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FULL RESEARCH PAPER

Biotic factors affecting the expression of partial resistance in pea to ascochyta blight in a detached stipule assay

Caroline Onfroy · Alain Baranger · Bernard Tivoli

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Abstract The expression of partial resistance in pea to ascochyta blight (caused by *Mycosphaerella pinodes*) was studied in a detached stipule assay by quantifying two resistance components (fleck coalescence and lesion expansion) using the method of point inoculation of stipules. Factors determining optimal conditions for the observation of partial resistance are spore concentration, the age of the fungal culture prior to spore harvest and the pathogenicity of the isolate used for testing. Partial resistance was not expressed when spore concentration was high or when the selected isolate was aggressive. Furthermore, assessments of components of partial resistance were highly correlated with disease severity in a seedling test. A screening protocol was developed based on inoculations of detached stipules to study partial resistance in pea. To simplify the rating process, a more comprehensive disease rating scale which took into account fleck coalescence and lesion expansion was tested by screening a large number of genotypes.

Keywords *Pisum sativum* · *Mycosphaerella pinodes* · *Phoma medicaginis* var. *pinodella* · Components of resistance · Fleck coalescence · Lesion extension · Screening test · Spore concentration · Age of spores

C. Onfroy · B. Tivoli (✉)
UMR INRA-Agrocampus Rennes BiO3P, BP 35327, 35653 Le Rheu cedex, France
e-mail: bernard.tivoli@rennes.inra.fr

C. Onfroy
Union Nationale Interprofessionnelle des Plantes riches en Protéines (UNIP), 12 avenue George V, 75008 Paris, France

A. Baranger
UMR INRA-Agrocampus Rennes APBV, BP 35327, 35653 Le Rheu cedex, France

Introduction

Ascochyta blight of pea (*Pisum sativum*) is caused by three related fungal species, commonly referred to as the Ascochyta complex: *Ascochyta pisi*, *Ascochyta pinodes* (teleomorph: *Mycosphaerella pinodes*) and *Phoma medicaginis* var. *pinodella*, formerly known as *Ascochyta pinodella* (Jones 1927). *Mycosphaerella pinodes* and *P. medicaginis* var. *pinodella* cause foot rot, and similar symptoms on leaves, stems, pods and seeds (Hare and Walker 1944) which can result in substantial yield and seed quality losses in France (Allard et al. 1993) and throughout the major pea cropping regions worldwide (Bretag and Ramsey 2001). The first studies on pea resistance to *M. pinodes* have shown the absence of specific resistance (Nasir et al. 1992; Clulow et al. 1992). Most recent studies on resistance to the ascochyta blight complex in pea have described the observed resistance as partial (Onfroy et al. 1999; Wroth and Khan 1999; Wang et al. 2000; Xue and Warkentin 2001;

Timmerman et al. 2002; Prioul et al. 2003, 2004; Fondevilla et al. 2005). Partial disease resistance is defined as an interference with one or more steps of the epidemic cycle, resulting in a slow-down of disease progress and/or a reduction in the pathogen multiplication (Parlevliet 1979). The growth of the pathogen can be assessed quantitatively both by directly assessing disease severity (symptoms) and disease development over time, or by considering disease severity as the result of different factors (Parlevliet 1979). These epidemiological components of quantitative resistance include resistance to infection (*i.e.,* reduced germination, appressorium formation or penetration), delayed incubation period (from inoculation to the occurrence of the first symptoms), delayed latency period (from inoculation to sporulation), reduced infectious period (sporulation duration), and reduced intensity of spore production (spore quantity per time unit).

Specific and reliable methodologies are needed for the assessment of these components of resistance under field or controlled conditions. The use of point inoculation on leaflets, either detached or in situ under controlled conditions, can be helpful in dissecting plant reactions and for providing insight into the different steps of the epidemic cycle. In the *Botrytis fabae*/faba bean pathosystem, Tivoli et al. (1986) used a detached leaf assay to determine three main epidemic phases, namely appearance of symptoms (number of spots 15 h after inoculation, rate of new spot formation), disease development (disease severity score 6 days after inoculation), and sporulation (number of spores/leaflet 11 days after inoculation). More recently, Bouhassan et al. (2003), using this methodology in the same pathosystem, quantified five components of partial resistance: the incubation period, the number of spots, lesion diameter, the latency period and the intensity level of sporulation.

