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Original Paper

Macroscopic and histological characterisation of genes *er1* and *er2* for powdery mildew resistance in pea

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Abstract

In pea, two single recessive genes, *er1* and *er2*, have been identified for resistance to powdery mildew caused by *Erysiphe pisi*, but little is known about their mode of action. Pea accessions carrying the genes *er1* or *er2* and other accessions displaying resistance to powdery mildew in the field were studied. In accessions carrying gene *er1*, epidermal cell penetration was prevented and very few haustoria or colonies were formed. Under controlled conditions, *er1* conferred complete or almost complete resistance to the fungal isolates used and this resistance was not associated with macroscopically visible necrosis. Under field conditions these accessions developed a low level of disease. Resistance in line JI2480 (carrying *er2*) increased with temperature and leaf age, and complete resistance was expressed only at high temperature (25 °C) or in mature leaves. This resistance was based mainly on post-penetration cell death, complemented by a reduction of percentage penetration success in mature leaves. Combining the resistance provided by gene *er1* and by line JI2480 into new cultivars is likely to increase their level of resistance and enhance durability of the protection.

Introduction

Dry pea (*Pisum sativum* L.) is the most widely grown grain legume in Europe and second-most in the world (FAOSTAT data, 2004). Powdery mildew, caused by *Erysiphe pisi*, affects all green parts of pea plants and can cause 25–50% yield losses (Munjal et al., 1963; Warkenting et al., 1996). The disease reduces not only seed yield but also seed quality (Tiwary et al., 1997a, b). It spreads rapidly during dry weather when nights are cool (Reiling, 1984). *Erysiphe pisi* overwinters on infected pea debris or on alternative hosts (Falloon and Viljanen-Rollinson, 2001). Secondary spread occurs via airborne conidia (Warkentin et al., 1996). Conidia germinate producing a germ tube with a lobed primary appressorium. A penetration peg emerges and if it penetrates successfully through the host cuticle and cell wall, a primary haustorium forms within the epidermal cell. Nutrient uptake from the plant cell through the haustorium supports development of secondary hyphae that radiate across the host epidermis forming hyphal appressoria from which secondary haustoria are formed (Falloon et al., 1989; Smith et al., 1996). Finally, aerial conidiophores emerge from surface hyphae producing conidia capable of initiating a new cycle of infection (Falloon et al., 1989).

The resistance genes to powdery mildew so far described are recessive, with many studies suggesting single gene inheritance (Harland, 1948; Pierce, 1948; Saxena et al., 1975). Heringa et al.

(1969) reported two independent recessive genes termed *er1* and *er2*. Genetic analyses have shown that gene *er1* is present in many resistant lines (Tiwari et al., 1997a).

Previous studies relied on genetic and macroscopic assessment of disease development, but the histological basis of the resistance conferred by the genes *er1* and *er2* remains unknown. The objective of the present investigation was to study the cellular mechanisms governed by these genes.

Materials and methods

Plant material

Eleven accession lines of pea reported to be resistant to powdery mildew were used. They were kindly provided by John Innes Centre, Norwich, UK, and are listed, together with their suggested genotypes (where known) in Table 1. Accession JI502 (cv. Rondo) and the commercial pea cv. Messire were included as susceptible controls. For all experiments involving controlled conditions, seedlings were grown, 3 per pot, in 1 l capacity pots filled with 1:1 sand-peat mixture (v/v) plus a fertiliser containing nitrogen, phosphorous, potassium, magnesium and trace elements (Osmocote Exact Mini; Scotts International), in ventilated glasshouses situated in Córdoba (Spain) or in Aberystwyth (UK) with no additional control of the environment.

Fungal material

Some experiments were performed in Aberystwyth (UK) and others in Córdoba (Spain). In the experiments carried out in Aberystwyth, an English *E. pisi* isolate was used. This isolate, termed JG, was supplied by JR Green, University of Birmingham, UK and maintained on susceptible pea cv. Kelvedon Wonder. In Spain, a local isolate termed CO-01 was used. Isolate CO-01 was derived from a population collected from naturally infected field plants at Córdoba (Spain) in 2001, and maintained on susceptible pea cv. Messire.

Experiments performed with detached leaves

Inoculation and incubation

Detached leaves were placed on agar technical 4 g l^{-1} containing benzimidazole (62.5 mg l^{-1}) (Rubiales et al., 1993) in Petri dishes and inoculated

Table 1. Mean powdery mildew severity (percentage of leaf or plant area covered by mycelium) developing after inoculation with *E. pisi* isolate JG or CO-01 on detached fourth-formed leaves or whole plants

