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Histological responses of host and non-host plants to *Hyaloperonospora parasitica*

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Abstract Differences in *Hyaloperonospora parasitica* development and plant tissue responses were compared for 10 cruciferous hosts (including both resistant and susceptible genotypes), 3 leguminous and 1 graminaceous non-host species. Cotyledons, or true leaves in the case of *Triticum aestivum* and *Pisum sativum,* were studied at 2, 8, 24 h and 3, 5, 7 days post inoculation (dpi). The high levels of zoosporangial germination observed on all species tested, as well as on glass slides, suggested that inhibition of germination did not play a significant role in distinguishing host *versus* non-host resistance. During the early stages of infection, at spore germination and host penetration, there was no evidence of a clear-cut difference between *Brassica* host species which displayed a hypersensitive, partially resistant or susceptible reaction compared with non-host species. Haustoria formation was the key infection phase for the establishment of biotrophy. Across all tested species, haustoria were initiated inside the epidermal cells. However, there were significant differences in the frequency and timing of haustorial formation and the final size of haustoria among the tested species at early infection stage. Fully developed haustoria were never observed in *Raphanus raphanistrum*, *Triticum aestivum, Lupinus angustifolius* nor *Trifolium subterraneum*. Instead, the haustorium development appears to abort in the penetrated epidermal cells of these species. Although haustoria were formed in the epidermal and mesophyll cells of *Sinapsis alba* and *Pisum sativum,* subsequent hyphal growth and/or continued haustoria formation were rare or few, respectively. Hypersensitive reaction was the key resistance response observed among the host and non-host resistant species tested. It is noteworthy that, in the initial stages of pathogenesis, there was no differentiating point that separated the non-host species from those that were hosts.

Keywords Biotrophy · Brassicaceae · Downy mildew · Fabaceae · Hypersensitive response · Host-pathogen interaction · Resistance

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Introduction

Oomycete pathogens such as species of *Phytophthora*, *Pythium, Albugo* and the various downy-mildew genera, form a unique branch of eukaryotic plant pathogens with an independent evolutionary history (Kamoun et al. 1999). Among these oomycetes, downy-mildew species cause some of the most destructive plant diseases in the world (Viennot-

Bourgin 1981). *Hyaloperonospora* (formerly *Peronospora*) *parasitica*, the causal agent of downy mildew of cruciferous vegetables and field crops, is endemic across the oilseed rape-growing regions in Australia (Howlett et al. 1999; Ge et al. 2008), Europe (Paul et al. 1998), China and Japan (Satou and Fukumoto 1996) and, in particular, on the Indian subcontinent (Nashaat et al. 2004). While young *Brassica* plants are most susceptible to *H. parasitica* (Channon 1981; Silué et al. 1996), infection at a later stage of plant development can also result in yield and quality reductions (Channon 1981). As fungicides are often expensive and lead to the development of pathogen resistance to such chemicals, identification and deployment of host resistance has increasingly become the most efficient and cost-effective means for managing this disease. Host resistance has been studied from many perspectives in the *Arabidopsis-H. parasitica* pathosystem (Mauch-Mani and Slusarenko 1993; 1996; Donofrio and Delaney 2001) including the role of signalling pathways. For example, McDowell et al. (2000) reported that the *RPP7* gene initiated host resistance through a novel signalling pathway that functions independently of salicylic acid accumulation or jasmonic acid response components.

In nature, plants are exposed to many potential pathogens. For this reason, plants possess different constitutive and inducible mechanisms to recognize and defend themselves against pathogens. While a wide range of pathogens may attempt to penetrate plant tissues, only a limited number of these invasion interactions are successful, suggesting that plants can exhibit a 'general' host resistance, defined by Heath (1980) and Jahnen and Hahlbrock (1988) as 'non-host' resistance. Non-host resistance is highly effective and durable, and hence it is often suggested that the mechanisms of non-host resistance could be exploited to generate disease resistant crop plants. Several components of non-host disease resistance have been identified (Zhou et al. 1999; Mellersh and Heath 2003; Yun et al. 2003). Heath (2000) and Holub and Cooper (2004) pointed out that in spite of tremendous progress in plant science, non-host resistance is still poorly understood in contrast with host resistance. It is still not clear why a pathogen can be highly virulent on one plant species while having no effect on others. Understanding non-host resistance is key to deciphering the complex plant defence mechanisms against pathogens.

An understanding of the mechanisms of host and non-host resistance to *H. parasitica* would not only provide information about the way in which host-parasite specificity is determined but it also offers potential for future more precise and long-lasting control of this disease through development of new cultivars with more effective and durable host resistance. The determination of both the factors responsible for non-host resistance and the racial status of this pathogen could together lead to identification of physiological and molecular markers for use in securing effective resistance to this disease against the pathotypes/races of this pathogen.

In this paper we investigate the early infection events of hypersensitive, partially resistant or susceptible *Brassica* and other crucifer host species by *H. parasitica*. These responses are compared with those of the pathogen on three legumes and a cereal species included as non-cruciferous non-host plants, in order to provide additional understanding of the interactions involved in the determination of host-*H. parasitica* specificity.

Materials and methods

Plant materials

Plant materials used in this study, including 10 cruciferous hosts (including both resistant and susceptible genotypes), viz. two *B. napus*, one each of *B. oleracea* var. *italica, B. nigra*, *B. juncea*, *B. rapa*, *B. carinata*, *Eruca vesicaria* ssp. *sativa*, *Raphanus raphanistrum* and *Sinapis alba*; 3 leguminous, viz. *Lupinus angustifolius, Pisum sativum* and *Trifolium subterraneum*, and 1 graminaceous, viz. *Triticum aestivum* (Table 1). All cruciferous plants used in this study were considered as hosts, while three leguminous and one graminaceous hosts were considered to be non-host species, based on previous reports.

Fifteen seeds of each genotype were sown into 150×70×70 mm pot, 6 pots per genotype. After emergence, the seedlings were thinned to 10 plants/pot. Samples for histological study were taken randomly from different plants from different pots. A total of 24 plants per genotype (6 replicates with 4 plants in each replicate) were assessed for disease severity at 7 dpi.

