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Phenotypic and histological expression of different genetic backgrounds in interactions between lettuce, wild *Lactuca* spp., *L. sativa* × *L. serriola* hybrids and *Bremia lactucae*

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Abstract

Phenotypic and histological responses of cultivated lettuce (*Lactuca sativa*) and wild relatives *L. saligna, L. virosa* as well as interspecific crosses derived from *L. sativa* × *L. serriola* to two races of *Bremia lactucae* (CS2, CS9) were investigated. With the exception of *L. sativa* genotypes, all accessions and hybrids expressed incomplete or complete resistance to both pathogen races, with slight differences at seedling and adult plant stages, respectively. Histological features of the interactions (development of pathogen infection structures and host hypersensitive response to attempted infection) were studied on leaf discs 48 h after inoculation. Interactions with similar phenotypic expression of resistance were characterized by significant variation in rate of development of pathogen infection structures and hypersensitive reactions. Differences found within eight *Lactuca* spp. accessions and hybrids challenged by two distinct pathogen races are interpreted and discussed.

Abbreviations: dai – days after inoculation; hai – hours after inoculation; HR – hypersensitive reaction; ID – infection degree; IH – intercellular hyphae; IMD – irreversible membrane damage; IS – infection site; ITRH – intracellular hyphae; ITERH – intercellular hyphae; NC – number of necrotic epidermal cells per infection site; PV – primary vesicle; SV – secondary vesicle; SEN – subepidermal necrosis; PN – proportion of infection sites with necrotic epidermal cells

Introduction

Breeding for resistance to lettuce downy mildew (*Bremia lactucae*) has mostly been based on utilizing race-specific *Dm* genes and/or R-factors (resistance factors) (Lebeda et al., 2002). To date, at least 20 *Dm* genes or R-factors have been introduced into lettuce cultivars (Pink, 2002; Lebeda and Zinkernagel, 2003a) and more than 40 *Dm* genes and/or R-factors are known (Lebeda et al., 2002; Michelmore et al., 2003). The lack of durability of race-specific resistance (conferred mostly by single dominant genes) in commercial cultivars is caused by the rapid occurrence of new 'matching' virulence factors (v-factors), driven by the fact that the genetic structure of pathogen populations evolves in a tight micro-coevolutionary process with that of its host (Lebeda and Zinkernagel, 2003b). The high evolutionary potential of *B. lactucae* (*sensu* McDonald and Linde, 2002) for rapid adaptation to new *Dm genes* is determined by a number of factors: a short period

of asexual reproduction (ca. 1 week) which conditions early selection of aggressive races, rapid spread of conidia by wind (migration and gene-flow between pathogen populations in crop pathosystems (*L. sativa*) and also possibly between the crop and wild hosts (*L. serriola*) pathosystems) and a heterothallic sexual mating system (conservation of mutations and new recombinations) (Lebeda et al., 2002).

Recently, lettuce breeding strategies have focused on obtaining more durable resistance to *B. lactucae*. One approach is the utilization of field (rate-reducing), or race-nonspecific or non-host resistance (Jeuken and Lindhout, 2002; Lebeda et al., 2002; Mauch-Mani, 2002) through marker-assisted selection (MAS) for quantitative trait loci (QTLs) (e.g. Jeuken and Lindhout, 2002; Hand et al., 2003). An alternative strategy to obtain durable resistance is through the managed deployment of R-factors (e.g. Pink, 2002; Pink and Hand, 2002). Deployment strategies include growing of multilines and/or cultivar mixtures (Pink and Puddephat, 1999) and the accumulation (pyramiding) of several resistance genes in a lettuce cultivar (Pink, 2002). The latter approach would be particularly effective if the different R-factors determined different resistance mechanisms. The exploitation and utilization in lettuce breeding of wild *Lactuca* germplasm offers a source of resistance genes which may determine different resistance mechanisms (Lebeda et al., 2002; Beharav et al., 2006).

Bremia lactucae as a biotrophic parasite grows in the living cells of its host. Host reactions following contact with the pathogen vary according to diverse genetic backgrounds of *Lactuca* spp.–*B. lactucae* interactions (Lebeda et al., 2001, 2002; Mansfield et al., 1997). The different mechanisms of resistance in accessions of *Lactuca* spp. (race-specific, race-non-specific) are characterized by sequences of events that cannot be distinguished macroscopically. However, there are substantial observable differences at the tissue and cell levels including timing and rate of pathogen infection structure development and occurrence of hypersensitive response (HR) (Maclean and Tommerup, 1979; Lebeda et al., 2001, 2002), irreversible membrane damage (IMD) (Woods et al., 1988), accumulation of phenolics (Bennet et al., 1996; Sedlářová and Lebeda, 2001) and occurrence of oxidative stress (Bestwick et al., 2001). Previous time-course experiments showed the broad variation of responses in the *Lactuca* spp.–*B. lactucae* pathosystem and possible relationship between development of infection structures and host defence (for review see Lebeda et al., 2001, 2002)). Data concerning histological components of host–pathogen interactions thus provide important background information for the utilization of wild *Lactuca* spp. and their hybrids in lettuce breeding.

