar results (Fig. 7C–F). Extracts from infected Sarpo Mira leaves strongly inhibited zoospore germination and hyphal growth (Fig. 7F). A weaker inhibitory activity was observed when extracts from mock-inoculated Sarpo Mira leaves were used (Fig. 7E).

These results demonstrated that the late blight infection induces the production of antimicrobial compounds in Sarpo Mira plants. The activity of these compounds increases in both leaves and roots upon infection, but a basal level of antimicrobial activity is already present in leaves of mock-inoculated Sarpo Mira plants.

Discussion

Even with intensive fungicide usage and the introgression of *R* genes into new potato cultivars, *P. infestans* still causes massive losses to the potato industry (Haverkort *et al.*, 2009). There are potato genotypes that are extremely resistant to this pathogen, but they lack properties attractive for consumers and growers. Discovering the mechanisms that confer resistance against late blight may provide valuable information for breeding high value resistant cultivars and for disease control alternatives. In the present study, the highly resistant Sarpo Mira cultivar was used to reveal the impact of plant integrity on the responses to *P. infestans*. Sarpo Mira contains at least five *R* genes (Rietman, 2011), and several *P. infestans*-responsive genes were differentially regulated in Sarpo Mira plants compared with the susceptible Bintje cultivar (Orłowska *et al.*, 2012). Although *R3a*, *R3b*, and *R4* genes were shown to be present in the Sarpo Mira cultivar (Rietman, 2011), the results of the present field studies indicate that *R3* and *R4* genes are not solely responsible for high late blight resistance in Sarpo Mira. It is possible that the other two *R* genes found in Sarpo Mira (*Rpi-Smira1* and *Rpi-Smira2*) (Rietman, 2011) confer resistance to *P. infestans.* Based on the strategy used for breeding the Sarpo Mira cultivar, it is possible that this cultivar has even more *R* genes than the five already characterized (Rietman, 2011). Because potato differentials carrying either the *R8* or the *R9* genes were fully resistant in the performed field trials, it is possible that these genes could be involved in the high resistance of Sarpo Mira, but the resistance of Sarpo Mira could be caused by other *R* genes not included in the differential set. Furthermore, crossing populations with Sarpo Mira as a parent showed a 1:1 segregation, indicating that only one *R* gene or closely linked *R* genes were providing resistance in the trials at the LKF-Vandel Breeding Foundation (Hanne-Grethe Kirk, Vandel, personal communication).

Differential responses to the pathogen in detached leaves compared with leaves of whole plants

Experimental approaches using detached leaves have been widely used to study biochemical and gene regulation aspects of host–pathogen interactions (Beyer *et al.*, 2001; Ros *et al.*, 2005, 2008; Wang *et al.*, 2005; Tian *et al.*, 2006), although it has previously been shown that detached potato leaves can be more susceptible to *Phytophthora* infection than leaves of whole plants (Stewart, 1990; Stewart and Gourlay, 1995; Vleeshouwers *et al.*, 1999; Wang *et al.*, 2004; Brooks, 2007). The observed differences were attributed to differences in experimental conditions such as humidity or wounding (Stewart, 1990; Stewart and Gourlay, 1995; Vleeshouwers *et al.*, 1999; Wang *et al.*, 2004; Brooks, 2007). The results of this study show that the progress of infection in leaves of *in vitro* grown whole Sarpo Mira plants is different from that in detached leaves. This is in accordance with recent finding of Rietman (2011). The differences between the symptoms in detached leaves and those of leaves in whole plants included not only differences in the growth of *P. infestans*, but also differences in the appearance of the HR. The first differences were already observed at 65 hai. No HR lesions were observed in detached leaves at any screening within 6 dai, and these leaves became overgrown with *P. infestans* mycelia within 6 dai. In comparison, >40% of the leaves of whole plants had HR lesions at 65 hai and *P. infestans* mycelium was visible only on 10% of them at 3 and 6 dai.

These differences could be attributed to the presence of specific *R* genes as previously reported (Stewart, 1990; Stewart and Gourlay, 1995). Among the five *R* genes discovered to be present in Sarpo Mira, *Rpi-Smira2* contributes to field resistance and, interestingly, *Rpi-Smira2*-mediated resistance was not detectable in detached leaf assays for any of the nine *P. infestans* isolates that were tested (Rietman, 2011). The author suggests three possible explanations: one is that the resistance is linked to a different avirulence candidate from that used in the study (PBHR_099), the second is that the resistance is dependent on the modulation of the *Rpi* gene upon infection, and the third is that the expression of the *Rpi* gene and of other defence-related genes is different between detached leaves and field-grown plants (Rietman, 2011).