Few references pertaining to the use of point inoculation of leaves to study ascochyta blight on pea are available. Heath and Wood (1969) used excised leaves to determine the factors acting on the phases of the epidemic cycles of *M. pinodes* and *A. pisi* (spore concentration, leaf age, water content of the leaf). This method has also been used to screen for cultivar susceptibility and/or pathogenicity of isolates. Wang et al. (2000), using excised leaves to study susceptibility in pea to *A. pisi*, reported significant isolate × genotype interactions. Based on point inoculation of leaves in situ on plants, Nasir et al. (1992) described the development of different *M. pinodes* pathotype groups in susceptible and partially resistant pea genotypes. Wroth (1998a, b) also used in situ inoculations to screen progeny families for their resistance to *M. pinodes*, and to study variation in pathogenicity among and within *M. pinodes* populations.

To date, no study has specifically focused on factors affecting the expression of partial resistance to ascochyta blight in pea. We therefore carried out experiments to identify which factors influence the expression of partial resistance to *M. pinodes* and *P. medicaginis* var *pinodella* in pea, and to determine optimum screening conditions to achieve maximum levels of differentiation among pea genotypes. We focused our study on two main components of partial resistance which are key factors in disease expression, namely fleck coalescence and lesion expansion. Fleck coalescence takes into account the early stages of interaction, from the inoculation to the first typical necrotic symptom, corresponding to the hemibiotrophic phase of the pathogen (Clulow et al. 1991), where different mechanisms of resistance are involved (Wroth 1998a). Lesion expansion reflects the growth rate of the pathogen in the host during the necrotrophic phase (Parlevliet 1979). A set of six genotypes differing in their levels of susceptibility to *M. pinodes* and *P. medicaginis* var. *pinodella* as determined by Onfroy et al. (1999), was used to define the effects of spore concentrations, fungal colony age prior to harvest of spores, and pathogenicity of isolates on these components of partial resistance assessed on detached leaves. As a result, a protocol is proposed for a reliable screening test to identify and quantify partial resistance to ascochyta blight in pea.

Materials and methods

Plant material

A set of six genotypes differing in their levels of susceptibility to *M. pinodes* and *P. medicaginis* var. *pinodella* (Onfroy et al. 1999) were used to test the effect of different factors on the expression of resistance. The germplasm line DP and the breeding

line FP (synonym CE101, Baranger et al. 2004) were defined as having a high level of partial resistance. The cv. Melrose was defined as partially resistant, the germplasm line JI 252 and the field pea cv. Solara (afila type) were found to be moderately susceptible, and the line JI 296 (garden pea cv. Chemin long) was highly susceptible. Seven other genotypes were included to study the correlation between disease reaction on detached stipules and seedlings, chosen on the basis of screening results for plantlet or adult plant resistance (Onfroy, unpublished results; Baranger, unpublished results): breeding lines CP and GP (synonym CF100, Baranger et al. 2004), germplasm lines JI96, GSP935 (PI288025) and GSP940a (PI343292), and winter pea cvs Champagne and Froidure. Origin and morphology data for all genotypes are described in Baranger et al. (2004), except for GSP935 (PI288025) and GSP940a (PI343292), which are described on the Pullman genebank website (http://www.ars-grin.gov). Three seeds of each genotype were planted in 9 cm diam pots containing a mixture of unsterilised soil/sand/peat (1:1:1). The soil originated from an experimental plot at the INRA research centre in Le Rheu. Six plants were used per genotype for the detached stipule assays. The pots were placed in trays in a growth chamber with a temperature of 15°C night/18°C day and a 14 h photoperiod with a light intensity of 160 ± 2 $\mu Em^{-2}s^{-1}$, until the plants reached the 5–6 leaf stage. For the seedling test, plant preparation and experimental design were carried out according to Onfroy et al. (1999).