Table 1 Plant material tested in this study and its reaction to *Hyaloperonospora parasitica* (isolate UWA DM 53) expressed as the severity of downy mildew disease 7 days post inoculation, on cotyledons/true leaves (0-9 scale was applied, where 0 = no symptoms of downy mildew; 9 = heavy sporulation, cotyledon collapsed; for details see Williams 1985)

Species	Genotype /cultivar	Host/non-host	Reaction to host	Disease severity
Brassica napus	Thunder TT	host	S	8.33
B. oleracea var. *italica*	Shogun	host	S	6.97
B. nigra	P23845	host	PR	3.63
B. napus	Pioneer 46Y78	host	PR	3.43
B. juncea	Dune	host	HR	1.13
Eruca vesicaria ssp. *sativa*	MJB 1A	host	HR	0.67
B. rapa	Xi Shui Bai	host	HR	0.57
B. carinata	ATC94011	host	HR	0.53
Sinapis alba	Concerta	host	NR	0
Raphanus raphanistrum	Wild radish	host	NR	0
Pisum sativum	Dunwar	non-host	NR	0
Lupinus angustifolius	Tanjil	non-host	NR	0
Trifolium subterraneum	Woogenellup	non-host	NR	0
Triticum aestivum	Gamenya	non-host	NR	0
Significance $P<0.01$	LSD			0.44

Disease severity data were analysed using two-way analysis of variance (ANOVA)

Significancy of mean of disease severity differences within species or genotype were assessed by test for least significant difference (LSD)

Reaction to host within parenthesis indicates: *S* susceptible; *PR* partially resistant; *HR* hypersensitive response; *NR* no response

Pathogen isolate and inoculum preparation

A single zoosporangial isolate of *H. parasitica* (UWA DM 53) originating from *B. napus* was used for this study. Inoculum was prepared from a culture of this isolate maintained on cotyledons of oilseed rape (*B. napus cv.* Thunder TT). Cotyledons that supported abundant sporulation by the pathogen were collected and placed in 50 ml of distilled water in a 200 ml glass flask. The flask was shaken vigorously to dislodge the zoosporangia. The resulting zoosporangial suspension was filtered through a single layer of cheesecloth and the concentration determined with a haemocytometer counting chamber and adjusted to 1×10^5 zoosporangia ml^{-1}.

Inoculation

The experiment was conducted in a phytotron growth cabinet where air temperature was maintained at 15 (night)-20 (day) °C, photoperiod of 12/12 h, light intensity was 150 μE m^{-2} s^{-1} and plants were watered daily with deionised water. After 10 d, a 10 μl droplet of zoosporangial suspension was deposited on the each lobe of 10 day old cotyledons of each test genotype, except for wheat and pea where the suspension was applied to the upper leaf surface of 10 day old first true leaf. A 10 μl droplet of water was deposited at the same inoculation sites on control plants. Inoculated and control plants were placed into plastic boxes with sealed lids in order to maintain high humidity post-inoculation for a period of 24 h. Subsequently, the lids were removed and plants watered daily. At 6 dpi, the plants in the plastic boxes were again covered by lids for a further 24 h to maintain high humidity.

Disease severity assessment

Disease severity on cotyledons/true leaves was assessed at 7 dpi using the 0–9 scale as defined by Williams (1985); where 0 = no symptoms or signs of *H. parasitica*; 1 = minute scattered necrotic flecks under the inoculum drop, no sporulation; 2 = larger necrotic flecks under the inoculum drop, no sporulation; 3 = very sparse sporulation, one to a few conidiophores,

necrotic flecking but often with tissue necrosis evident; 5 = sparse sporulation, tissue necrosis; 7 = abundant sporulation, tissue necrosis and chlorosis may be present; and, 9 = heavy sporulation, cotyledon collapsed.

Histological studies

Epifluorescence microscopy

Plants inoculated with zoosporangia were sampled 0, 2, 8 h and 1, 3, 5 and 7 dpi. Four leaf segments taken from different plants at each time-point were decolorized in 10% KOH over night and stained with aniline blue in 0.07 M K_2HPO_4 for UV light observation as described by Heath (1974). A zoosporangium was considered as germinated if it produced either a germ tube of at least half of the diameter of the zoosporangium or a germ tube with a primary appressorium. A total of 200 zoosporangia per leaf segment were scored. The total number of zoosporangia, germinated zoosporangia, appressoria, and haustoria were recorded, as was the presence of any cell necrosis, at the penetration sites and in cells. The samples were examined and photographed using a Olympus BX 51 microscope photograph system with an excitation filter (G365) and an emission filter (LP 420) inserted into a beam of incident light from a mercury vapor lamp.

Bright-field microscopy

Infected cotyledons/true leaves were sampled 0, 2, 8 h and 1, 3, 5 and 7 dpi. Four cotyledon/true leaf segments (taken from different plants) per time-point were cleared in 95% ethanol over night and stained with 1% cotton blue and whole, wet mounts examined and photographed using same microscopy photograph system as above with bright field lighting. Drops of zoosporangial suspension were deposited on glass slides in moist chambers as comparisons. One hundred zoosporangia were assessed at five random fields of view on each of five inoculation sites per cultivar. The number of zoosporangia, germinated zoosporangia, appressoria, and haustoria were recorded, as was the presence of any cell necrosis, at the penetration sites and within cells.

Scanning electron microscopy

Plants were sampled at 2 and 4 hpi for this experiment. The infected region of the tissue was cut and fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer pH 7 at room temperature. Samples were further processed by using PELCO BioWave Microwave Processor. Tissues were vacuum infiltrated for 6 min (2 min on / 2 min off / 2 min on) at 80 W. Specimens were then washed with 0.05 M phosphate buffer for 40 s at 80 W, dehydrated in a series of ethanol solutions ranging from 50% to 100% (dry ethanol) and twice with 100 % dry acetone again for 40 s at 250 W. Samples were then critically point dried (PELCO Critical Point Dryer) using liquid carbon dioxide, sputter coated with gold and examined and photographed using the JEOL 6400 SEM.

Statistical analysis

Disease severity data were analysed using GenStat® Release 9.1 (Lawes Agricultural Trust, Rothamsted Experimental Station). The data were analysed using two-way analysis of variance (ANOVA). Significant differences between mean disease severities within species or genotype were assessed by testing for least significant difference (LSD).