The objective of our work was to advance the knowledge of infection processes and defence responses (pathogen infection structures formation, HR occurrence) that determine host resistance or susceptibility to highly virulent races of *B. lactucae* in well-defined *Dm*(R)–*Avr* gene combinations and in the reaction of *L. sativa* × *L. serriola* hybrids.

Materials and methods

Plant material and cultivation

Following preliminary research carried out at Warwick HRI Wellesbourne (UK) eight genotypes of *Lactuca sativa*, *L. serriola*, *L. saligna*, *L. virosa* and breeding lines derived from *L. sativa* × *L. serriola* crosses were selected for detailed histological studies. The basic characteristics of the plant material and reactions of the genotypes to the two races of *B. lactucae* used in this study are presented in Table 1.

Seedlings were grown as described by Lebeda and Pink (1998). Six weeks after sowing four plants per accession and four leaves from each plant (4th–6th youngest true leaves) were excised to prepare leaf discs of 10 mm diam (5 per leaf). Discs were randomized within inoculation boxes (Ward Propagator 35.5 × 21.5 × 17.0 cm; Ward of Darlaston, UK) and sampled for histological analysis (48 hai) and macroscopic assessment of symptoms and sporulation intensity. Two boxes were used for each set of observations. Moreover, 20 whole seedlings (7 days after sowing) per accession were used to compare *Lactuca* spp. cotyledon tissue reactions.

Pathogen isolates, maintenance, inoculation and incubation

Screening of *Lactuca* spp. accession responses was performed with two *B. lactucae* races – CS2

Table 1. Description of *Lactuca* spp. and *L. sativa* × *L. serriola* interspecific hybrids regarding country of origin, principal mechanism of resistance to *Bremia lactucae*, known genetic background and phenotypic responses to races CS2 and CS9

Lactuca spp./ interspecific cross	Cultivar/line/ accession	Country of origin	Type of resistance	*Dm* gene (R-factor)	Response[a] to		References
					CS2	CS9	
L. sativa	Cobham Green	UK	Race-specific	*Dm* ? (*Dm* 0?)	+	+	Lebeda and Blok (1991)
L. sativa	Saladin	USA (Salinas)	Race-specific	*Dm*5/8,7,13	+	+	Gordon (pers. comm.)
L. sativa	Mariska	Holland	Race-specific	R18	+[b]	−	Lebeda and Blok (1991)
L. saligna	LSA/6 (HRIGRU 10.006386)	Czechoslovakia	?	*Dm* ?	−	−	Lebeda (1986)
L. virosa	LVIR/57/1	UK	Race-specific	*Dm* ?	−	−	Lebeda (1986)
Hilde × *L. serriola* (Swedish) F_7BC_1	CSP 93289	UK (HRI)[c]	Race-specific	*Dm* ?	(−)	−	
L. sativa	Hilde	Belgium	Race-specific	R12	+	+	Lebeda and Blok (1991)
L. serriola	'Swedish'	Sweden [d]	Race-specific	*Dm* ?	−	−	Lebeda and Zinkernagel (2003a)
Malika × (Hilde × *L. serriola* (Swedish) F_8BC_3	CLX 1364	UK (HRI)	Race-specific	*Dm* ?	(+)	−	
L. sativa	Malika	UK	Race-specific	*Dm*5/8,7	+	+	Pink (unpubl.)
Malika × (Hilde × *L. serriola* (Swedish) F_8BC_3	CLX 1366	UK (HRI)	Race-specific	*Dm* ?	(+)	−	

[a]Scoring of interactions: −, incompatible (no sporulation); (−), incompletely incompatible (very limited sporulation (1–25%), mostly followed by tissue necrosis or chlorosis); (+), incompletely compatible (reduced sporulation (25–50%) found in the tested set of seedlings and leaf discs); +, compatible (profuse sporulation).
[b]Resistant in the seedling stage.
[c]Material of the same genetic background was described as CS-RL (Lebeda and Blok, 1991; Lebeda and Zinkernagel, 2003a).
[d]The original parental accession of *L. serriola* ("Swedish") is not available (see Lebeda and Blok, 1991). ? Reaction not known; expected type of resistance; *Dm* gene(s) not characterized.