The expression of some *PR* genes was also found to be different between detached leaves and leaves of whole plants at early hours after the inoculation with *P. infestans*. Both the acidic and the basic chitinases (*ChtA* and *Cht*B) and the *PR-1* gene were

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more highly induced by the pathogen inoculation in leaves of whole plants than in detached leaves at 1 hai. Similarly, it was previously shown that these genes were induced earlier in resistant plants than in susceptible plants (Orłowska *et al.*, 2012). To exclude the possibility that the observed changes in gene expression were attributed to wounding during the detachment of the leaves, the expression of these genes was also studied in detached mock-inoculated leaves. All but one of the selected genes were found not to be regulated by the treatment at the analysed time points. A patatin-like gene was the only exception, in agreement with a previous work showing that a patatin-like protein was rapidly induced by wounding in *Ricinus communis* (Domingues *et al.*, 2007).

Taken together, the obtained data suggest that the response to late blight infection in leaves of whole plants differs from that in detached leaves and that the very early responses can have an impact on the resistance to *P. infestans*.

Meristems might be important for HR in Sarpo Mira

The differences in pathogen resistance between detached leaves and whole plants led to the study of the pathogen resistance in other parts of the Sarpo Mira cultivar. First, the impact of removing the plants' apical meristems and axillary meristems on the resistance to *P. infestans* was studied. Detached leaves with axillary meristems showed HR lesions, while no HR lesions were observed in the absence of meristems. Furthermore, the number of HR lesions increased when more meristems were attached to the infected plant parts. This indicates that the meristems or substances produced by the meristem might be necessary for the development of HR. It is also possible that the stem plays a role in the development of HR. Stems were not included in the detached leaves without meristems in which no HR lesions were observed. In addition, the detached leaves with meristems, that showed few HR lesions upon infection, had a short fragment of the stem. However, literature searches did not lead to information indicating that stems or compounds produced in the stems could be responsible for the formation of HR lesions. In contrast, active meristems are rich sources of auxin (Vernoux *et al.*, 2010), and auxin was shown to induce the formation of HR lesions in grapevine rootstocks (Dietrich *et al.*, 2010). Indole acetic acid (IAA) has been suggested to act through the regulation of glutathione *S*-transferase in the potato–*P. infestans* interaction (Hahn and Strittmatter, 1994). The inhibition or modification of the glutathione *S*-transferase activity by IAA might contribute to necrosis of the host tissue in the vicinity of infection sites, a typical symptom of HR (Hahn and Strittmatter, 1994). Recent studies showed that IAA inhibited the growth of *P. infestans in vitro*, and its application to detached potato leaves prior to inoculation with *P. infestans* also inhibited pathogen growth (Martínez-Noël *et al.*, 2001). It is possible that the removal of the meristems in Sarpo Mira could cause a reduction in auxin production and thereby a reduction in HR responses. Additional studies will be needed to identify the role of the meristems, auxin, and the stems in the HR responses of this cultivar.

Sarpo Mira roots are important for foliar resistance to P. infestans

It is evident from the presented data that the meristems are important for the formation of HR lesions, although detached leaves could be infected by *P. infestans* even when HR lesions were present. Furthermore, rooted plants in which the apical meristems were removed had fewer HR lesions but a similar level of resistance to *P. infestans* compared with whole Sarpo Mira plants. To address the impact of roots on the levels of pathogen resistance in the Sarpo Mira cultivar, two experimental set-ups were used. In the first one, whole plants as well as de-rooted shoots were inoculated with the pathogen. In the second one, Sarpo Mira shoots and roots were grafted with roots and shoots of the susceptible cultivar Bintje.

The number of HR lesions was not directly linked to the level of resistance in the Sarpo Mira cultivar. The whole plants had the highest number of HR lesions and showed the highest resistance. However, de-rooted shoots were significantly less resistant to *P. infestans* compared with plants without apical meristems, although they had a similar number of visible HR lesions. The induction of HR is often preceded by the activation of effector-triggered immunity upon recognition of the effector by the R protein (Jones and Dangl, 2006). R proteins induce responses that stop pathogen infection locally and induce signals that enhance defence responses in distal tissues, which is known as SAR (Vlot *et al.*, 2008). Thus, the increased disease severity in plant tissues with reduced HR may not only be caused by a failure in the restriction of pathogen spread due to cell death but could also be caused by an impaired SAR.