Results

Disease intensity

Disease severities across the tested genotypes differed significantly ($P<0.01$) (Table 1). The highest score (8.3) for disease was recorded on the highly susceptible *B. napus* cv. Thunder TT, where the infected cotyledons showed heavy sporulation and collapsed. The partially resistant *B. napus* cv. Pioneer 46Y78 had a disease score of 3.4, while *B. oleracea* and *B. nigra* had disease scores 7 and 3.6, respectively. However, other crucifer species tested, including *B. carinata*, *B. rapa*, *B. juncea* and *E. vesicaria* ssp. *sativa*, all showed only a hypersensitive response on the inoculated cotyledons. No symptoms were evident on host cruciferous species *S. alba* or *R. raphanistrum*, nor on any of the non-host genotypes tested.

Infection progress

A summary of the development of *H. parasitica* on the various host and non-host plants is shown in

Table 2. At 2 h post inoculation (hpi), zoosporangia germinated equally well (>76%) on all host and non-host species tested, but zoosporangial germination rates were lower on glass slides (43%). Appressoria formed at the tip of the germ-tube on all host, non-host species and even on the glass slides. Appressoria formation resulted in the direct penetration of epidermal cells for *Brassica* and other cruciferous species (Fig. 1a) and also for *P. sativum*. Hyphae penetrated mostly between anticlinalwalls of epidermal cells, but infrequently into the lumen of an epidermal cell. On the cotyledons of *L. angustifolius* and *Trifolium subterraneum*, penetrations were direct and/or indirect (i.e., through stomata). However, there was no penetration of the leaves of the monocotyledon species (*Triticum aestivum*). Compared with the *Brassica* and other cruciferous host plants, there was a significantly lower number of penetrations by the pathogen on all non-host plants, with the exception of *P. sativum* and the cruciferous host *R. raphanistrum.* Following penetration, haustoria formation was observed to varying degrees on all of the host species, but not observed on *B. juncea* and *B. carinata* at this stage. Often the first haustorium was formed in one of the epidermal cells adjoining to where the hypha had penetrated and, frequently, one haustorium was observed in each of the epidermal cells proximal to the penetrating hypha (Fig. 1b). Haustoria also formed in the non-host species *P. sativum* and *L. angustifolius*, but were not formed in *Trifolium subterraneum* or *Triticum aestivum.* Infected cells in *B. rapa* developed a distinctive fluorescence under UV light (Fig. 1c).

At 8 hpi, on all host species, except for *R. raphanistrum*, as well as on *P. sativum*, the penetrating hypha lay in between the anticlinal cell walls of two neighbouring epidermal cells within which one, two or three haustoria were formed. The penetrating hypha usually reached as far as the immediately adjacent mesophyll cell and haustoria was sometimes formed in this mesophyll cell, as well as in the epidermal cells. There were, however, no significant differences among these species in relation to the number and size of the haustoria formed. Infected cells developed a distinctive fluorescence in *B. carinata*, *B. rapa, B. oleracea*, *B. nigra* and *S. alba*, and of these the fluorescence was most intense in *S. alba* and *B. carinata*. There was significantly fewer haustoria and less depth of penetration in the host *R. raphanistrum* and in the three non-hosts *L. angustifolius, Trifolium subterraneum* and *Triticum aestivum*, compared with species described above. Further, the haustoria in *R. raphanistrum, L. angustifolius*, *Trifolium subterraneum* and *Triticum aestivum* were relatively small, infrequent and were formed only in the epidermal cells. On all host species except *R. raphanistrum,* and on the non-host *P. sativum*, 100% of zoosporangia germinated, and in all cases, penetration was direct. In contrast, there were only 46, 12, 9 and 7% penetrated directly, and 0, 25, 65 and 0% penetrated through stomata, for *R. raphanistrum, L. angustifolius*, *Trifolium subterraneum* (Fig. 1d) and *Triticum aestivum*, respectively. On the host *R. raphanistrum* and non-host species *Triticum aestivum* and *Trifolium subterraneum* (Fig. 1d), as well as on the glass slide, germ-tubes were longer than for all the other species tested.

At 24 hpi, there were significant differences in the extent of tissue colonisation by the pathogen among the different hosts. In the highly susceptible *B. napus* cv. Thunder TT, hyphae grew past approximately 5–7 cells forming more than 10 haustoria inside the proximal mesophyll cells. On the partially resistant *B. napus* cv. Pioneer and on *B. rapa*, hyphae of the pathogen grew past approximately 3-4 cells and formed roughly 5-6 haustoria inside the proximal mesophyll cells. On all other host species and on the non-host species *P. sativum,* there were about 3-4 haustoria formed inside the initially-penetrated epidermal cells. In contrast, in *R. raphanistrum, Triticum aestivum*, *Trifolium subterraneum* and *L. angustifolius*, further invasion was restricted to the first epidermal cell penetrated. In these cells, the small haustoria (approximately 3 μm wide ×7 μm long) were evident at 8 hpi. At 24 hpi however, the appressoria on the surface of epidermal cell expanded to about 15–20 (wide)× 30–40 (long) μm with no evidence of the original haustorium. The haustorium development appeared to have aborted/collapsed at this point. Instead, a newly emerging hypha was seen to extend from the appressorium growing out, without any evidence of penetration, up to 180 μm on the surface of the epidermis (Fig. 1e). Infected cells of *E. vesicaria* ssp. *sativa* and *S. alba* showed a distinctive fluorescence. Hypersensitive cell death was readily observed among the species which showed distinctive fluorescence and also in *R. raphanistrum*. The mesophyll cell containing the first (oldest) haustorium to be produced, close to the penetrated

Table 2 Development of *Hyaloperonospora parasitica* isolate UWA DM 53 on host and non-host cotyledons/true leaves tested