Production of inoculum

Three *M. pinodes* isolates (Mp1, Mp2, Mp3), originating from different regions in France (Midi-Pyrénées, Normandy, Champagne), were compared for their effect on resistance expression to a *P. medicaginis* var. *pinodella* isolate (Pm1) originating from the central region of France. Subcultures of the isolates were taken from malt agar slants and grown on V8 medium (99 ml V8 vegetable juice (Campbell, France), 35 g agar, 801 ml distilled water, autoclaved at 105°C for 30 min) under white light with a 12 h photoperiod at 20°C (wavelengths between 350 and 750 nm). Pycnidiospore suspensions were prepared by flooding the surface of 10 day-old cultures with sterile distilled water, gently scraping the colony with a glass rod and filtering the suspension through two layers of sterile cheesecloth (except for the experiment testing the age of the spores where 7-, 10- and 14 day-old cultures were used). The concentration of spores was determined with a haemocytometer and was adjusted to the required spore concentration (100, 500, 1000 and 5000 10 μl^{-1}). Tween 20 (VWR International SAS, Strasbourg, France) was added as a wetting agent (two drops 500 ml^{-1} spore suspension).

Inoculation and disease assessment on detached leaflets and stipules

The inoculation method used was based on that proposed by Heath and Wood (1969), consisting of depositing a drop of spore suspension on detached leaflets. Preliminary studies with the six genotypes used by Onfroy et al. (1999) revealed that (1) the reaction to ascochyta blight was identical on detached leaflets and on detached stipules, (2) the largest range between resistant and susceptible genotypes was observed on stipules from nodes 2, 3 or 4 of seedlings with 5–6 nodes (node 1 generally showed early senescence), and (3) a drop of 10 µl was optimal for inoculation (a drop of 5 µl evaporated too quickly, a drop of 20 µl induced lesions too large for accurate assessments). Short stem segments with attached stipules (referred to as detached stipules hereafter) from nodes 3 or 4 were used in all subsequent experiments because the cv. Solara is semi-leafless, and therefore lacks leaflets. After cutting, the detached stipules were floated, lower surface down, on tap water in a compartmented square Petri dish (12 cm side, Gosselin, France). Inoculation was with a drop of 10 µl of spore suspension placed on the upper surface of the stipules, avoiding the main veins. To avoid drop evaporation, Petri dishes were placed into large transparent plastic boxes.

From the six plants per genotype, two stipules were detached and inoculated each with a drop of the spore suspension resulting in 12 replicate assessments for each genotype. Detached stipules were incubated in a climatic chamber for an initial period of 18 h in the dark, followed subsequently by 7 days with a continuous cycle of 14 h light and 10 h darkness at 20°C. Symptom appearance on detached stipules was assessed each day after inoculation (dai) using a 0–3 semi-quantitative scale (fleck coalescence scale):

0 = symptom-free; 1 = flecks appearing; 2 = flecks covering half of the area of drop deposition; 3 = coalescence of the flecks within the area of drop deposition (approx. 3 mm).

Once necrosis had developed beyond the borders of each drop deposit, disease progress was assessed by measuring lesion diameter (mm) daily, with a graduated ruler, and was summarized as Area Under the Disease Progression Curve (AUDPC) calculated by plotting mean disease expansion against time according to the formulae proposed by Shaner and Finney (1977). In addition, the 0–7 scale based on different types of symptoms as described by Wroth (1998a) was adapted to our experimental conditions on detached stipules: 0 = symptom-free; 1 = flecks appearing; 2 = flecks covering half of the drop deposit; 3 = coalescence of the flecks in the area of the drop deposit (approx. 3 mm diam); 4 = 3–6 mm lesion diam; 5 = 6–9 mm lesion diam; 6 = 9–12 mm lesion diam, 7 = superior to 12mn lesion diam.

Inoculation and disease assessment on plantlets

Inoculation of seedlings by spraying spore suspensions of *M. pinodes* or *P. medicaginis* var *pinodella* was conducted as described by Onfroy et al. (1999). A spore suspension of 10^5 spores ml^{-1} was applied to plants at the 4–5 leaf stage using a hand-held garden sprayer and plants were incubated under a continuous cycle of 14 h at 18°C in light and 10 h at 15°C in darkness. Disease severity was assessed daily after inoculation using a 0–5 disease scale described previously (Onfroy et al. 1999). AUDPC was calculated using the formula proposed by Shaner and Finney (1977).