Accession	Genotype	Reference	JG		CO-01	
			Detached 4th leaves[a]	Whole mature plant in greenhouse[b]	Detached 4th leaves[a]	Mature plants in the field[c]
JI502	*Er1Er2*	Heringa et al. (1969)	90 (1.25)[d]	100 (1.57)	60 (0.89)	87 (1.20)
JI1760	Unknown		100 (1.57)	60 (0.89)	–	70 (0.99)
JI2480	*Er1er2*	Tiwari et al. (1997a)	9 (0.31)	46 (0.75)	20 (0.46)	2 (0.08)
JI1559	*er1Er2*	Tiwari et al. (1997a)	0 (0.00)	1 (0.10)	0 (0.00)	2 (0.14)
JI2302	*er1Er2*	Heringa et al., 1969	0 (0.00)	0 (0.00)	0 (0.00)	33 (0.61)
JI210	*er1Er2*	Tiwari et al. (1997a)	0 (0.00)	5 (0.23)	–	38 (0.66)
JI1210	*er1Er2*	Tiwari et al., 1997a	0 (0.00)	0 (0.00)	–	37 (0.65)
JI1951	*er1Er2*	Tiwari et al. (1997a)	0 (0.00)	0 (0.00)	–	4 (0.20)
JI1213	Unknown		0 (0.00)	1 (0.10)	–	50 (0.79)
JI1412	Unknown		1 (0.10)	5 (0.23)	–	37 (0.65)
JI1566	Unknown		0 (0.00)	1 (0.10)	–	32 (0.60)
JI1747	Unknown		0 (0.00)	4 (0.20)	–	47 (0.76)
P-value			<0.001	<0.001	<0.001	<0.001
$LSD_{Bonferroni}$[e]			(0.18)	(0.08)	(0.13)	(0.55)

[a]Detached fourth-formed leaves incubated in a growth chamber at 20 °C for 8 dai.
[b]Whole plants inoculated in a greenhouse at Aberystwyth in August 2001 and scored 10 dai.
[c]Mean disease severity at the end of the crop cycle of plants naturally infected in the field (Córdoba) during 2000, 2001 and 2002.
[d]Figures in parentheses are angular transformation of percentage data.
[e]LDS critical value for comparison using Bonferroni correction.
– Indicates that the accession was not included in the experiment.

using a settling tower to give an inoculum density of about five conidia mm^{-2}. After inoculation, lids were fitted to the Petri dishes which were placed in a cabinet at the temperature required for each experiment. Incubation commenced with a 6 h light period (250 μmol m^{-2} s^{-1}) followed by cycles of 12 h darkness/12 h light.

Histological studies

Leaflets were laid, adaxial surface up, on filter paper moistened with a 1:3 (v/v) mixture of glacial acetic acid:absolute ethanol for fixation. When bleached, leaflets were transferred to filter paper moistened with tap water, left for 2 h to soften the tissues and then transferred to filter paper moistened with lactoglycerol (1:1:1, lactic acid:glycerol:water, v/v) until cleared (2 h) and for storage (Rubiales and Carver, 2000). For light microscope examination of fungal development, fungal structures were first stained so as to avoid displacing ungerminated spores, by spraying leaves lightly with a solution of 0.2% methyl blue in 95% ethanol (Carver et al., 2001) and observed without a coverslip (200× magnification). For every leaf, percentage germination was calculated by scoring each of 100 conidia for the presence of a germ tube. To assess further development, another 100 germinated sporelings were examined and classified according to whether they had each formed a simple germ tube but no appressorium, had formed an appressorium but no secondary hyphae (failed appressoria), or a colony had established as indicated by secondary hyphae emerging from the appressorial germ tube or mother conidium. Percentages were calculated for spore germination, for germinated sporelings forming an appressorium and for sporelings with an appressorium that established a colony. As a measure of colony size, the number of hyphal tips produced by each established colony (indicating the total number of hyphae) was counted for 20 randomly selected colonies.

To assess host cell death, indicating a hypersensitive response (HR) as a result of pathogen attack, 50 sporelings that had failed to establish a colony (sporelings that had formed an appressorium but no secondary hyphae) and a further 100 established colonies (sporelings with secondary hyphae) were examined on every leaf. Leaves were observed using bright field and differential interference contrast (DIC) microscopy. By bright field microscopy, the walls and contents of dead cells were discoloured yellow or brown, and by DIC the cell contents appeared granular and disorganised. Spray staining was not adequate to reveal fungal haustoria located within leaflet epidermal cells, so after initial assessments leaflets were immersed in trypan blue (0.05%) in lactophenol:ethanol (1:2 v/v) for 1 h at 60 °C to stain the haustoria. After fitting a coverslip, 20 colonies selected at random were examined (400× magnification) and counts made of the numbers of haustoria formed in each colony.

Comparison of E. pisi *development on accessions incubated at 20 °C*

All accessions were inoculated with isolate JG. As no important differences were observed among accessions having gene *er1* at the histological level (Table 2), only one accession representing each genotype [JI2302 (*er1Er2*), JI2480 (*Er1er2*) and JI502 (*Er1Er2*)] was studied with isolate CO-01.

Fourth-formed leaves, cut from plants that had formed their fifth leaves, were placed in Petri dishes using a randomised design with four replicates, each having three leaves of every accession line. They were inoculated with isolate CO-01 or JG in different experiments and incubated at 20 ± 0.5 °C. Preliminary studies indicated that hypersensitive response due to *E. pisi* attack is best observed at 3 days after inoculation (dai) when cytoplasmic discolouration and granulation in dead cells is fully developed so that death can be recognised with confidence and genotypic differences are maximised. Therefore, assessments of cell death were made using one leaf from each replicate fixed 3 dai. However, by this time colonies on susceptible lines were extremely well developed, with hyphae of neighbouring colonies becoming intertwined, making developmental assessments complex and inaccurate. Therefore, observations on spore germination, appressorium and haustorium formation, and colony size were performed on one leaf from each replicate fixed 2 dai. The remainder were incubated for 8 d, and then scored visually for powdery mildew severity (percentage of leaf area covered by mycelium).