(*Avr*6 + *Avr*11 + *Avr*16 + *Avr*17 + *Avr*18 + *Avr*36 + *Avr*37 + *Avr*38) (sextet code according to IBEB denomination system: EU-A-31/59/01/00) (Lebeda and Blok, 1991) and CS9(*Avr*16 + *Avr*17 + *Avr*18 + *Avr*36 + *Avr*37 + *Avr*38) (EU-A-63/63/01/00) (Van Ettekoven and van der Arend, 1999; modified for *Dm*7).

The pathogen was maintained and multiplied on seedlings of *L. sativa* L. cv. Cobham Green (*Dm*0) grown in glass crystallizing dishes in controlled conditions of a growth chamber (15 °C, 12 h photoperiod, 100 μ Em^{-2} s^{-1} provided by warm-white fluorescent GroLux tubes (F65W/840, Polylux XL, UK). Inoculum was prepared by washing 2–3 day-old conidia off infected seedlings in distilled water. As phenotype responses may vary with different ontogenetic stages, both seedlings and discs from mature true leaf tissue were examined for each interaction. Either cotyledons (for sporulation assessment) or undersides of leaf discs (for both sporulation assessment and histological studies) were inoculated with a suspension of freshly harvested spores (concentration 5×10^4 ml^{-1}) using a glass sprayer. Boxes with seedlings/leaf discs were then incubated at 15 °C, those for the histological studies were kept for 48 hai in the dark, and those for evaluation of sporulation intensity were transferred after 24 hai in darkness to the growth chamber with 12 h photoperiod (Lebeda and Pink, 1998).

Evaluation of intensity of sporulation

Data on sporulation intensity were recorded at 2-day intervals up to 14 days after inoculation (Table 2), using a 0–3 scale (0 = symptomless, 1 = isolated sporophores present, 2 = < 50% and 3 = > 50% cotyledon area covered with sporophores) for cotyledons and a 0–4 scale (0 = symptomless; 1 = < 25%, 2 = < 50%, 3 = < 75% and 4 = > 75% disc area covered with sporophores) for true leaf discs (Lebeda, 1986). Different scoring of seedlings is commonly used to diminish the error in semiquantitative evaluation on small areas of cotyledons. Intensity of sporulation (the degree of infection, DI) was expressed as a percentage of the maximum scores (Lebeda, 1986) according to the following formula: $P = \sum (n \cdot v) \cdot 100/x \cdot N$; where: P = the total degree of infection, n = number of cotyledons/discs in each category of infection, v = the category of infection, x = the range of the scale (i.e. $x = 3$ for 0–3 and $x = 4$ for 0–4 scale), N = the total number of evaluated discs.

Histological analysis

Leaf discs (20 per genotype/isolate combination) were collected 48 hai, fixed for 2 days in 100% acetic acid, cleared and stored in an aqueous solution of chloral hydrate (1.7 g ml^{-1}). For microscopic observation the discs were mounted in

Table 2. Sporulation intensity of *Bremia lactucae* (race CS2 and CS9) on seedlings (cotyledons) and leaf discs (derived from 4th to 6th youngest true leaves of 6 weeks old plants) of studied *Lactuca* spp. genotypes 14 days after inoculation

Lactuca spp./interspecific cross	Sporulation (%)[a]			
	Cotyledons		Leaf discs	
	Race of *Bremia lactucae*			
	CS2	CS9	CS2	CS9
L. sativa cv. Cobham Green	93.4	92.3 C	92.5	100.0
L. sativa cv. Saladin	46.6	75.3 C, HR	96.2	100.0
L. sativa cv. Mariska	6.9 C, HR	0.0 C, HR	72.5 N	0.0
L. saligna (LSA/6)	0.0	0.0	0.0	0.0
L. virosa (LVIR/57/1)	0.0	0.0	0.0 N, SE	1.2 N, SE
Hilde × *L. serriola* (Swedish), CSP 93289	4.6 C, HR	0.0	22.5 N, SE	0.0
Malika × *L. serriola* (Swedish), CLX 1364	50.0 N	0.0	35.0 N, SE	0.0
Malika × *L. serriola* (Swedish), CLX 1366	50.5 N	0.0	37.5 N, SE, SN	0.0

[a]Mean values; C, Chlorosis of seedlings; HR, Hypersensitive response (local necrotic spots on cotyledons); N, Necrosis of seedlings/Necrotic response; SE, Sporulation along the cut surface of leaf discs; SN, Sporulation mostly from the necrotic spots.