Data analysis

The effect of various factors on fleck coalescence and lesion expansion (including AUDPC) were analysed by ANOVA using the General Linear Model (GLM) procedure of the statistical package SAS version 8.1 (SAS 1988). The Student Newman-Keul's test ($P = 0.05$) was used to determine whether differences between plant genotypes, between fungal species or between isolates were statistically significant. Relationships between scoring criteria were tested by Pearson correlation analysis (SAS 1988).

Results

Effect of spore concentrations

The effect of inoculum pressure on partial resistance expression was investigated by inoculating detached stipules with different numbers of spores per drop: 100, 500, 1000, and 5000 (Fig. 1; Table 1). This experiment showed that, as expected, a drop containing 100 spores induced a slow appearance of symptoms. Two dai, the first flecks appeared only in the most susceptible genotypes JI296 and Solara. On the other hand, a drop containing 5000 spores induced a very fast development of symptoms. Disease severity was already very high at two dai and the area covered by the inoculation drop of all the genotypes was almost entirely necrotic, and no differences among the genotypes could be discerned. Concentrations of 500 and 1000 spores $drop^{-1}$ allowed differences between genotypes to be distinguished based on their partial resistance (Fig. 1). Expanding lesions were first observed on genotypes JI 296, Solara and JI252 for 100, 500 and 1000 spores $drop^{-1}$. With 100 spores $drop^{-1}$, only the genotypes Solara (at 5 and 7 dai) and JI296 (at 7 dai) reached the lesion expansion phase. On the other hand, a dose of 5000 spores $drop^{-1}$ differentiated susceptible and

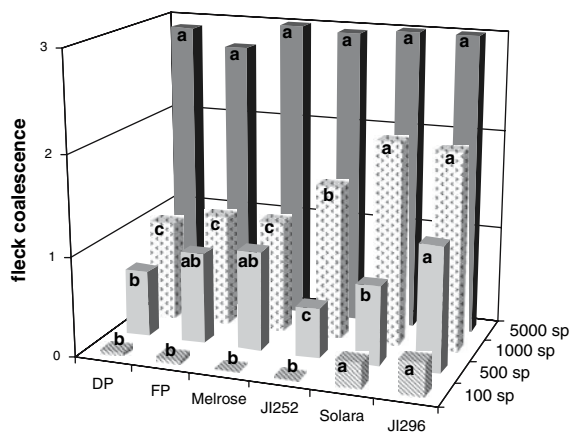

Fig. 1 Mean fleck coalescence scores (scale 0–3) on detached stipules of a set of six pea genotypes, 2 days after point inoculation with spore suspensions of *Mycosphaerella pinodes* isolate Mp1 at four concentrations. For each spore concentration (sp), fleck coalescence means of genotypes showing the same letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

Table 1 Mean lesion diameters (mm) on detached stipules of a set of six pea genotypes at 3, 5 and 7 days after point inoculation (dai) with spore suspensions of *Mycosphaerella pinodes* isolate (Mp1) at four concentrations

No. spores drop^{-1}	dai	Genotypes					
		DP	FP	Melrose	JI252	Solara	JI296
100	3	*fc*	*fc*	*fc*	*fc*	*fc*	*fc*
	5	*fc*	*fc*	*fc*	*fc*	4.7	fc
	7	*fc*	*fc*	*fc*	*fc*	9.3	8.9
500	3	*fc*	*fc*	*fc*	*fc*	*fc*	*fc*
	5	*fc*	*fc*	*fc*	3.9	6.2	5.8
	7	6.2 c	4.2 d	5.9 c	7.0 c	10.5 b	15.3 a
1000	3	*fc*	*fc*	*fc*	*fc*	3.1	3.1
	5	5.6 b	4.5 c	5.6 b	5.9 b	7.0 a	6.9 a
	7	10.0	*owa*	10.0	*owa*	11.6	16.1
5000	3	4.4 d	3.0 e	5.4 c	7.2 a	6.7 b	7.2 a
	5	*owa*	6.6 ± 0.7	*owa*	*owa*	10.9	11.3
	7	*owa*	*owa*	*owa*	*owa*	16.8	18.3

fc = fleck coalescence; *owa* = necrosis spreading over whole area of the stipule