Effect of temperature on resistance expression

Incubation temperature affects the speed of fungal growth (Paulech and Herrera, 1969) and may also affect the timing of plant responses to pathogen attack. To standardise timings of fixation in

Table 2. Mean values for developmental stages of *E. pisi* (isolate JG) and host responses assessed by light microscopy following 2 or 3 days of incubation on detached fourth-formed leaves of different pea accession lines

Accession Line	Genotype	2 Days of incubation					3 Days of incubation	
		Percentage of germination	Percentage of germinated sporelings forming appressorium	Percentage of germinated sporelings with appressorium that established a colony	Number of hyphal tips/colony	Number of haustoria/ colony	Percentage of failed appressoria[a] associated with epidermal cell death	Percentage of established colonies associated with epidermal cell death
JI502	*Er1Er2*	86.8 (1.20)[b]	98.7 (1.46)	90.9 (1.26)	5.1	2.6	44.5 (0.73)	3.5 (0.19)
JI1760	unknown	84.2 (1.16)	98.3 (1.44)	86.3 (1.19)	5.2	2.7	65.0 (0.94)	4.0 (0.20)
JI2480	*Er1er2*	81.0 (1.12)	97.5 (1.41)	77.1 (1.07)	1.5	1.4	40.0 (0.68)	2.0 (0.14)
JI1559	*er1Er2*	76.5 (1.07)	95.8 (1.36)	1.1 (0.11)	3.4[d]	1.0[d]	33.0 (0.61)	†
JI2302	*er1Er2*	89.2 (1.24)	99.3 (1.49)	0.0 (0.00)	†	†	34.5 (0.63)	†
JI210	*er1Er2*	90.7 (1.26)	100.0 (1.57)	0.0 (0.00)	†	†	36.0 (0.64)	†
JI1210	*er1Er2*	87.2 (1.21)	98.8 (1.46)	0.3 (0.06)	†	†	27.0 (0.55)	†
JI1951	*er1Er2*	82.2 (1.14)	96.8 (1.39)	1.0 (0.10)	†	†	18.5 (0.45)	†
JI1213	unknown	83.7 (1.16)	97.8 (1.42)	0.3 (0.06)	†	†	47.0 (0.76)	†
JI1412	unknown	81.2 (1.12)	97.8 (1.42)	2.3 (0.15)	3.2[d]	1.1[d]	13.5 (0.38)	†
JI1566	unknown	86.7 (1.20)	99.0 (1.47)	0.0 (0.00)	4.3[d]	1.0[d]	8.5 (0.30)	†
JI1747	unknown	77.2 (1.07)	97.8 (1.42)	0.0 (0.00)	†	†	22.5 (0.49)	†
P-value		0.055	0.057	<0.001	< 0.001	0.003	<0.001	0.084
$LSD_{Bonferroni}$[c]		–	–	0.059	1.97	0.82	(0.39)	–

[a]Sporelings that had formed an appressorium but no secondary hyphae.
[b]Figures in parentheses are angular transformation of percentage data.
[c]LDS critical value for comparison using Bonferroni correction.
[d]In accessions JI1412, JI1566 and JI1559 number of hyphal tips per colony and number of haustoria per colony data were excluded from statistical analysis because no colonies were formed in three of the replicates, and only few in the fourth one.
† indicates data omitted from analysis because too few colonies were formed to give reliable data.

relation to disease development, four detached fourth-formed leaves of JI502 (susceptible) for each incubation temperature and time of fixation combination were incubated at 15, 20 or 25 °C and fixed 1, 2 or 3 dai. Colony size was measured by counting numbers of hyphal tips for 20 colonies on each leaf. This showed that colonies incubated for 3 days at 15 °C were of similar size to those incubated for 2 days at 25 °C (LSD-test, data not shown). Therefore, these incubation periods were used to study the effect of temperature on resistance expression. Thus, four replicate detached leaves of accession JI502 (*Er1Er2*), JI2302 (*er1Er2*) and JI2480 (*Er1er2*) were inoculated (as above) with isolate JG and fixed for histological examination after incubation for 3 days at 15 °C and 2 days at 25 °C.

Four other leaves per isolate, genotype and temperature combination (15, 20 and 25 °C) were inoculated with isolates JG or CO-01 in different experiments and used for visual estimation of powdery mildew severity (percentage of leaf area covered by mycelium) and infection type (IT) 10 dai using a 0–4 scale (Moseman et al., 1965), where 0 = no visible sign of disease, and 4 = well developed, freely sporulating colonies.