This defence response can be achieved by two mechanisms. First, it can be caused by the *de novo* induction of resistance mechanisms activated by translocated signals from the stress-exposed tissues. Secondly, it can result from the systemic transport of antimicrobial metabolites. Recent evidence supports a crucial role for both mechanisms (Heil and Ton, 2008). Several candidate signalling molecules have been studied in the past few years, including SA (salicylic acid), JA (jasmonic acid), methyl salicylate, an as yet undefined glycerolipid-derived factor, and a group of peptides that are involved in cell–cell basal defence signalling (Vlot *et al.*, 2008). Furthermore, it has been suggested that the SAR signal produced in infected leaves travels mostly with the phloem to the upper and lower leaves, but part of the signal might also move by a different route (Guedes *et al.*, 1980; Heil and Ton, 2008).

SAR is characterized by the coordinated activation of a specific set of genes, including those encoding PR proteins in both local and distal tissues (Durrant and Dong, 2004). Many PR proteins possess antimicrobial activity that leads to an enhanced resistance that spreads to distal organs (Vleeshouwers *et al.*, 2000; van Loon *et al.*, 2006). As presented, PR-1, ChtA, and ChtB were more highly induced at 1 hai in whole plants compared with detached leaves. PR-1 expression is often used as a marker of SAR (Vleeshouwers *et al.*, 2000). These results, together with previous findings (Orłowska *et al.*, 2012), suggest that SAR, activated by *R* genes upon pathogen attack, contributes to the resistance of Sarpo Mira against *P. infestans*.

The contribution of roots to leaf defence has not been widely recognized, probably because leaves and roots are

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spatially separated from one another, often by great distances (Kaplan *et al.*, 2008). In *Nicotiana sylvestris* and *N. tabacum*, nicotine is produced in roots upon leaf damage and is translocated to the above-ground tissues where it provides foliar protection against herbivores (Baldwin *et al.*, 1997; Kaplan *et al.*, 2008). To assay whether only resistant roots were able to provide improved resistance in Sarpo Mira plants, susceptible roots from Bintje plants were grafted with Sarpo Mira shoots and Sarpo Mira roots with Bintje shoots. The highest resistance was observed when Sarpo Mira roots were grafted on Sarpo Mira shoots (S/S). In comparison with de-rooted shoots, Sarpo Mira shoots with Bintje roots (S/B) had a lower percentage of infected leaves at 3 dai. No difference was observed between S/B and S/S plants at 3 dai; however, at 6 dai, ~30% of S/B plants showed infection symptoms. In addition, all the S/S grafted plants survived the infection, while 70% of S/B plants survived. Interestingly, all the S/B plants that survived at 6 dai were alive at 10 dai, indicating that the first days after infection were crucial for the survival of the S/B plants. This suggests that the Sarpo Mira shoot needs to mobilize a signal or an active compound to or through the roots to provide complete resistance. It is possible that these molecules provide resistance by themselves or that they act as signals that cause an increased production of antimicrobial compounds in the roots that could lead to enhanced resistance to the pathogen. The results suggest that the movement or induction of defence-associated compounds was delayed in some of the Bintje roots compared with Sarpo Mira roots and, therefore, the pathogen was able to overcome the plant defences. This suggests that either the Bintje roots may not recognize the defence-associated compounds as efficiently as Sarpo Mira roots, that they may have a lower production of these compounds, or that the defence-associated compounds produced by Bintje roots are less effective than those produced by Sarpo Mira roots. Grafting Bintje shoots into Sarpo Mira roots (B/S) resulted in a reduced number of infected leaves at the early stages of infection in comparison with grafted Bintje shoots with Bintje roots (B/B). Six days after inoculation, the level of infection in B/S plants was similar to that in Bintje shoots with Bintje roots (B/B). The Sarpo Mira roots did provide an increased resistance to Bintje shoots, but this resistance was short lived. A possible explanation is that Sarpo Mira roots already contained defence-associated compounds that were transferred to the Bintje shoots, but that the Bintje shoots were not able to induce the production of this compound and, therefore, the pathogen was able to overcome its effect. Alternatively, it is possible that the signals from the infected Bintje shoots were delayed or too weak to induce the production of sufficient defence-associated compounds.