For each spore concentration × dai combination (i.e., for each line of the table), lesion diameter means of genotypes showing the same lower case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

resistant genotypes only at 3 dai, whereas longer periods of incubation led to the rapid development of necrosis on the stipule surfaces. With 500 and 1000 spores drop^{-1}, lesion diameters discriminated better between genotypes and were significantly larger in genotypes JI 296 and Solara, and significantly smaller in genotype FP (Table 1). Strong effects of spore concentrations were observed both on fleck coalescence and lesion expansion. Concentrations too low (drops containing 100 spores) or too high (drops of 5000 spores) were inadequate for monitoring any component of resistance. Drops containing 500 or 1000 spores were more likely to reveal a range of partial resistance of both components. With drops containing 500 spores, the standard deviations were greater than with drops containing 1000 spores both for fleck coalescence and lesion expansion.

A further experiment was carried out, consisting of daily assessments of lesion diameters from 2 to 7 dai on stipules inoculated with 500 or 1000 spores drop^{-1} (Fig. 2). Because of the small size of its stipules, lesion diameters on genotype JI252 were measured only up to 5 dai. Differences between susceptible and resistant genotypes were mainly due to a delay in the onset of lesion expansion (3 or 4 dai depending on the genotype), whereas the slopes of plots of lesion expansion (i.e., increase in diameter) against time were similar for the all six genotypes tested ($P > 0.05$).

AUDPC based on increases in lesion diameter from 4 to 7 dai, revealed significant differences among the five genotypes (Table 2). Lesion diameters assessed 5 dai allowed for comparisons between the six genotypes including JI252. The results showed that both spore concentrations were adequate in revealing differences in partial resistance of genotypes FP and DP. Genotypes Solara and JI 296 were highly susceptible, while genotypes Melrose and JI252 showed an intermediate reaction. A concentration of 500 spores drop^{-1} allowed slightly better discrimination within these intermediate genotypes than 1000 spores, indicating that JI252 is more resistant than Melrose.

Effect of fungal colony age on the pathogenicity of spores and expression of partial resistance

This experiment aimed at assessing the effect of the age (7, 10 or 14 day-old) of colonies from which spores for inoculation were harvested, on the expression of partial resistance on detached stipules. Spores harvested from a 7 day-old colony were significantly more aggressive than spores from older cultures,

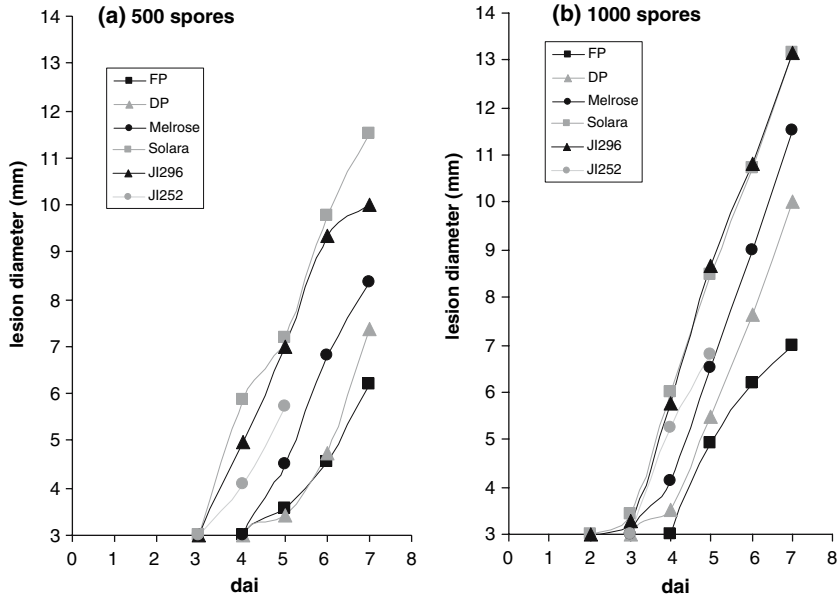

Fig. 2 Disease progress curves based on mean lesion diameters on detached stipules of a set of six pea genotypes after point inoculation with spore suspensions of *Mycosphaerella pinodes* isolate Mp1 at inoculum concentrations of (**a**) 500 spores and (**b**) 1000 spores drop^{-1}. Sp: spores