Effect of leaf age on resistance expression

Accessions JI2302 (*er1Er2*), JI2480 (*Er1er2*) and the susceptible commercial pea cv. Messire were used. Eight plants of each accession were sown in pots (8 l capacity), one plant per pot, and grown in a greenhouse. A second sowing of eight plants per accession was made 12 weeks later (in 0.5 l capacity pots) and grown in the same greenhouse. When plants of the first sowing were at the pod filling stage and the second were at the fifth leaf stage, the fourth-formed leaf of each plant of both plantings was excised. In this way, fourth formed leaves of different ages were collected, the fourth leaf of pod-filling plants being fully mature while that of the second planting being only recently emerged. The detached leaves were inoculated in the settling tower in a series of four replicates, each having four detached leaflets (two leaves) of each accession and each age. Incubation was at 20 °C and one leaflet per accession and leaf age per replicate was fixed and stained at 2 dai, a second at 3 dai, while the remaining two leaflets of each treatment combination were maintained in the plates until 7 dai when disease severity was estimated visually.

Experiments performed with intact plants

To check the agreement between data from detached leaf tests with disease development on whole plants, accessions evaluated in detached leaves with isolate JG were also evaluated with this isolate using glasshouse-grown whole plants in Aberystwyth. In addition, to study the effectiveness of resistance conferred by genes *er1* and *er2* under Spanish field conditions, the collection of pea accessions was also evaluated in an experimental field plot located in Córdoba (Spain).

Disease assessments of glasshouse-grown whole plants

Two replicates of three plants of each accession were grown in a completely randomised block in a ventilated glasshouse with no additional control of environmental conditions, during August of 2001. During this month average air temperature at Aberystwyth was around 20/10 °C day/night. Plants were inoculated at flowering with isolate JG by shaking heavily sporulating plants over them. Disease severity (percentage of plant area covered by mycelium) was scored 10 dai.

Field experiments

Each accession was evaluated during 2000, 2001 and 2002 under field conditions at Córdoba (Spain) where heavy natural powdery mildew epidemics occur every year. Plants were sown in January in a deep loam soil (typical xerofluvent) in a randomised complete block design with two blocks, each block having 1 m rows with ten plants of each accession. No fertilisers or pesticides were used. During 2000 and 2001, only final disease severity was recorded at the end of the crop cycle, before plants senesced (early June). In 2002, disease severity (percentage of plant area covered by mycelium) was recorded three times at weekly intervals starting in mid May.

Statistical analyses

Statistical analyses were performed using Statistix 8.0 statistical package (Analytical Software, Tallahase, USA). Before performing analyses of variance, the normality and equality of variances

were checked using Shapiro–Wilk's (Shapiro and Wilk, 1965) and Bartlett's tests (Little and Hills, 1972) respectively. When necessary, percentage data were transformed to angles (y = arcsine$\sqrt{x/100}$) and again checked before applying analysis of variance. When statistically significant interactions between temperature and accession, or leaf age and accession, were detected, differences due to temperature or leaf age were analysed separately for each accession. Comparisons of means were performed by LSD (Least Significant Difference) test (Little and Hills, 1972) when a low number of means were compared. When a high number of means were compared Bonferroni correction (Dunn, 1961) was used. Null hypotheses were rejected when $P \leq 0.05$.

Results

Visual assessments of disease development under controlled conditions at 20 °C, in the greenhouse and in the field

Accession JI502 (genotype *Er1Er2*) was highly susceptible to both *E. pisi* isolates under all situations (Table 1) as was accession JI1760 (genotype unknown). Against isolate JG, accession JI2480, reported to carry gene *er2*, showed lower disease severity than the susceptible control in detached leaf and whole plant experiments (9% and 46%, respectively). Disease was also less severe in this accession when it was inoculated with isolate CO-01 under controlled conditions at 20 °C. In the field, leaves of JI2480 remained almost disease free even though stems of this line were infected. Accessions JI210, JI1210, JI1559, JI1951, JI2302 (reported to carry gene *er1*), and JI1213, JI1412, JI1566 and JI1747 (genotype unknown) were either completely resistant or highly resistant to isolate JG in both experiments. Thus, only a few colonies developed in accessions JI1559, JI210, JI1213, JI1412, JI1566 and JI1747 at the end of disease assessment period in the greenhouse-grown whole plants experiment. With isolate CO-01, JI2302 and JI1559 were completely resistant under controlled conditions. In the field, accessions JI1951 and JI1559 were highly resistant with little disease symptoms (2% and 4% final disease severity). The remaining accessions carrying gene *er1* showed incomplete resistance in the field showing a significant reduction in disease severity compared with the susceptible check. Thus, the susceptible control JI502 reached a final disease severity value of 87% while accessions carrying gene *er1*, excepting JI1951 and JI1559, reached 33–38% of plant area affected at the final scoring. With regard to the accessions with unknown genotype, final disease severity was also lower in accessions JI1412 and JI1566 than in JI502 whereas accessions JI1213, JI1747 and JI1760 did not differ significantly from JI502 in this parameter.