Host		2 hpi						8 hpi					24 hpi		
Species	Genotype/cultivar	% germ	appre.	penet.	haust.	% germ	% appre.	% direct./stomatal penetration	haust.	flu.	appre.	penet. mesophyll cell	haust.	cd	callose
B. napus	Thunder TT	99	+	+	0-1	100	100	100/0	2-3	–	+	+	10–15	–	–
B. oleracea	Shogun	99	+	+	0-1	100	100	100/0	2-3	+	+	+	3-4	–	–
B. nigra	P23845	99	+	+	0–2	100	100	100/0	2-3	+	+	+	3–5	–	–
B. napus	Pioneer 46Y78	99	+	+	1-2	100	100	100/0	2-3	–	+	+	5-6	–	–
B. juncea	Dune	98	+	+	0	100	100	100/0	2	–	+	+	3–5	+	–
E. vesicaria ssp. *sativa*	MJB 1A	99	+	+	1-2	100	100	100/0	2	–	+	+	2-3	+	–
B. rapa	Xi Shui Bai	99	+	+	0-1	100	100	100/0	2-3	+	+	+	5 (3–8)	+	+
B. carinata	ATC94011	99	+	+	0	100	100	100/0	2-3	+	+	+	3–5	+	+
S. alba	Concerta	98	+	+	0-1	100	100	100/0	2-3	+	+	+	3–5	+	–
R. raphanistrum	Wild radish	76	+	+	0	82	35	46/0	0-1	–	+	–	0	+	–
P. sativum	Dunwar	90	+	+	0-1	100	100	100/0	2	–	+	+	2-3	+	+
L. angustifolius	Tanjil	89	+	+	1-2	92	74	12/25	0-1	–	+	+	0-1	–	–
Trifolium subterraneum	Woogenellup	89	+	+	0	93	30	9/65	0-1	–	+	+	0-1	–	–
Triticum aestivum	Gamenya	88	+	–	0	86	29	7/0	0	–	+	+	0-1	–	–
Glass slides		43	+	–	–	72	+	–	–	–	100	–	–	–	–

appre. appressorium, *haust.* haustoria, *germ* germination, *cd* cell death associated with HR, *flu* fluorescence

+ present, – absent

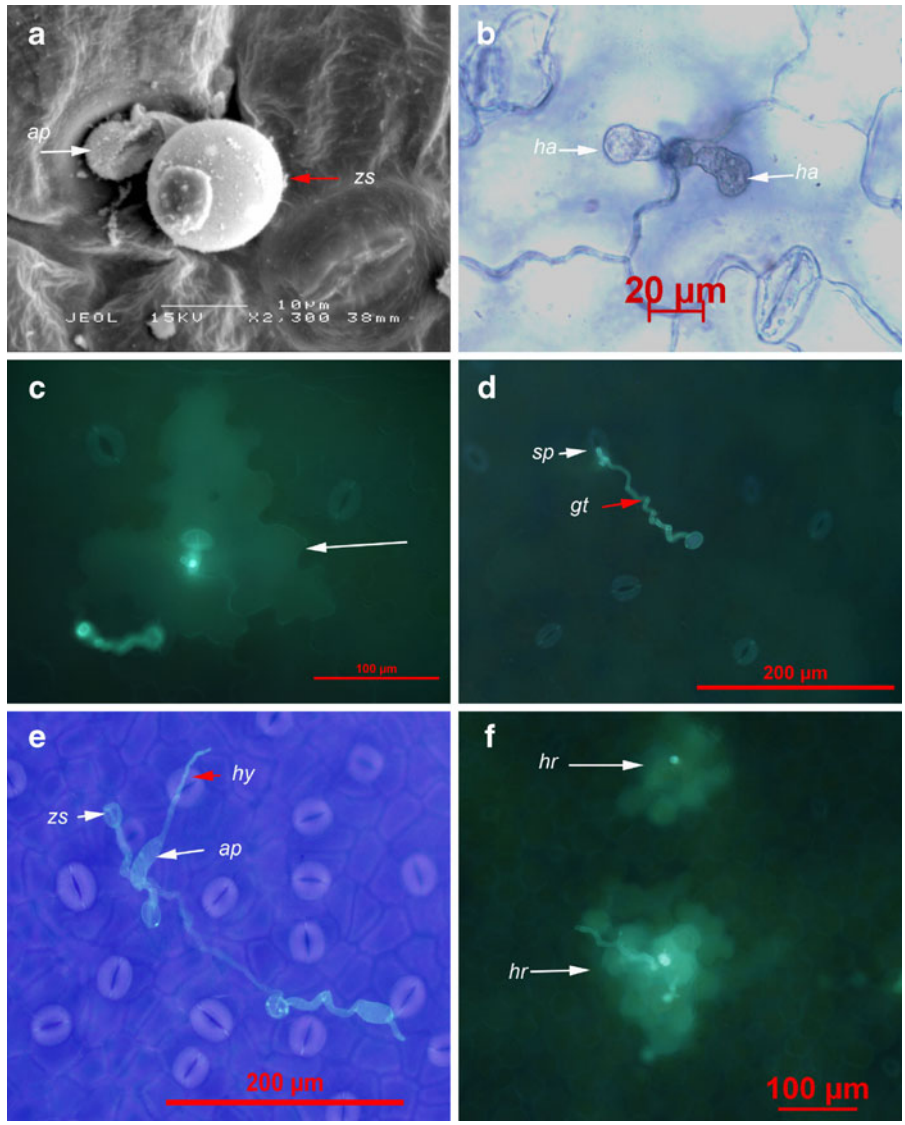

Fig. 1 Formation of infection structures of *Hyaloperonospora parasitica* on host and non-host plant. Samples for Fig. 1. **a**, scanning electron microscopy image; **b,** fresh cotyledon/leaf cleared in boiling 95% ethanol, stained with 1% cotton blue; **c**, **d, e** and **f**, fresh cotyledon/leaf cleared in 10% KOH and stained with 0.05% Aniline Blue in 0.067 M K2HPO4 (pH 9). **a,** direct penetration (*white arrow*) of epidermal cell of *Brassica napus* cv Thunder TT by a germ tube from a zoosporangium (*red arrow*). **b**, one haustorium (*arrows*) was observed in each of the epidermal cells proximal to the penetrating hypha of *B. rapa* after 4 hpi. **c**, infected cells in *B. rapa* developed a distinctive fluorescence (*arrow*) under uv light 4 hpi. **d**, indirect penetration through stomata (*white arrow*) of *Trifolium subterraneum* from a long germ tube (red arrow). **e**, failure of penetration, the appressorium getting larger (*white arrow*) with a hypha growing out of the appressorium, on *Lupinus angustifolius*. **f,** typical hypersensitive reaction (*arrows*) on *B. carinata* 3 dpi. *ap*: appressorium, *gt*: germ tube, *sp*: stomatal penetration, *ha*: haustorium, *hy*: hypha, *zs*: zoosporangia

epidermal cell, was the first to show a hypersensitive reaction. Callose deposition at the point of penetration was observed in the host species *B. carinata* and *B. rapa* and in the non-host *P. sativum*.