Table 2 Mean lesion diameters (mm) at 5 days after inoculation (dai) and AUDPC calculated from increasing lesion diameters from 4 to 7 dai on detached stipules of a set of six pea genotypes after point inoculation with spore suspensions of *Mycosphaerella pinodes* isolate (Mp1) at two concentrations

	No. spores drop^{-1}	Genotypes					
		DP	FP	Melrose	JI252	Solara	JI296
Lesion diameter	500	3.4 d	3.5 d	4.5 c	5.8 b	7.2 a	7.0 a
	1000	5.5 c	4.9 c	6.5 b	6.8 b	8.5 a	8.7 a
AUDPC	500	4.4 d	3.7 d	8.0 c	–	16.6 a	14.8 b
	1000	10.8 c	7.1 d	14.3 b	–	19.8 a	20.0 a

For each spore concentration (i.e., for each line of the table), lesion diameter and AUDPC means of genotypes showing the same lower case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

irrespective of spore concentration (Table 3). For example, the average fleck coalescence scores for the six genotypes 2 dai were 1.3, 0.8 and 0.7 for spores obtained from 7, 10 and 14 day-old colonies, respectively, when inoculated at 500 spores drop^{-1}. Extensive lesions in the most susceptible genotypes were already observed at 3 dai when using inoculum from 7 day-old colonies (genotype JI296), whereas inoculum from 10 and 14 day-old colonies allowed data to be obtained for all genotypes both at 2 and 3 dai. Furthermore, ranges for partial resistance and differentiation among genotypes were best for inoculum from 10 and 14 day-old colonies. At 1000 spores drop^{-1}, fleck coalescence and expansion of lesions occurred more rapidly and data could be obtained for all genotypes only at 2 dai. Differentiation among genotypes was not as accurate as with a drop containing 500 spores.

A very clear effect of colony age was also observed for lesion expansion over time, summarized as AUDPC. Average AUDPC was significantly higher for inoculum from 7 day-old colonies than from 10 or 14 day-old colonies (Table 3). Thus, for drops containing 500 spores, lesion diameter mean values for AUDPC over all genotypes were 8.4, 5.3 and 5.7, respectively, and for drops containing 1000 spores, these values were 12.4, 10.0 and 9.9 for inoculum from 7, 10 and 14 day-old colonies, respectively (data not shown). Irrespective of colony age, differences among genotypes with regard to partial resistance were observed, but the expression of partial resistance was better displayed with spores

Table 3 Mean fleck coalescence scores for detached stipules of a set of six pea genotypes at 2 and 3 days after point inoculation (dai) with two concentrations of spore suspension of *Mycosphaerella pinodes* isolate (Mp1) harvested from 7, 10 and 14 day-old colonies

No. spores drop^{-1}	Age of the colony (days)	dai	Genotypes						
			DP	FP	Melrose	JI252	Solara	JI296	Overall mean
500	7	2	1.0 bc	0.8 c	1.0 bc	1.8 a	1.4 b	1.9 a	1.3 A
		3	1.4	1.7	1.3	2.9	3.0	*le*	
	10	2	0.3 c	0.3 c	0.4 c	0.8 bc	1.1 b	1.8 a	0.8 B
		3	1.0 c	1.2 c	1.0 c	1.8 b	2.6 a	3.0 a	
	14	2	0.0 c	0.2 c	0.8 b	0.8 ab	1.0 ab	1.3 a	0.7 B
		3	1.0 c	1.0 c	1.0 c	2.4 b	2.7 b	3.0 a	
1000	7	2	2.0 bc	1.8 c	2.0 bc	2.6 ab	2.3 abc	2.8 a	2.3 A
		3	2.9	3.0	3.0	*le*	*le*	*le*	
	10	2	1.0 b	0.9 b	1.1 b	1.7 a	2.1 a	2.1 a	1.5 B
		3	2.0	2.3	2.0	3.0	3.0	*le*	
	14	2	0.8 b	0.8 b	1.0 b	1.8 a	1.7 a	1.7 a	1.3 C
		3	1.9	2.4	2.1	3.0	3.0	*le*	

le = lesion expansion

For each spore concentration × age of the colony combination (i.e., for each line of the table), lesion diameter means of genotypes showing the same lower case letter are not significantly different; SNK test ($P = 0.05$)