Assessments of powdery mildew development in the field (Figure 1) indicated that the disease developed rapidly in the susceptible accession JI502 and JI1760, whereas development was delayed on accessions JI1210, JI2302 (reported to carry gene *er1*), and JI1213, JI1412 and JI1566 (genotype unknown). Accessions JI1747 and JI210 were also less affected by the disease than JI502 until the second disease assessment date, when they were already almost senescent. JI2480 (reported to carry gene *er2*), JI1559 and JI1951 (reported to carry gene *er1*) remained almost disease free until the end of the cycle.

Histological analyses of pathogen development and host response at 20 °C

Histological results were similar for both *E. pisi* isolates and therefore data from only the most extensive experiment using isolate JG are shown in Table 2. There were no significant differences in the early stages of fungal development on the 12 accessions studied, i.e. in percentages of spores that germinated or of germinated conidia that formed appressoria. However, there were significant differences between accessions in the percentage of germinated sporelings with an appressorium that established a colony, in the size of colonies (indicated by the numbers of hyphal tips) and in number of haustoria formed per colony.

On the susceptible accession JI502, on JI1760 (genotype unknown), and on JI2480 (carrying *er2*), the vast majority of sporelings that formed appressoria went on to form colonies, whereas significantly fewer did so on accessions carrying the gene *er1* (JI1559, JI2302, JI210, JI1210, JI1951,), or on JI1213, JI1412, JI1566 and JI1747 (genotypes unknown). Colonies formed on the susceptible accessions JI502 and JI1760 developed

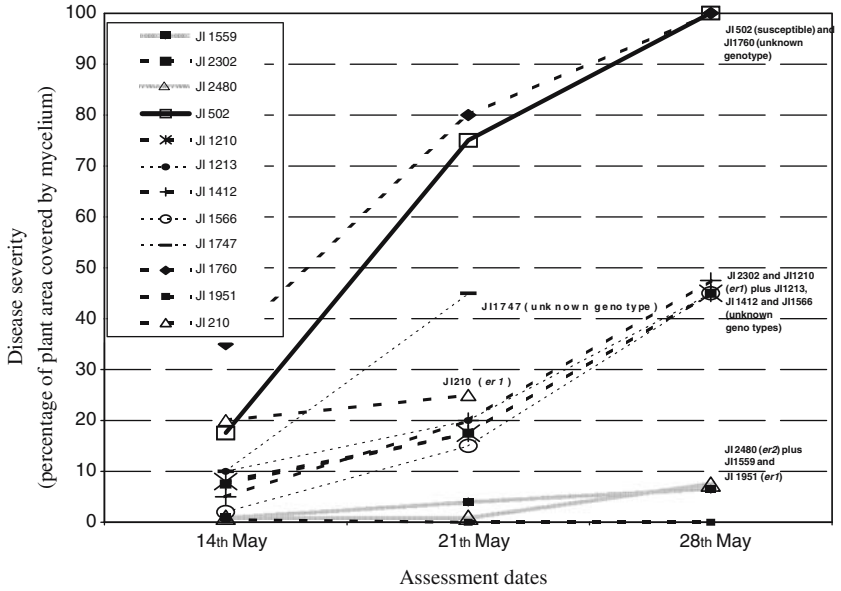

Figure 1. Development of powdery mildew severity scored weekly in a collection of eleven accession lines of pea reported to be resistant to this disease and the susceptible control JI502, under field conditions in Córdoba (Spain) during 2002. Accessions JI1747 and JI210 died after the second disease assessment date, and therefore, were not scored at the third assessment date.

well having more than five hyphal tips and an average of more than 2.5 haustoria (Table 2) after 2 days of incubation. After 3 days of incubation, only a very small percentage of colonies were associated with host cell death (Figure 2), indicating that most had established fully functional biotrophic relationships with their host. It was not possible to obtain data for colony development on most of the resistant lines because none or very few were formed. However, on JI2480 where sufficient colonies did form to give reliable data, they produced few haustoria at 2 dai (most had no more than the single primary haustorium). As a consequence, in JI2480, colonies were small and produced fewer secondary hyphae than on susceptible accessions. In accessions JI1559, JI1412 and JI1566, colonies were formed only in one of the four replicates performed. Colonies formed in this replicate tended to be smaller than in the susceptible check.

Effect of temperature on resistance expression

Results obtained from the macroscopic evaluation of disease severity were similar for both *E. pisi* isolates. Therefore we only present data obtained with isolate JG (Table 3), with which histological studies were also carried out. Accession JI502 was susceptible at all temperatures studied so that by 10 dai, a similar, high percentage of leaf area was covered by mycelium in all cases; colonies were well developed and sporulating freely, and there was no macroscopic evidence of necrosis in the host tissue. Accession JI2302 (*er1Er2*) was completely resistant at all temperatures, and again, no necrosis was visible on the leaves (infection type 0). On the other hand, disease development was clearly affected by temperature in accession JI2480 (*Er1er2*). At 15 °C, disease severity was higher than on the control JI502 and infection type was 4, as on JI502. At 20 °C, the percentage leaf area covered by mycelium was much lower than on JI502, but still there was no macroscopically visible necrosis. At 25 °C no visible mycelium was formed and many small necrotic flecks were visible on the leaf surfaces (infection type 1).