50% glycerol. Formation of infection structures (germ-tubes, primary and secondary vesicles, haustoria, infection hyphae) and responses of host tissue (HR of epidermal and mesophyll cells) were recorded on the leaf disc area, with the exception of a 250 μm edge along the cut surface (Lebeda and Reinink, 1994; Lebeda and Pink, 1998). Four randomly selected leaf discs were used for microscopic evaluation.

Length of *B. lactucae* germ-tubes formed on the surface of *Lactuca* spp. leaf discs was measured microscopically 48 hai in μm. At least 60 measurements were performed for each host (genotype)–pathogen (race) interaction, i.e. at least 15 values of germ-tube length were measured per leaf disc.

On each leaf disc the frequency (total numbers) of infection structures were recorded. Except primary vesicles (PV, occurrence of a PV means successful penetration of *B. lactucae*), the frequency of other infection structures was calculated as a proportion (percentages) of the structures from which they were derived (Tables 4, 5). Plant tissue response was recorded as frequency of hypersensitive reactions (HR, hypersensitively reacting cells were distinguished by cytoplasm granulation and darkening) associated with individual penetrations (PV formation); number of necrotic epidermal cells per necrotic spot (NNC), occurrence of subepidermal necrosis (SEN), expressed as proportion of infection sites producing SEN; and degree of SEN (DSEN), recorded on the scale: 0 = no necrotic cells present, 1 = one to three necrotic cells, 2 = four to five necrotic cells, 3 = six or more necrotic cells (Lebeda and Pink, 1998).

Statistical analysis

The data on germ-tube lengths were analysed by maximum likelihood. Separate variance components were fitted for variability in germ-tube lengths within and between the different plants/leaf discs. The hypothesis that all accessions had the same germ-tube length was tested by comparing the change in the deviance of the model due to varying accessions means to a Chi-square distribution with seven degrees of freedom.

Differences in the number of primary vesicles per leaf disc were tested using a generalized linear model (McGullagh and Nelder, 1989) with a Poisson distribution and log link function. For proportions (percentages), a generalized linear model with a binomial distribution and probit link was used (Lebeda and Pink, 1998). Significance levels are given for comparison with *L. sativa* cv. Cobham Green and *L. saligna* (LSA/6), the susceptible and resistant controls, respectively. All programmes used to analyse data were written in Genstat.

Results

Intensity of sporulation

The reaction phenotypes of the *Lactuca* spp. differed substantially over 14 dai. The results for sporulation intensity and macroscopic symptoms of resistance are summarized in Table 2 for both isolates (CS2 and CS9), for cotyledons and for leaf discs. The first sporulation was observed 6 days after inoculation. The breeding lines (Hilde × *L. serriola* (Swedish), Malika × *L. serriola* (Swedish) (CLX 1364 and CLX 1366)) showed complete resistance to race CS9 and an intermediate resistance to CS2 compared to the parental genotypes.

Generally, in interactions with race CS2, partially resistant genotypes and hybrids expressed a delay and reduction in sporulation. Cultivar Mariska was resistant at the seedling stage but susceptible at the adult stage.

Interactions with race CS9 were incompatible in all accessions except the known susceptible *L. sativa* cvs. Cobham Green and Saladin. Differences were recorded between leaf discs from adult plants compared to cotyledons. Cotyledons of cvs. Saladin, Mariska (*L. sativa*) and Hilde × *L. serriola* (Swedish) showed a higher degree of resistance than leaf discs. Both Mariska (resistant) and Saladin (susceptible) showed chlorosis and HR of cotyledons but no HR was observed in adult plants of cv. Saladin. In *L. virosa* a weak sporulation with necrosis occurred on leaf discs along the cut surface (Table 2).

Formation of pathogen infection structures and expression of host resistance

The infection processes of the host–pathogen interactions (*Lactuca* spp. and *L. sativa* × *L. serriola* hybrids–*B. lactucae*) were investigated 48 hai. Values were compared to the susceptible (*L. sativa* cv. Cobham Green) and the resistant (*L. saligna* LSA/6) controls, respectively.

Germination

Germ-tube length was highly variable for both pathogen races (CS2 and CS9). Variation in length of germ-tubes did not relate to host resistance, i.e. to *Dm* gene expression. However it appear to be specific for each host genotype–pathogen race interaction (Table 3).