The presented results indicate that roots play a direct role in foliar plant defences and that these leaf–root connections have an important effect on the resistance against *P. infestans* in the Sarpo Mira potato cultivar. It can be hypothesized that the SAR signals travel through the long-distance signalling system as previously described (Lucas and Lee, 2004). The signals enter the phloem at the infection site and are delivered to the shoot apical meristem, axillary meristems, and to the root tissues through the petiole and stem. The likely disruption of the movement of defence-associated compounds by detaching the leaves could also explain the high susceptibility to *P. infestans* of detached leaves compared with the high resistance of leaves of whole plants.

Sarpo Mira tissue extracts have antimicrobial activity

The obtained results suggested that the production of antimicrobial compounds could be involved in the resistance of Sarpo Mira to *P. infestans*. Therefore, the antimicrobial activity of Sarpo Mira and Bintje tissue extracts against *P. infestans* was analysed. The weak inhibitory effect of the extract from non-infected Sarpo Mira leaves suggests that compounds with antimicrobial activity are already present in the leaves of these plants. No antimicrobial activity was observed in extracts from roots of mock-inoculated plants, indicating that roots of non-infected plants might contain no or lower amounts of these compounds, or that these compounds are less active in roots of non-infected plants. The antimicrobial activity of the extracts increased both in leaves and in roots upon infection. The extracts lost their antimicrobial activity by heat treatment but their activity was not affected by freezing. Therefore, although further studies will be necessary to corroborate this, it is possible that the antimicrobial activity is, at least in part, of protein nature.

Many PR proteins are known for their antimicrobial activity *in vitro* or in transgenic plants (Woloshuk *et al.*, 1991; Beerhues and Kombrink, 1994; Liu *et al.*, 1994; Niderman *et al.*, 1995; Vleeshouwers *et al.*, 2000). The potato PR-1 had an inhibitory effect on zoospore germination and mycelial growth (Niderman *et al.*, 1995). Similarly, several purified plant chitinases were able to inhibit the growth of pathogens (Mauch *et al.*, 1988; Graham and Sticklen, 1994; Kombrink and Somssich, 1997). The higher expression of both chitinases (*ChtA* and *ChtB*) and the *PR-1* genes in leaves of whole plants after infection with *P. infestans* suggests that the proteins encoded by these genes could represent the antimicrobial compounds contributing to the strong resistance in Sarpo Mira.

The change of colour of Sarpo Mira leaves after late blight infection may suggest the production of pathogen-induced phenolic compounds. Phytoalexins, phenolics, and glycoalkaloids with antimicrobial activity are frequently produced upon pathogen attack (Matern and Kneusel, 1988; Bennett and Wallsgrove, 1994). It has previously been shown that these compounds accumulated in late blight-infected leaves of a resistant but not of a susceptible potato cultivar (Andreu *et al.*, 2001).

In addition to the production of antimicrobial compounds, it is likely that physical barriers are increased in Sarpo Mira plants upon pathogen infection. This is suggested because the leaves of infected Sarpo Mira plants became thicker and harder, probably due to changes in the plant cell walls. Many plant species react to pathogen or insect attack by synthesizing lignin and callose that serve as a protective physical barrier (Matern and Kneusel, 1988; Bhuiyan *et al.*, 2009; Luna *et al.*, 2010).

Therefore, it is possible that Sarpo Mira plants, upon pathogen attack, are able to induce a coordinated defence consisting of the production of different defence-associated compounds such as antimicrobial molecules, PR proteins, and phenolic compounds, as well as compounds that increase the cell wall stability.

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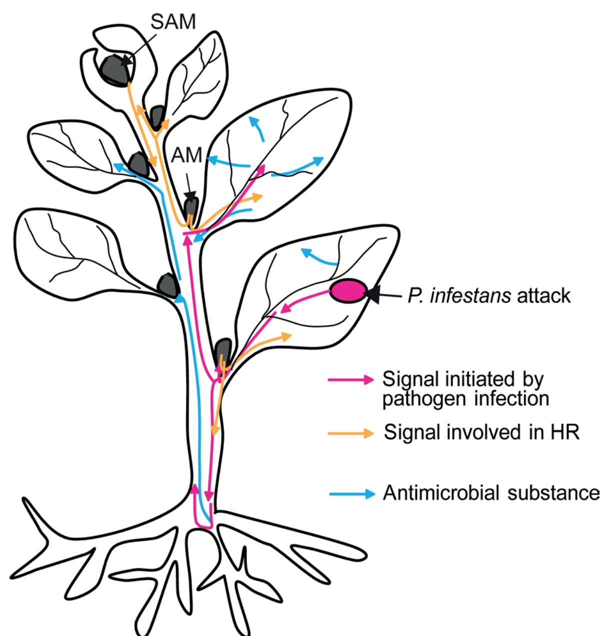