Colony size in the susceptible control was similar after 3 days of incubation at 15 °C and 2 days of incubation at 25 °C. Therefore, these incubation periods were used for histological comparisons between temperatures (Table 4). No differences in percentages of spore germination, of germinated sporelings forming appressoria (data not shown) or of germinated sporelings with an

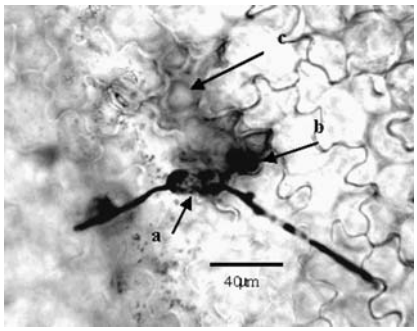

Figure 2. Hypersensitive response to *Erysiphe pisi* in accession JI2480 after 2 days of incubation at 25 °C. Observation was made using bright field and differential interference contrast. (a) Germinated spore. (b) Appressorium. (c) Dead epidermal cell.

appressorium that established a colony were observed in any of the accessions between 15 °C and 25 °C. There was a significant statistical interaction between accession and temperature in percentage of failed appressoria (sporelings with appressoria that failed to establish a colony) associated with epidermal cell death, established colonies associated with epidermal cell death and colony size (number of hyphal tips/colony). Thus, the effect of incubation temperature was analysed separately for each genotype. In the susceptible accession JI502, the percentage of failed appressoria where the host epidermal cell died as a result of attack, was similar at both temperatures. Furthermore, in this accession, very few established colonies were associated with cell death, irrespective of temperature. However, in accession JI2302 (*er1Er2*) the proportion of failed appressoria associated with epidermal cell death was significantly higher at 25 °C than at 15 °C. By contrast, in JI2480 (*Er1er2*) the percentage of failed appressoria associated with epidermal cell death did not increase with temperature. Here, however, there was a substantial increase in the percentage of established colonies associated with cell death (Figure 2) at 25 °C and this increased post-penetration cell death at 25 °C was associated with a reduction in colony size (number of hyphal tips per colony).

Effect of leaf age on resistance expression

Both recently expanded and fully mature fourth formed leaves of cv. Messire were susceptible although a lower percentage of leaf area was affected by *E. pisi* in the older leaves (Table 5). Leaves of accession JI2302 (*er1Er2*) were completely resistant at both ages. Accession JI2480 (*Er1er2*) displayed incomplete resistance in young leaves, with a reduced disease severity compared to Messire. Older, fully mature leaves, were completely resistant and no visible disease symptoms developed.

Statistically significant interactions were detected between genotypes and leaf age in all parameters studied. Therefore, the effect of leaf age in these parameters was analysed separately for each accession. The percentages of germinated sporelings with appressoria that established a colony, of established colonies associated with epidermal cell death and the size of the colonies were not significantly affected by leaf age in the susceptible cv. Messire. By contrast, in accession JI2480, significantly fewer germinated sporeling appressoria succeeded in establishing colonies on old compared to young leaves. In addition, colo-

Table 3. Mean disease severity (DS; percentage of leaf area covered by mycelium) of powdery mildew (*E. pisi* isolate JG) and infection type (IT; based on the 0–4 scale) 10 days after inoculation, on pea accessions incubated at different temperatures

Accession	Genotype	Incubation temperature					
		15 °C		20 °C		25 °C	
		DS	IT	DS	IT	DS	IT
JI502	*Er1Er2*	65 (0.94)[a]	4	65 (0.94)	4	73	4
JI2302	*er1Er2*	0 (0.00)	0	0 (0.00)	0	0	0
JI2480	*Er1er2*	73 (1.02)	4	25 (0.52)	4	0	1
P-value		<0.001	–	<0.001	–	–	–
LSD[b]		0.26	–	0.08	–	–	–

[a]Figures in parentheses are angular transformation of percentage data.
[b]LSD critical value for comparison.
– Indicates that the accession was not included in the experiments.

Table 4. Mean values for developmental stages of *E. pisi* (isolate JG) and host responses of pea accessions, incubated at 15 °C and studied 3 dai or incubated at 25 °C and studied 2 dai

Temperature	Accession (genotype)											
	JI502 (*Er1Er2*)				JI2302 (*er1Er2*)				JI2480 (*Er1er2*)			
	Percentage of germinated sporelings with appressorium that established a colony	Number of hyphal tips/ colony	Percentage of failed appressoria[a] associated with epidermal cell death	Percentage of established colonies associated with epidermal cell death	Percentage of germinated sporelings with appressorium that established a colony	Number of hyphal tips/ colony	Percentage of failed appressoria associated with epidermal cell death	Percentage of established colonies associated with epidermal cell death	Percentage of germinated sporelings with appressorium that established a colony	Number of hyphal tips/ colony	Percentage of failed appressoria associated with epidermal cell death	Percentage of established colonies associated with epidermal cell death
15 °C	94 (1.32)[b]	9	39 (0.67)	2 (0.14)	3 (0.17)	†	32 (0.60)	†	94 (1.32)	8	44 (0.73)	4 (0.20)
25 °C	83 (1.15)	8	41 (0.70)	1 (0.10)	0 (0.00)	†	63 (0.92)	†	86 (1.19)	3	41 (0.70)	32 (0.60)
P-value	0.111	0.174	0.885	<0.001	0.136	–	0.002	–	0.645	0.006	0.646	0.023
LSD[c]	–	–	–	(0.04)	–	–	(0.15)	–	–	3	–	(0.34)

[a]Sporelings that had formed an appressorium but no secondary hyphae.
[b]Figures in parentheses are angular transformation of percentage data.
[c]LSD critical value for comparison.
† Indicates that data were not analysed because too few colonies were formed to give reliable data.