At 3 dpi, there were significant differences in the colonisation development of the pathogen across the different host species. In the highly susceptible *B. napus* cv. Thunder TT, the pathogen produced intercellular hyphae that ramified around many cells with multiple branches and haustoria, eventually colonizing much of the cotyledon tissues. By this stage, haustoria were broad and conspicuous, reach-

ing 20 μm in length and occupying much of the lumen of individual infected host cells. On the partially resistant *B. napus* cv. Pioneer, *B. oleracea* and *B. nigra*, the hyphae grew extensively producing fully formed haustoria, although the area of tissue colonised was less than that observed for *B. napus* cv. Thunder TT. However, at this stage, in the host species *B. juncea*, *B. carinata*, *B. rapa,* and *E. vesicaria* ssp. *sativa,* the hyphae still remained non-branched and had only grown a little longer than at 24 hpi. In these host species, not many new haustoria developed from the intercellular hyphae and penetrated cells showed a typical hypersensitive reaction (HR) (Fig. 1f), and a great of number of cells displayed a HR, including those adjacent to the penetrated cells (Fig. 2a). Intensive callose deposition at the point of penetration was observed on *B. carinata* and *S. alba*. On *S. alba* and *P. sativum*, there were little difference compared with what was observed at 24 hpi in relation to either the length of the hyphae or the number of haustoria present, but fully developed haustoria were observed in the proximal mesophyll cells (2b). On the host *R. raphanistrum* and on the other three non-hosts and on the glass slides, at this point of time (3dpi), appressoria were significantly larger than those observed at 24 hpi, with hyphae growing out from the appressoria. However, these hyphae rarely penetrated or produced huastoria in these particular species.

At 5 dpi, in the highly susceptible *B. napus* cv. Thunder TT, hyphae with many branches and many haustoria were observed, and the whole cotyledon was colonised and hyphae had proceeded to grow into the petiole and towards the stem. On the partially resistant *B. napus* cv. Pioneer, and on *B. oleracea* and *B. nigra,* hyphae were seen to have colonised most of the cotyledon with branches and many haustoria present. There were, however, no visible external disease symptoms evident to the naked eye on these three species at this point of time. On *B. juncea*, *B. carinata*, *B. rapa*, *S. alba, E. vesicaria* ssp. *sativa* and the non-host *P. sativum,* there were lesions typical of hypersensitive reaction. No further progression of infection structures was evident in either the host *R. raphanistrum* or in the three non-host species.

At 7 dpi, the sporangiophores were fully developed on *B. napus*, *B. oleracea* and *B. nigra*, and hyphae were also observed growing within the petiole and towards the stem. However, most severe disease symptoms were observed on *B. napus* cv. Thunder TT. Hypersensitive reaction lesions were clearly evident on the host species *B. juncea*, *B. carinata*, *B. rapa*, and *E. vesicaria* ssp. *sativa*. No sporangiophores were visible on the plants that showed hypersensitive reactions. Visible hypersensitive reactions were not evident on *S. alba* or *P. sativum.* No disease symptoms were observed on the host species *S. alba*, *R. raphanistrum* nor on any of the non-host species studied.

Discussion

This study showed clear separation of the reactions of the tested species to *H. parasitica* into four groups.

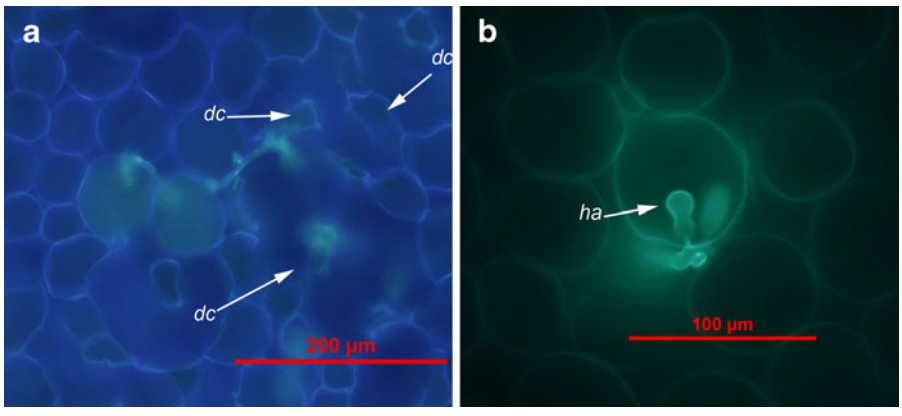

Fig. 2 Formation of infection structures of *Hyaloperonospora parasitica* on host plant. All samples cleared in 10% KOH and stained with 0.05% Aniline Blue in 0.067 M K2HPO4 (pH 9). **a,** additional cells dead adjacent to the penetrated cells (*arrows*) on *B. carinata* 3 dpi. **b**, a fully developed haustorium in a *Sinapis alba* mesophyll cell 3 dpi. *dc*: dead cells associate HR, *ha*: haustorium

Group 1, comprised of the highly susceptible hosts, represented by *B. napus* cv. Thunder TT and *B. oleracea*, with highest disease scores. Group 2, comprised of susceptible hosts, including *B. napus* cv. Pioneer, and *B. nigra* that responded with moderate disease scores. This group of hosts reacted to *H. parasitica* similarly to Group1, but overall disease severity was lower and disease progression was slower. Group 3, showed HR to *H. parasitica*, represented by host species *B. juncea*, *B. carinata*, *B. rapa*, and *E. vesicaria* ssp. *sativa*. Group 4, included host crucifer species *S. alba*, *R. raphanistrum* and all non-host species, all of which showed no disease symptoms. *Hyaloperonospora* has only recently been separated from *Peronospora* (Constantinescu and Fatehi 2002). *H. parasitica* contains isolates from diverse species of Brassicaeae, and also of other closely related families such as Resedaceae and Capparidaceae and, remarkably, even the unrelated Cistaceae (Constantinescu and Fatehi 2002). While the pathogenicity of *H. parasitica* varies at both the host generic and the species levels, the basis for its particular host range still remains un-clear. For example, *S. alba* and *R. raphanistrum* have been reported as host plants for *H. parasitica* (Dickinson and Greenhalgh 1977). However, in our study, *R. raphanistrum* had a similar response to *H. parasitica* as the non-host legumes *Trifolium subterraneum* and *L. angustifolius*, while *S. alba* responded similarly to the non-host legume *P. sativum*. Whether the reaction of *S. alba* or *R. raphanistrum* to this isolate of *H. parasitica* is truly pathotype specific still remains to be clarified. Based on infection progression and disease severity from our study, there were no clear cut-offs at the anatomical level which separate species that are hosts of *H. parasitica* from species that are non-hosts to *H. parasitica*.