For each spore concentration, lesion diameter means over all genotypes (i.e., for the last column of the table) for each age of the colony showing the same upper case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

from 10 and 14 day-old colonies (Fig. 3). For instance, mean lesion diameter values for AUDPC for genotype DP using drops containing 1000 spores were 4.5 and 4.3 for spores harvested from 10 and 14 day-old colonies, but had already reached 8.6 for spores obtained from 7 day-old colonies. Furthermore, results from this experiment indicate that the expression of partial resistance in the genotype JI252 collapsed with drops containing 1000 spores.

Effect of the isolate

Three *M. pinodes* and one *P. medicaginis* var. *pinodella* isolates were considered for their effects on the expression of partial resistance. At 2 dai, significant differences in fleck coalescence were observed between isolates (Table 4). The *P. medicaginis* var. *pinodella* isolate was generally far less aggressive than the *M. pinodes* isolates. Significant differences in fleck coalescence were also observed among the three *M. pinodes* isolates, with Mp1 and Mp2 being the least and Mp3 the most aggressive isolate. Although the disease symptoms appeared later with the *P. medicaginis* var. *pinodella* isolate, it was still possible to discern significant differences between resistant and susceptible genotypes 2 dai with drops containing 1000 spores. Irrespective of the *M. pinodes* isolate and inoculum concentration, differences among genotypes could only be observed at 2 dai, since at 3 dai the most susceptible genotypes had always reached a mean fleck coalescence of 3.

AUDPC calculated from lesion diameters between 3 and 6 dai confirmed significant differences in pathogenicity among *M. pinodes*, and between *M. pinodes* and *P. medicaginis* var. *pinodella* isolates (Fig. 4). Thus, inoculations with Mp1, Mp2, Mp3 and Pm1 resulted in AUDPC means of all genotypes of 6.2, 6.5, 9.0 and 2.1, respectively, for drops containing 500 spores, and 9.2, 11.3, 13.7 and 5.6, respectively, for drops containing 1000 spores (data not shown). Furthermore, statistically significant differences between susceptible and resistant genotypes were displayed irrespective of the *M. pinodes* isolate and spore concentrations (Fig. 4). For the *P. medicaginis* var. *pinodella* isolate, differences between genotypes were best displayed with drops containing 1000 spores. No specific effect of any *M. pinodes* isolate was observed on disease progress (data not

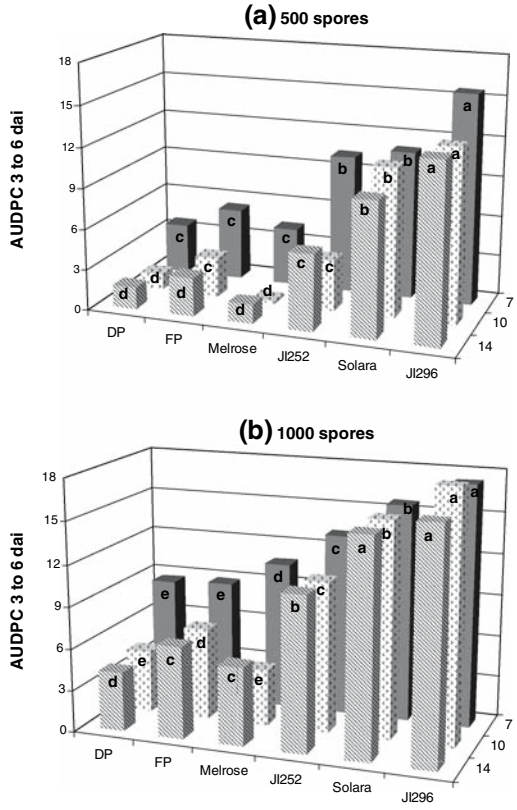

Fig. 3 Mean AUDPC calculated from lesion diameters from 3 to 6 days on detached stipules of a set of six pea genotypes after point inoculation with spore suspensions of *Mycosphaerella pinodes* isolate Mp1 at inoculum concentrations of (**a**) 500 spores and (**b**) 1000 spores drop^{-1}, from 7, 10 and 14 day-old colonies. For each age of the colony, AUDPC means of genotypes showing the same letter are not significantly different; Student Newman-Keul's test (P = 0.05)