Table 5. Mean values for developmental stages of *E. pisi* (isolate CO-01) and host responses 2 dai or 3 dai, and disease severity (percentage of leaf area covered by mycelium) 7 dai, in recently expanded and fully mature leaves of three accessions of pea incubated at 20 °C

Leaf age	Accession (genotype)											
	Messire (*Er1Er2*)				JI2302 (*er1Er2*)				JI2480 (*Er1er2*)			
	Percentage of germinated sporelings with appressorium that established a colony	Percentage of established colonies associated with epidermal cell death	Number of hyphal tips/colony	Disease severity (%)	Percentage of germinated sporelings with appressorium that established a colony	Percentage of established colonies associated with epidermal cell death	Number of hyphal tips/colony	Disease severity (%)	Percentage of germinated sporelings with appressorium that established a colony	Percentage of established colonies associated with epidermal cell death	Number of hyphal tips/colony	Disease severity (%)
Recently expanded	83 (1.15)[a]	20 (0.46)	7	66 (0.95)	0	†	†	0	87 (1.20)	8 (0.29)	5	26 (0.54)
Fully mature	72 (1.01)	35 (0.63)	6	42 (0.70)	0	†	†	0	20 (0.46)	70 (0.99)	1	0 (0.00)
P-value	0.265	0.055	0.252	0.003	–	–	–	–	0.010	<0.001	0.002	<0.001
LSD[b]	–	–	–	(0.13)	–	–	–	–	(0.58)	(0.22)	2	(0.10)

[a]Figures in parentheses are angular transformation of percentage data.
[b]LSD critical value for comparison.
† Indicates data were not analysed because too few colonies were formed to give reliable data.

nies formed on old leaves were smaller and a higher proportion was associated with epidermal cell death. In accession JI2302 no colonies were formed, irrespective of leaf age.

Discussion

Although a number of previous studies on genes *er1* and *er2* for powdery mildew resistance in pea used macroscopic assessment of disease development, this is the first study of the resistance conferred by these genes at the histological level. Intact plants gave data comparable to detached leaves indicating the validity of their use in large scale experiments where close control of incubation environment is required.

Histological investigation showed that in the susceptible control most (99%) penetration attempts results in successful colony formation. This was also the case (77%) in the *er2* carrying accession JI2480. Nevertheless, even in these lines a proportion of appressoria failed to establish colonies and a high proportion of these failures were in contact with host epidermal cells that had apparently died as a result of attack. Thus, in susceptible leaves lacking known resistance genes, the *E. pisi*-host cell interaction can lead to cell death similar to the hypersensitive response. This is also the case in cereal-*Blumeria graminis* interactions where some cell death is seen even in genetically 'compatible' situations (e.g. Carver et al., 1992). In comparison with the susceptible line, however, colony formation was extremely low in accessions carrying gene *er1* (JI1559, JI2302, JI210, JI1210, JI1951) where the vast majority of *E. pisi* sporelings formed an appressorium but no, or very few, colonies developed. The percentage of failed appressoria associated with host epidermal cell death in *er1*-carrying accessions was similar to, or lower than, the frequency seen in the susceptible control and in the *er2*-carrying accession. Therefore, although hypersensitivity is effective in preventing colony formation, in lines carrying gene *er1* the very low frequency of appressoria that established a colony seems to depend on a form of resistance acting in addition to the rapid death of attacked host cells. In these lines, no haustoria were visible using the microscope and this suggests a barrier to penetration. In cereals, epidermal cells attacked by *B. graminis* react to attack from appressoria by depositing epidermal cell wall appositions, termed papillae, which are thought to play a key role in penetration resistance (Zeyen et al., 2002). Using our microscope techniques, we could not detect equivalent structures in attacked pea cells. This, however, does not rule out the possibility that minute local wall modifications, undetectable by light microscopy or obscured by overlying appressoria, may be involved in penetration resistance by pea and influenced by the activity of *er1*.

Accession JI2302 (*er1Er2*) was completely resistant to both isolates under controlled conditions and in the greenhouse. The resistance was also totally effective at all temperatures studied and in both young and old leaves. Tiwari et al. (1997b) also reported that this accession showed almost complete resistance when inoculated with 26 different isolates of *E. pisi* in a detached leaf assay. In the field trials at Cordoba, most other accessions carrying *er1* showed effective but incomplete resistance, as they developed less powdery mildew than susceptible lines. In previous studies, JI2302 was also resistant in the field, being described as 'slightly susceptible' to 'fully resistant' (Cousing, 1965; Heringa et al., 1969; Tiwary et al., 1997a, b), and Tiwary et al. (1997b) reported that lines JI210 and JI1210 (also carrying *er1*) were highly to moderately resistant under field conditions at various locations. The reasons why most *er1* accessions showed near complete resistance in the growth chamber under controlled conditions and in the greenhouse, with the isolates used in the current investigation, and yet display only incomplete field resistance may be that inoculum load and disease pressure in the field is greater than that we employed under controlled conditions, and that this overcomes the resistance to some degree. Alternatively, it may be that other untested environmental factors have effects on expression of resistance. This remains to be elucidated.