The high levels of zoosporangial germination on all species tested as well as on glass slides suggest that inhibition of germination does not play a significant role in the expression of host and/or non-host resistance. A similar observation was made by Heath (1974) in relation to non-host resistance to cowpea rust (*Uromyces phaseoli* var. *vignae*). In our study, appressoria were formed on all tested species as well as on glass slides by 8 hpi. However, there were significant differences in terms of both the number and the size of appressoria among the host and non-host species. On all host species and on the non-host *P. sativum,* zoosporangia sometimes formed germ tubes and occasionally failed to do so, but, in all cases where direct penetration was initiated, apressoria in the form of swollen discs were formed. In contrast, while zoosporangia formed germ tubes on *R. raphanistrum*, *L. angustifolius, Trifolium subterraneum*, *Triticum aestivum* and on glass slides, apressoria, however, were either absent or only in low numbers but with an average size greater than that those on other plant species tested. For *L. angustifolius* and *Trifolium subterraneum,* penetration occurred directly or through stomata, but for *Triticum aestivum* there was only very low occurrence (7%) of direct penetration. In *R. raphanistrum*, *L. angustifolius, Trifolium subterraneum*, *Triticum aestivum*, the attempted penetration appears to have been initiated. However, no further developments were observed. The appressoria on the surface of epidermal cell were expanded and a new hypha extended from the appressoria growing out on the surface of the epidermis. Such restriction in progression of penetration was previously reported for bean rust on bean by Wynn (1976). Wynn (1976) observed when penetration failed with the first appressoria over stomata, hypha continued to grow toward a second stomata to form another appressoria. The branched infection hyphae were unable to penetrate the plant and remained on the surface. In our study, the formation of larger appressoria and the growth of a hypha growing out of each of the appressoria on glass slides and on these four species, together suggest that at least the development processes of the pathogen up to this point may be primarily determined by factors other than specific signals from plants. Little is known about the signalling associated with or required for appressorium formation on plant surfaces. Although surface hydrophobicity and hardness can be sufficient to stimulate appressorium development in some pathogens (e.g., Carzaniga et al. 2001; Tucker and Talbot 2001), certain pathogens are known to require specific signals to develop infection structures (e.g., the sensing of cutin by *Colletotrichum* spp. (Dickman et al. 2003), or sensing the height of the guard cell lip by *Uromyces appendiculatus* (Allen et al. 1991). In our study, *H. parasitica* was able to penetrate both host and non-host plants, suggesting that the penetration stage is not critical in determining the success or failure of pathogenesis. However, there were significant differences in relation to the frequency and

timing of penetration among the tested species. Interestingly, biotrophy requires an impressive series of cellular interactions between the plant and the parasite (Lebeda et al. 2001; Mendgen and Hahn 2002).

Haustoria were formed either inside the epidermal and/or the mesophyll cells across all tested species at different stages post inoculation. However, there were significant differences in terms of the frequency and timing of haustorial formation and in the final size of haustoria among the tested species. Fully developed haustoria were never observed in *R. raphanistrum*, *Triticum aestivum, L. angustifolius* or *Trifolium subterraneum*. Instead, the haustorium development appears to cease, collapse or abort in penetrated epidermal and/or the mesophyll cells of these species. Although haustoria formed in the epidermal and/or mesophyll cells of *S. alba* and *P. sativum,* further hyphal growth was rare and only a few additional haustoria developed. Taken together, this suggests that there is some inhibition to haustorium formation on these particular species. Overall, our findings suggest that *H. parasitica* is able to initiate an infection of plant tissue up to the stage at or immediately following first formation of one or more haustoria, which then induces a rapid activation of defence response(s) in infected cells or tissues. This results in the cessation of further progress in infection. This is in agreement with Lebeda et al. (2008) that plant resistance is mostly related to recognition (pre-haustorial or post-haustorial) limiting pathogen intercellular hypha growth and haustoria formation. The restriction of haustoria formation may be a crucial limiting factor in host–pathogen communication and play an important role in expression of resistance (Lebeda et al. 2006).

Callose deposition was detectable after KOH treatment and aniline blue staining of plant tissues. In *B. carinata*, the rapid accumulation of callose at the point of penetration of the cell wall of infected cotyledons was observed as early as 8 hpi. After 24 h, callose deposits were easily visible under fluorescence at the point of penetration of hosts *B. carinata*, *B. rapa*, and in the non-host *P. sativum*. No callose deposition was evident in un-inoculated control plants nor in inoculated *R. raphanistrum*. Similar observations were made by Gindro et al. (2003) in and around the stomatal cells of grapevine infected by *P. viticola*; by Diez-Navajas et al. (2008) in non-host infected by *P. viticola*; by Sedlářová and Lebeda (2001) for lettuce (*Lactuca* spp.) infected by *B. lactucae*; and by Heintz and Blaich (1990) around haustoria of *Uncinula necator* in grapevines resistant to powdery mildew.

Hypersensitive reaction was found to be a typical response to infection by *H. parasitica* in resistant hosts and in the non-host *P. sativum*. In host species such as *B. rapa*, *B. carinata, B. juncea*, *B. nigra*, *B. oleracea* or *S. alba*, a significant proportion of penetrated cells showed a yellow fluorescence by 8 hpi, and for *E. vesicaria* ssp. *sativa* and in non-host *P. sativum* at 24 hpi. The yellow fluorescence observed in these species was strongly associated with a hypersensitive reaction. Similar observations were made by Dai et al. (1995) in grapevine infected by *Plasmopara viticola* and by others (Bennett et al. 1996; Lebeda et al. 2008) in lettuce cells challenged by *Bremia lactucae*. This fluorescence may have been caused by the accumulation of phenolics (Bennett et al. 1996) and of lignin-like deposits produced by peroxidase mediated cross-linking (Bolwell 1993; Fry 1986; Graham and Graham 1991; Scalbert et al. 1985). In partially resistant species like *Vitis rupestris* (Dai et al. 1995; Unger et al. 2007) as well as in the partially resistant grapevine cultivar Solaris (Gindro et al. 2003), necrosis appeared as necrotic spots only several days post infection by *P. viticola*. The hypersensitive response is an effective defence mechanism against both biotrophs and non-biotrophs alike because of the accompanying up-regulation of a multitude of ‘defence’ responses that produce a strongly antimicrobial environment in and around the dead cells (Koch and Slusarenko 1990; Goodman and Novacky 1994; Hua Li et al. 2004; 2007). Interestingly, in our study, the hypersensitive reaction-like events (e.g., early enhancement of yellow fluorescence, and cell death around the penetrated cells) were observed in *S. alba* and *P. sativum* despite the absence of a visible hypersensitive response. At all stages of the infection processes we observed, susceptible responses were characterized by the absence of those reactions seen in incompatible interactions. Hypersensitive responses in the resistant host species, and also in one non-host, typically occurred during the early stages of infection. This is in contrast to the rapid colonisation of plant tissue, pathogen reproduction and secondary infection cycles that were observed on susceptible *B. napus* cv. Thunder TT.