Fig. 8. Model of the signalling path induced by the late blight infection in Sarpo Mira. Based on the literature and on the presented results, a model of the signalling path that occurs when *P. infestans* infects Sarpo Mira is proposed (see text in the discussion for more details). *Phytophthora infestans* penetrates the plant and translocates avirulence (*Avr*) factors that activate corresponding *R* genes. R proteins induce the production of defence-associated compounds (magenta arrows) that are transported in the plant and stimulate the meristems to produce molecules (yellow arrows) involved in the hypersensitive reaction (HR) and stimulate the roots to produce antimicrobial compounds (light blue arrows). Both the roots and the shoots of Sarpo Mira plants produce defence-associated compounds, some of them with antimicrobial activity. The results from the grafting experiments indicate that these compounds are mobilized within the plant from the roots to the shoots (light blue arrows) and probably circulate to the roots again and provide increased resistance to the whole plant. The systemic reaction seems to affect the timing of the induction of pathogenesis-related genes, and the rapid induction of genes such as *PR-1*, *ChtA*, and *ChtB* could have an impact in pathogen resistance.

Model of the signalling path induced by the late blight infection of Sarpo Mira

Based on the literature and on the presented results, a model of the signalling path that occurs when *P. infestans* infects Sarpo Mira is proposed (Fig. 8). At the first stages of infection, *P. infestans* penetrates the plant and translocates *Avr* factors that activate corresponding *R* genes (Flor, 1971; Dangl and Jones, 2001; Collier and Moffett, 2009). R proteins induce the production of defence-associated compounds that are transported in the plant and stimulate the meristems or stems to produce molecules involved in the HR and stimulate the roots to produce antimicrobial compounds. This is in agreement with the suggestion that signals responsible for SAR are produced in infected tissue and travel to distal parts of the plant (Guedes *et al.*, 1980; Heil and Ton, 2008). It is possible that auxin could be one of the defence-associated compounds produced by the meristems of Sarpo Mira plants upon infection. Auxin has been shown to inhibit pathogen growth and induce HR lesions, and active meristems are a rich source of this plant hormone (Hahn and Strittmatter, 1994; Martínez-Noël *et al.*, 2001; Dietrich *et al.*, 2010; Vernoux *et al.*, 2010). Both the roots and the shoots of Sarpo Mira plants produce defence-associated compounds, some of them with antimicrobial activity. There is increasing evidence that roots play a role in plant defence (reviewed by Erb *et al.*, 2009). The results from the grafting experiments indicate that defence-associated compounds are mobilized within the plant from the roots to the shoots and probably circulate back to the roots and provide increased resistance to the whole plant.

The difference between detached leaves and leaves of whole plants indicates that the systemic reaction affects the timing of the induction of *PR* genes, and the rapid induction of genes such as *PR-1*, *ChtA*, and *ChtB* could have an impact in pathogen resistance. The products of some of these genes have already been shown to have antimicrobial activity (Vleeshouwers *et al.*, 2000; van Loon *et al.*, 2006).

In addition, the presented results indicate that the infection of Sarpo Mira plants may induce the production of polyphenolic compounds. Polyphenolic compounds have previously been shown to have antimicrobial activity (Matern and Kneusel, 1988; Bennett and Wallsgrove, 1994), and their distribution in all tissues could protect the plant against further infections.

Conclusion

The presented results suggest that Sarpo Mira plants are able to use a combination of strategies to avoid infection by the late blight pathogen. This work showed that the resistance might rely on a systemic response, which increases the production of antimicrobial compounds and probably also strengthens the physical barrier against the pathogen. The presented results show that meristems, stems, or both are required for the HR of Sarpo Mira leaves and that roots contribute to the resistance of Sarpo Mira plants against the late blight pathogen. If confirmed in other species, the involvement of meristems and roots in pathogen defence responses could represent a general mechanism in plants.

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