Development of infection structures

Primary vesicles
The number of primary vesicles (PV) was examined 48 hai and data are given in Table 4. The highest number of PV formed by either pathogen race was found in the susceptible cv. Saladin (*L. sativa*). In contrast a very low number of PV was recorded in resistant control LSA/6 (*L. saligna*). In interactions with race CS2 the lowest number of PV was recorded in Hilde × *L. serriola* (Swedish), CSP 93289 which showed an incompletely resistant phenotype. The number of PV was also low in the compatible interaction with cv. Mariska (*L. sativa*). For *Lactuca* spp. interactions with race CS9, low numbers of PV correlated with resistance (Table 4). Only in the resistant CLX 1366 (*L. sativa* × *L. serriola* hybrid) the number of PV approached the values found for the susceptible control cultivar Cobham Green.

Secondary vesicles
The frequency of other infection structures was calculated as a percentage of the structures from which they were derived (Tables 4, 5). The frequency of secondary vesicles (SV), given as the percentage of PV developing SV, is summarized in Table 4. The lowest frequencies were recorded in *L. saligna* (LSA/6) for both isolates.

A surprisingly high frequency of PV developing SV was recorded on *L. virosa* (LVIR/57/1) and the three hybrids (CSP 93289, CLX 1364, CLX 1366) which all displayed a resistant phenotype to CS9. Values for race CS2 and formation of PV and SV in both hybrids Malika × (Hilde × *L. serriola*) (CLX 1364 and CLX 1366) were similar to those found for CS9 (Table 4) despite the fact that these two hybrids displayed a partially susceptible phenotype to this isolate (Table 2).

Hyphae
Formation of hyphae was expressed as the percentage of secondary vesicles forming intra- (ITRH) and intercellular (ITERH) hyphae (Table 5). In *L. saligna* (LSA/6) several ITRH of

Table 3. Length of germ-tubes of races CS2 and CS9 of *B. lactucae* in leaf discs of different *Lactuca* spp. 48 h after inoculation (hai)

Lactuca spp./cultivar/accession/interspecific cross	Length (μm)[a]	
	Race of *Bremia lactucae*	
	CS2	CS9
L. sativa		
Cobham Green	63.2	81.8[s]
Saladin	56.3	44.4[c]
Mariska	76.7[s]	79.4[s]
L. saligna (LSA/6)	55.5	29.0[c]
L. virosa (LVIR/57/1)	55.6	31.9[c]
Hilde × *L. serriola* (Swedish), CSP 93289	87.8[c,s]	86.0[s]
Malika × (Hilde × *L. serriola* (Swedish)), CLX 1364	51.3	64.5[s]
Malika × (Hilde × *L. serriola* (Swedish)), CLX 1366	61.0	64.9[s]

[a]Mean values; [c,s]Significantly different ($P \leq 0.05$) from *L. sativa* cv. Cobham Green and *L. saligna* (LSA/6), susceptible and resistant control, respectively.

Table 4. Development of *B. lactucae* races CS2 and CS9: number of primary vesicles (PV) and percentage of primary vesicles forming a secondary vesicle (SV) 48 h after inoculation (hai)

Lactuca spp./cultivar/accession/interspecific cross	Number of PV/leaf disc		Percentage SV/PV	
	Race of *Bremia lactucae*			
	CS2	CS9	CS2	CS9
L. sativa				
Cobham Green	82.7^{s}	132.7^{s}	94^{s}	97^{s}
Saladin	151.5^{c,s}	198.0^{c,s}	95^{s}	96^{s}
Mariska	31.5^{c}	42.0^{c,s}	81^{c,s}	86^{c,s}
L. saligna (LSA/6)	27.2^{c}	11.7^{c}	32^{c}	21^{c}
L. virosa (LVIR/57/1)	83.7^{s}	73.0^{c,s}	83^{c,s}	80^{c,s}
Hilde × *L. serriola* (Swedish), CSP 93289	23.7^{c}	62.0^{c,s}	79^{c,s}	90^{c,s}
Malika × (Hilde × *L. serriola* (Swedish)), CLX 1364	59.7^{s}	65.5^{c,s}	87^{s}	87^{c,s}
Malika × (Hilde × *L. serriola* (Swedish)), CLX 1366	53.7^{s}	111.7^{s}	87^{s}	93^{c,s}

^{c,s}Significantly different ($P \leq 0.05$) from *L. sativa* cv. Cobham Green and *L. saligna* (LSA/6), respectively.

CS2 emerged with no ITERH; no development of hyphae was observed of race CS9. Generally, in the resistant genotypes (*L. virosa* (LVIR/57/1) and the interspecific hybrids CSP 93289, CLX 1364, CLX 1366) a high percentage of intracellular hyphae was recorded but with a relatively low percentage of intercellular hyphae compared to the number found in susceptible cvs Cobham Green and Saladin.