In conclusion, the comparison of infection processes on different cruciferous host plants (resistant and susceptible genotypes) and on leguminous and graminaceous non-host species by *H. parasitica* indicated the critical stages during infection which determine the outcome of pathogenesis. We propose that each stage beyond initial penetration could represent a 'switching point' (Heath 1974) at which the plant response(s) determines the subsequent progress or limitation of the infection resulting in compatible or incompatible interactions. Even though both the host and non-host resistance responses appear to have certain similarities, it remains to be determined if these responses involve the same or different signal transduction pathways. Although the interactions of *H. parasitica* with *Arabidopsis thaliana* have been used as model for studies on signaling pathways it is clear that such studies should first be conducted on the pathogen interaction with closely related host species as well as non-host plants, both at phenotypic as well as histological levels.

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References

Allen, E. A., Hazen, B. E., Hoch, H. C., Kwon, Y., Leinhos, G. M. E., Staples, R. C., et al. (1991). Appressorium formation in response to topographical signals by 27 rust species. *Phytopathology, 81*, 323–331.

Bennett, M., Gallagher, M., Fagg, J., Bestwick, C., Paul, T., Beale, M., et al. (1996). The hypersensitive reaction, membrane damage and accummulation of autofluorescent phenolics in lettuce cells challenged by *Bremia lactucae*. *The Plant Journal, 9*, 851–865.

Bolwell, G. P. (1993). Dynamic aspects of the plant extracellular matrix. *InternationalReview of Cytology, 146*, 261–324.

Carzaniga, R., Bowyer, P., & O'Connell, R. J. (2001). Production of extracellular matrices during development of infection structures by the downy mildew *Peronospora parasitica*. *New Phytologist, 149*, 83–93.

Channon, A. G. (1981). Downy mildew of Brassicas. In D. M. Spencer (Ed.), *The downy mildews* (pp. 321–336). London: Academic.

Constantinescu, O., & Fatehi, J. (2002). *Peronospora*-like fungi (Chromista, Peronosporales) parasitic on Brassicaceae and related hosts. *Nova Hedwigia, 74*, 291–338.

Dai, G. H., Andary, C., Mondolot-Cosson, L., & Boubals, D. (1995). Histochemical studies on the interaction between three species of grapevine, *Vitis vinifera*, *V. rupestris* and *V. rotundifolia* and the downy mildew fungus, *Plasmopara viticola*. *Physiological and Molecular Plant Pathology, 46*, 177–188.

Dickinson, C. H., & Greenhalgh, J. R. (1977). Host range and taxonomy of *Peronospora* on crucifers. *Transactions of the British Mycological Society, 69*, 111–116.

Dickman, M. B., Ha, Y. S., Yang, Z., Adams, B., & Huang, C. (2003). A protein kinase from *Colletotrichum trifolii* is induced by plant cutin and is required for appressorium formation. *Molecular Plant-Microbe Interactions, 16*, 411–421.

Diez-Navajas, A. M., Wiedemann-Merdinoglu, S., Greif, C., & Merdinoglu, D. (2008). Nonhost versus host resistance to the grapevine downy mildew, *Plasmopara viticola*, studied at the tissue level. *Phytopathology, 98*, 776–780.

Donofrio, N. M., & Delaney, T. P. (2001). Abnormal callose response phenotype and hypersensitivity to *Peronospora parasitica* in defense-compromised Arabidopsis *nim1-1* and salicylate hydroxylase-expressing plants. *Molecular Plant-Microbe Interactions, 14*, 439–450.

Fry, S. C. (1986). Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Annual Review of Plant Physiology, 37*, 165–186.

Ge, X. T., Li, H., Han, S., Sivasithamparam, K., & Barbetti, M. J. (2008). Evaluation of Australian *Brassica napus* genotypes for resistance to the downy mildew pathogen, *Hyaloperonospora parasitica*. *Australian Journal of Agricultural Research, 59*, 1030–1034.

Gindro, K., Pezet, R., & Viret, O. (2003). Histological study of the responses of two *Vitis vinifera* cultivars (resistant and susceptible) to *Plasmopara viticola* infections. *Plant Physiology and Biochemistry, 41*, 846–853.

Goodman, R. N., & Novacky, A. J. (1994). *The hypersensitive reaction in plants to pathogens. A resistance phenomenon*. St. Paul: APS.

Graham, M. Y., & Graham, T. L. (1991). Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytophthora megasperma* f. sp. *glycinea* wall glucan. *Plant Physiology, 97*, 1445–1455.

Heath, M. C. (1974). Light and electron microscope studies of the interactions of host and non-host plants with cowpea rust-*Uromyces phaseoli* var. *vignae*. *Physiological Plant Pathology, 4*, 403–414.

Heath, C. M. (1980). Reactions of nonsuscepts to fungal pathogens. *Annual Review of Phytopathology, 18*, 211–236.

Heath, M. C. (2000). Nonhost resistance and nonspecific plant defences. *Current Opinion in Plant Biology, 3*, 315–319.

Heintz, C., & Blaich, R. (1990). Ultrastructural and histochemical studies on interactions between *Vitis vinifera* L. and *Uncinula necator* (Schw.) Burr. *New Phytologist, 115*, 107–117.

Holub, E., & Cooper, A. (2004). Matrix, reinvention in plants: how genetics is unveiling secrets of non-host disease resistance. *Trends in Plant Science, 9*, 211–214.