The fact that accessions JI1559 and JI1951 remained almost free from powdery mildew in field experiments suggests that these accessions may carry an unknown resistance gene or genes in addition to *er1*. Similarly, Tiwary et al. (1997b) suggested that the high level of resistance in accession JI1559, as compared to other lines carrying *er1*, could be due to other modifier genes present in this line. JI1951 was also highly resistant against 26 different isolates in a detached leaf

experiment, and completely or highly resistant under field conditions at various locations (Tiwary et al., 1997b). According to Tiwari et al. (1997a), both accessions possess *er1* but not *er2*. Heringa et al. (1969) reported that accession JI1559 possesses genes *er1* and *er2*, but this conclusion was based simply on macroscopic assessments and not on genetic analysis. Thus, a separate undiscovered gene might be present in these accessions, increasing the effect of *er1* by preventing the development of the few established colonies that were seen to form on other *er1*-containing lines in the field tests. Histological analyses and field tests of the accessions of unknown genotype showed that these lines also carry useful resistance that in all cases severely limited colony establishment. Further genetic analyses of these accessions would be worthwhile because they may carry new resistance genes. Such new resistance genes are clearly needed for the control of this important disease which occurs in many warm areas of the world where there is little alternative to host resistance as a means of disease control.

Accession JI2480 (*Er1er2*) showed high field resistance to powdery mildew although some infection occurred on its stems. This supports previous observations (Heringa et al., 1969; Tiwari et al., 1997a) showing that under controlled conditions stems of JI2480 were more heavily infected than leaves, although Tiwari et al. (1997a) reported that both leaves and stems remained disease free in the field.

Tiwari et al. (1997b) reported that JI2480 was completely resistant or highly resistant in the field at some locations but susceptible at others. A possible explanation for this could be the existence of races of *E. pisi*, although, to date, *E. pisi* races with specific virulence to the resistance genes of pea have not been identified. Thus, Tiwari et al. (1997a) found little variability in apparent virulence between 31 single colony isolates of *E. pisi* tested against a set of 14 pea lines that represented the known powdery mildew reaction genotypes. Similarly, Banyal and Tyagi (1997) found no differences in virulence between a number of isolates although they varied somewhat in their ability to produce conidiophores bearing conidia.

An alternative explanation for the differences of powdery mildew susceptibility in JI2480 at different locations may be the effect of different environments on the expression of the resistance gene or genes present in this line. This is supported by our findings. Unlike the susceptible accession JI502 or accession JI2302 (*er1Er2*), temperature and leaf age had dramatic effects on accession JI2480 (*Er1er2*), affecting both host cell death following establishment of young colonies and final symptom development in detached leaves. In addition, success in colony establishment was reduced on mature leaves. Our finding of temperature dependence of resistance in accession JI2480 (*er2*) is in agreement with Tiwari et al. (1997a) who reported that resistance of JI2480 was ineffective where temperature fluctuated between 15 °C and 20 °C. In southern Spain, *E. pisi* infection commences at the end of April when plants are at podding stage. From April to June temperatures can reach 35 °C and rarely fall below 20 °C. Under these conditions, the field resistance seen in JI2480, which appears to be due to post-penetration cell death response, would be expected to be effective. This suggestion is supported by incidental observations that some leaves of field-grown JI2480 examined at the end of the season showed necrotic responses associated with approximately 80% of *E. pisi* colonies.

Line JI2480 posses gene *er2*, thus the mechanisms of resistance observed in this line and the effect of temperature and leaf age on their expression could be conditioned by this gene. However, we can not exclude the possibility of other unknown resistance genes present in JI2480 that could influence the expression of its resistance. Studying additional lines having gene *er2* or evaluating separately each resistance mechanism in a segregating population obtained from a cross between JI2480 and a susceptible line would help us to clarify whether all mechanisms observed in JI2480 are caused only by one gene (*er2*) or by additional genes.

Combining resistances that act first to limit penetration (e.g. lines having gene *er1* and lines JI1213, JI1412, JI1566 and JI1747 with unknown genotypes) and, if this fails, to cause death of invaded cells (as in line JI2480) would provide a double barrier to disease development that should enhance the durability of resistance offered by either mechanism alone. However, the fact that the penetration resistance of *er1* hampers detection of the presence of post-penetration resistance in hybrid progeny, even at the histological level, makes difficult the introduction of both resistance

mechanisms in the same pea line. The identification of molecular markers associated with genes *er1* and *er2* (Timmerman et al., 1994; Tiwari et al., 1998a, b; Janila and Sharma, 2004) offers a useful tool to allow combination of both genes in a same line.

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