Haustoria

The data are expressed as the percentage of SV producing haustoria (Table 5). High values characterized susceptible *L. sativa* genotypes, and significantly lower values the resistant genotypes (Tables 2, 5). No haustoria were recorded in the resistant control *L. saligna* (LSA/6). Haustorium formation was lower for race CS9 than for race CS2.

Expression of host resistance at tissue and cell levels

The percentage of penetrations associated with hypersensitive reactions (HR) was counted to quantify the degree of HR in epidermal and subepidermal tissues (Table 6). The extent of the HR response was expressed as the number of necrotic/hypersensitive cells per infection site. The lowest percentage of infection sites (IS) with necrotic epidermal cells (4%) was found in *L. saligna* (LSA/6) with a single cell per IS (Table 6). In contrast

Table 5. Development of *B. lactucae* races CS2 and CS9: percentage of secondary vesicles forming intra- or intercellular hyphae and haustoria 48 hai

Lactuca spp./cultivar/accession/interspecific cross	ITRH/SV		ITERH/SV		H/SV	
	Race of *Bremia lactucae*					
	CS2	CS9	CS2	CS9	CS2	CS9
L. sativa						
Cobham Green	65^{s}	78^{s}	67^{s}	80^{s}	76^{s}	84^{s}
Saladin	58^{s}	77^{s}	67^{s}	80^{s}	75^{s}	83^{s}
Mariska	58	42^{c}	39^{c,s}	7^{c}	57^{s}	16^{c}
L. saligna (LSA/6)	31^{c}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}
L. virosa (LVIR/57/1)	52^{c}	49^{c,s}	30^{c,s}	22^{c}	36^{c,s}	36^{c}
Hilde × *L. serriola* (Swedish), CSP 93289	43^{c}	54^{c,s}	36^{c,s}	15^{c}	44^{c,s}	20^{c}
Malika × (Hilde × *L. serriola* (Swedish)), CLX 1364	44^{c}	67^{s}	41^{c,s}	17^{c}	56^{c,s}	39^{c}
Malika × (Hilde × *L. serriola* (Swedish)), CLX 1366	45^{c}	75^{s}	34^{c,s}	28^{c}	55^{c,s}	40^{c}

ITRH, Intracellular hyphae; ITERH, Intercellular hyphae; H, Haustoria

^{c,s}Significantly different ($P \leq 0.05$) from *L. sativa* cv. Cobham Green and *L. saligna* (LSA/6), respectively.

Table 6. Histological response of *Lactuca* spp. to infection by *B. lactucae* races CS2 and CS9: occurrence and extent of epidermal and subepidermal necrosis 48 hai

Lactuca spp./cultivar/accession/interspecific cross	HR (%)		NNC		SEN (%)		DSEN	
	Race of *Bremia lactucae*							
	CS2	CS9	CS2	CS9	CS2	CS9	CS2	CS9
L. sativa								
Cobham Green	37	63	1.0	1.0	0.9^{s}	0.4^{s}	0.2	0.2
Saladin	4^{c}	13^{c}	1.0	1.0	0.2^{s}	0.0	0.2	0.0
Mariska	22	85	1.0	1.2	1.6	20.2^{c}	0.2	1.5
L. saligna (LSA/6)	4^{c}	4	1.0	1.0	20.2^{c}	27.3^{c}	1.0	1.1
L. virosa (LVIR/57/1)	85^{c,s}	87	1.3	1.2	43.2^{c,s}	59.6^{c,s}	2.2	2.0
Hilde × *L. serriola* (Swedish), CSP 93289	49	80	1.1	1.2	12.6^{c}	9.7^{c}	0.9	1.4
Malika × (Hilde × *L. serriola* (Swedish)), CLX 1364	44	91^{s}	1.0	1.3	0.4^{s}	37.7^{c}	0.2	1.9
Malika × (Hilde × *L. serriola* (Swedish)), CLX 1366	61^{s}	82	1.3	1.3	12.5^{c}	34.6^{c}	1.3	1.9

HR, Hypersensitive response, percentage of infection sites with necrotic epidermal cells; NNC, Mean number of necrotic epidermal cells per infection site with a necrotic response; SEN, Subepidermal necrosis, percentage of infection sites producing subepidermal necrosis; DSEN, Degree of SEN (see Materials and methods) ^{c,s}Significantly different ($P \leq 0.05$) from *L. sativa* cv. Cobham Green and *L. saligna* (LSA/6), respectively.

this resistant genotype showed a relatively high percentage of subepidermal necrosis (SEN) (Table 6). With this exception, a high degree of epidermal HR was positively related with resistance to either race CS2 (*L. virosa* (LVIR/57/1)) or race CS9 (all genotypes except the susceptible cvs. Cobham Green and Saladin). The expression of HR to both races by more than one cell per IS was typical for *L. virosa* and the interspecific breeding lines and was also found in the response of cv. Mariska to race CS9.