Howlett, B. J., Ballinger, D. J., & Barbetti, M. J. (1999). Diseases of canola in Australia. In P. A. Salisbury, T. D. Potter, G. McDonald, & A. G. Green (Eds.), *Canola in Australia: the first thirty years* (pp. 47–52). Canberra: Organising Committee of the 10th International Rapeseed Congress.

Jahnen, W., & Hahlbrock, K. (1988). Differential regulation and tissue-specific distribution of enzymes of phenylpropanoid pathways in developing parsley seedlings. *Planta, 173*, 197–204.

Kamoun, S., Huitema, E., & Vleeshouwers, V. G. A. A. (1999). Resistance to oomycetes: a general role for the hypersensitive response? *Trends in Plant Science, 4*, 196–200.

Koch, E., & Slusarenko, A. (1990). Arabidopsis is susceptible to infection by a downy mildew fungus. *The Plant Cell, 2*, 437–445.

Lebeda, A., Pink, D. A. C., & Mieslerová, B. (2001). Host–parasite specificity and defense variability in the *Lactuca* spp.–*Bremia lactucae* pathosystem. *Journal of Plant Pathology, 83*, 25–35.

Lebeda, A., Sedlářová, M., Lynn, J., & Pink, D. A. C. (2006). Phenotypic and histological expression of different genetic backgrounds in interactions between lettuce, wild *Lactuca* spp., *L. sativa* x *L. serriola* hybrids and *Bremia lactucae*. *European Journal of Plant Pathology, 115*, 431–441.

Lebeda, A., Sedlářová, M., Petřivalský, M., & Prokopová, J. (2008). Diversity of defence mechanisms in plant-oomycete interactions: A case study of *Lactuca* spp.-*Bremia lactucae*. *European Journal of Plant Pathology, 122*, 71–89.

Li, H., Sivasithamparam, K., Barbetti, M. J., & Kuo, J. (2004). Differences in the germination and invasion by ascospores and pycnidiospores of *Leptosphaeria maculans* on canola varieties with varying susceptibility to blackleg. *Journal of General Plant Pathology, 70*, 261–269.

Li, H., Kuo, J., Barbetti, M. J., & Sivasithamparam, K. (2007). Differences in the responses of stem tissues of spring-type *Brassica napus* cultivars with polygenic resistance and single dominant gene-based resistance, to inoculation with *Leptosphaeria maculans*. *Canadian Journal of Botany, 85*, 191–203.

Mauch-Mani, B., & Slusarenko, A. J. (1993). *Arabidopsis* as a model host for studying plant-pathogen interactions. *Trends in Microbiology, 1*, 265–270.

Mauch-Mani, B., & Slusarenko, A. J. (1996). Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *The Plant Cell, 8*, 203–212.

McDowell, J., Cuzick, A., Can, C., Beynon, J., Dangl, J. L., & Holub, E. B. (2000). Downy mildew (*Peronospora parasitica*) resistance genes in *Arabidopsis* vary in functional requirements for *NDR1*, *EDS1*, *NPR1* and salicylic acid accumulation. *The Plant Journal, 22*, 523–529.

Mellersh, D. G., & Heath, M. C. (2003). An investigation into the involvement of defense signaling pathways in components of the nonhost resistance of *Arabidopsis thaliana* to rust fungi also reveals a model system for studying rust fungal compatibility. *Molecular Plant Microbe Interactions, 16*, 398–404.

Mendgen, K., & Hahn, M. (2002). Plant infection and the establishment of fungal biotrophy. *Trends in Plant Science, 7*, 352–356.

Nashaat, N. I., Heran, A., Awasthi, R. P., & Kolte, S. J. (2004). Differential response and genes for resistance to *Peronospora parasitica* (downy mildew) in *Brassica juncea* (mustard). *Plant Breeding, 123*, 512–515.

Paul, V. H., Klodt-Bussmann, E., Dapprich, P. D., Capelli, C., & Tewari, J. P. (1998). Results on preservation, epidemiology, and aggressiveness of *Peronospora parasitica* and results with regard to the disease resistance of the pathogen on *Brassica napus*. *Bulletin OILB/SROP, 21*, 49–56.

Satou, M., & Fukumoto, F. (1996). The host range of downy mildew, *Peronospora parasitica*, from *Brassica oleracea*, cabbage and broccoli crops. *Annals of the Phytopathological Society of Japan, 62*, 393–396.

Scalbert, A., Monties, B., Lallemand, J. Y., Guittet, E., & Rolando, C. (1985). Ether linkage between phenolic acids and lignin fractions from wheat straw. *Phytochemistry, 24*, 1359–1362.

Sedlářová, M., & Lebeda, A. (2001). Histochemical detection and role of phenolic compounds in defence response of *Lactuca* spp. to lettuce downy mildew (*Bremia lactucae*). *Journal of Phytopathology, 149*, 693–697.

Silue´, D., Nashaat, N. I., & Trilly, Y. (1996). Differential responses of *Brassica oleraceae* and *B. rapa* accessions to seven isolates of *Peronospora parasitica* at the cotyledon stage. *Plant Disease, 80*, 142–144.

Tucker, S. L., & Talbot, N. J. (2001). Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annual Review of Phytopathology, 39*, 385–417.

Unger, S., Büche, C., Boso, S., & Kassemeyer, H. H. (2007). The course of two different *Vitis* genotypes by *Plasmopara viticola* indicates compatible and incompatible host–pathogen interactions. *Phytopathology, 97*, 780–786.

Viennot-Bourgin, G. (1981). Downy mildew of Brassicas. In D. M. Spencer (Ed.), *The downy mildews* (pp. 1–15). London: Academic.

Williams, P. H. (1985). 'Downy mildew.' Crucifer Genetics Cooperative (CrGc) Resource, Madison WI, University of Wisconsin, USA.

Wynn, W. K. (1976). Appressorium formation over stomates by the bean rust fungus: response to a surface contact stimulus. *Phytopathology, 66*, 136–146.

Yun, B. W., Atkinson, H. A., Gaborit, C., Greenland, A., Read, N. D., Pallas, J. A., et al. (2003). Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in *Arabidopsis* against wheat powdery mildew. *The Plant Journal, 34*, 768–777.

Zhou, N., Tootle, T. L., & Glazebrook, J. (1999). *Arabidopsis* PAD3, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. *Plant Cell, 11*, 2419–2428.