Higher values for subepidermal necrosis (SEN) both in terms of occurrence and extent were associated with the incompatibility of the interaction. Highest values of SEN were recorded in *L. virosa* (LVIR/57/1) corresponding well with values of NNC and DSEN (Table 6).

Discussion

Macroscopic assessment of disease progress followed by detailed microscopical studies were performed to understand the development of two *B. lactucae* races (CS2, CS9) within tissues of *L. sativa, L. saligna, L. virosa* and of *L. sativa* × *L. serriola* hybrids expressing different level of resistance. *Lactuca serriola* and other wild *Lactuca* spp. are used in breeding programmes as donors of new resistance factors (Lebeda and Zinkernagel, 2003b). However, few data exist on histological aspects of the interactions and mechanisms of resistance.

The influence of plant ontogenic stage on disease development was observed in the interactions between *L. sativa* (cv. Mariska and cv. Saladin) and *B. lactucae* (race CS2) as increased sporulation intensity in adult plants (Table 2). A similar phenomenon was recorded previously in *L. sativa* (cvs. Iceberg and Great Lakes)–*B. lactucae* (race NL15) interactions and has been explained by incomplete expression of R-genes in young plants (Lebeda and Reinink, 1991).

No evidence was found that length of germ-tubes is directly associated with resistance as proposed in previous studies (Lebeda and Reinink, 1991; Lebeda et al., 2001). Variation in germ tube length (Table 3) may reflect rather leaf surface characteristics (e.g. composition and amount of waxes, number of trichomes) although these also relate to the level of resistance and influence the pathogen infection cycle, e.g. adhesion of spores, appressorium and penetration peg formation (Mieslerova et al., 2004). However, it is not possible to be definitive about this since knowledge of the chemical composition of *Lactuca* spp. epicuticular waxes is still lacking (Jenks and Ashworth, 1999).

Penetration rate, as seen from PV number (Table 4), differed between the two isolates and clearly demonstrated that this feature is specific for host genotype–pathogen isolate interaction. In *L. saligna* (LSA/6) an extremely low frequency of

penetrations was combined with a lack of haustorial formation thus resulting in efficient pathogen restriction at the stage of SV. A similar phenomenon was observed also in other *L. saligna–B. lactucae* interactions (Lebeda and Reinink, 1994) indicating that resistance distinct from classical race specificity with HR exists among *L. saligna* accessions (Lebeda et al., 2002).

The rate of pathogen SV formed from PV in susceptible plants (94–97%) was mostly significantly different in comparison with resistant genotypes (79–93%) (Table 4). However, the variation was not as great as found for PV indicating that there is no strict physiological repression inside a cell to the formation of SV (compare with Lebeda et al., 2002). The only exception was *L. saligna* (LSA/6) where only 21% and 32% of PV, respectively, were able to form SV. This supports previous conclusions that at least in some *L. saligna* genotypes specific cell mechanisms able to restrict pathogen development in the early stages of infection process are operating (Lebeda et al., 2001).

Mechanisms giving resistant phenotypes are active at various levels and stages of development. In our studies (Tables 2, 4), a good correlation was found between resistance and a low percentage of SV forming intercellular (not intracellular) hyphae. Development of ITRH and ITERH was recorded (Table 5) at a level differing significantly from the *L. sativa* susceptible controls in all resistant plants, except for *L. saligna*. However, in all cases the formation of ITERH was substantially lower in comparison with ITRH. This relationship was not described in previous studies (Lebeda and Reinink, 1994; Lebeda and Pink, 1998). In *L. virosa* (LVIR/57/1) development of lettuce downy mildew was inhibited in the later stage of the infection process (intercellular hyphae and haustoria) with a delay of necrosis. For some *L. saligna* accessions, Lebeda and Reinink (1994) reported differences in the extent of intercellular hyphae formation. This study clearly showed that at least in some *L. saligna–B. lactucae* interactions the formation of both types of hyphae is restricted or completely stopped. It has been proposed that ITERH of downy mildews provide an interface for nutrient uptake and general inter-communication (Spencer-Phillips, 1997). From this viewpoint, the restriction of ITRH/ITERH formation may be a crucial limiting factor in host–pathogen communication and play an important role in expression of resistance (Lebeda and Reinink, 1994; Lebeda and Pink, 1998). This assumption is supported by current data about the formation of haustoria. In this study, all resistant accessions were characterized by some degree of haustorial formation, at a level significantly different from the compatible controls (Table 5), the exception again being *L. saligna*, where development of haustoria was not recorded. The existence of qualitative as well as quantitative resistance in *L. saligna* is expected (Jeuken and Lindhout, 2002; Lebeda et al., 2002; Beharav et al., 2006).

For race-specific resistance, the significance of rapid cell death, i.e. hypersensitive reaction (HR) has been demonstrated (Kamoun et al., 1999; Jabs and Slusarenko, 2000). In contrast to other plant–pathogen interactions (e.g. *Arabidopsis thaliana–Hyaloperonospora parasitica*) (Mauch-Mani, 2002; Slusarenko and Schlaich, 2003), HR in the *Lactuca* spp. – *Bremia lactucae* pathosystem is mostly restricted to penetrated cells with primary infection structures (penetration peg and/or PV) (Lebeda et al., 2001), and is expressed in both compatible and incompatible interactions as a quantitative phenomenon but with significant contrasts in timing (Lebeda et al., 2002). In agreement with this, HR was recorded in all interactions in this study; there was significant quantitative variation (Table 6). All resistant interactions were usually associated with HR; however, the extent of the response was specific for each host genotype–pathogen isolate and in general, the occurrence of HR in compatible interactions was more frequent for CS9 than CS2. This phenomenon was closely associated with the number of necrotic cells per penetration site (Table 6) and the degree of subepidermal necrosis (SEN, DSEN; Table 6). These differences support the conclusion that the host–fungal race interaction could be highly specific (Sedlářová et al., 2001, 2002). The highest degree of HR was recorded by *L. virosa*, and closely associated with a high degree of NNC, SEN and DSEN (Table 6). These microscopical responses were also expressed on a phenotypic level as necrotic spots (Table 2). Our observations correspond very well with previous conclusions made for different *L. virosa* genotype–*B. lactucae* race interactions (Lebeda and Pink, 1998; Sedlářová et al., 2001), and support the previous assumption of race-specific

resistance in *L. virosa* (Lebeda and Boukema, 1991). However, the genetic and physiological basis of this resistance is not known (Lebeda et al., 2002). There have been attempts to relate the level of resistance in *L. virosa* and other wild *Lactuca* spp. to some antimicrobial compounds (Sessa et al., 2000). It was concluded that pathogen growth restriction cannot be explained by the presence of phenolic anticipins as no significant correlation was found in sesquiterpene lactone profile (compared in lettuce and wild *Lactuca* spp. genotypes) and resistance to *B. lactucae*.

Lactuca saligna is variable in terms of mechanisms of resistance to *B. lactucae* (Lebeda et al., 2002; Beharav et al., 2006). Lebeda and Reinink (1994) reported for the first time a very low frequency of HR in *L. saligna* (CGN 05271) inoculated with race NL5. A similar phenomenon was observed also by accession CGN 05271 (Sedlářová et al., 2001), and recently by LSA/6, where HR is accompanied by SEN (Table 6). These comprehensive data demonstrate that at least in some *L. saligna* accessions, resistance mechanisms are not based on microscopically evident HR during 48 hai. According to Mansfield et al. (1997), dead cells are not necessarily dark-pigmented until many hours after IMD. In the case of some *L. saligna* accessions the pathogen development is stopped very early during the first 24 hai (Lebeda and Reinink, 1994; Sedlářová et al., 2001) without microscopically evident destruction of cytoplasm until after 48 hai (Table 6). Some authors have discussed this form of resistance found in wild *Lactuca* spp. in terms of non-host resistance, generally accompanied by very weak epidermal cell necrosis. However, some reactions in *L. saligna* previously postulated as non-host (Lebeda, 1986; Lebeda and Boukema, 1991), have later been found to be race-specific controlled by 'major' qualitative genes (Beharav et al., 2006), or QTLs (Jeuken and Lindhout, 2002). Nevertheless, *L. saligna* remains a promising source of resistance to *B. lactucae* (Lebeda and Zinkernagel, 2003a; Beharav et al., 2006).

Further research of the various aspects of *Lactuca* spp.–*B. lactucae* pathosystem would disclose information important for understanding these interactions and inform the more efficient breeding of lettuce cultivars resistant to *B. lactucae* and the deployment of R factors to achieve durable resistance.

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