shown). However, a combination of a highly aggressive isolate (such as Mp3) and a high spore concentration did not allow differences in fleck coalescence to be observed among genotypes. Therefore the choice of a moderately aggressive *M. pinodes* isolate (such as Mp1) may allow discrimination between genotypes under a wider range of conditions.

Validating of conditions using an enlarged set of genotypes

We tested the conditions identified above for screening for partial resistance to *M. pinodes* on detached stipules (stipule or leaflet from node 3 or 4, drop size of 10 µl with 500 or 1000 spores obtained from colonies of 10–14 days, isolate moderately aggressive) using an enlarged set of 13 genotypes. Fleck coalescence (Table 5A) covered a rather large range both at concentrations of 500 spores drop^{-1} (from 0.5 to 1.9 at 2 dai, and from 1.3 to 3.0 at 3 dai) and of 1000 spores (from 1.0 to 3.0 at 2 dai). With 500 spores per drop, three distinct groups of genotypes could be distinguished at 3 dai, one with the most resistant genotypes (FP, GP and Champagne), one with the most susceptible genotypes (Solara, CP, JI96, JI296, 935 and JI252), and an intermediate group with moderately susceptible genotypes, including DP, 940a, Melrose, and Froidure). When inoculated with 1000 spores drop^{-1}, these groups could not be separated as easily 2 dai as was possible after inoculation with a lower concentration of spores. However, overall, the same genotype classification was observed for both inoculum concentrations.

AUDPC calculated from lesion diameters between 3 and 6 dai also showed differences between genotypes (Fig. 5A). Genotype groupings were consistent with those based on fleck coalescence. Genotypes showing a delay in fleck coalescence also displayed the lowest AUDPC. Correlation coefficients between both components of resistance (fleck coalescence and AUDPC based on lesion expansion) were highly significant. At 500 spores drop^{-1}, R^2 values were 0.73 and 0.89 at 2 dai and 3 dai, respectively, whereas at 1000 spores drop^{-1}, R^2 values were 0.83 and 0.77 at 2 dai and 3 dai, respectively.

Comparison between detached stipules and the seedling tests

To check if partial resistance observed on detached stipules was correlated with partial resistance displayed in a seedling test, the results obtained from both methods were compared for this enlarged set of 13 genotypes (Fig. 5B). On seedlings inoculated with a spore suspension of 10^5 spores ml^{-1}, AUDPC was calculated based on disease severity measured between 4 and 11 dai (Fig. 5B). The mean AUDPC values showed a large range among genotypes, from 15.7 to 34.7 for lines FP and JI296, respectively. Mean AUDPC on seedlings was significantly correlated to fleck coalescence on detached stipules (R^2 ranging from 0.65 to 0.79) depending on spore concentration × dai combination, and to AUDPC based on lesion expansion on detached stipules

Table 4 Mean fleck coalescence scores for detached stipules of a set of six pea genotypes at 2 and 3 days after point inoculation (dai) with spore suspensions of three isolates of *Mycosphaerella pinodes* (Mp1–3) and one of *Phoma medicaginis* var. *pinodella* (Pm1) at two concentrations

No. spores drop^{-1}	Isolate	dai	Genotypes						
			DP	FP	Melrose	JI252	Solara	JI296	Overall mean
500	Mp 1	2	1.0 b	1.2 b	0.9 b	0.8 b	1.8 a	2.0 a	1.3 B
		3	1.6 b	2.3 ab	2.0 b	2.8 a	*le*	*le*	
	Mp2	2	0.8 b	1.3 b	1.0 b	1.0 b	1.8 a	1.9 a	1.3 B
		3	1.9 b	2.8 a	1.9 b	2.6 a	*le*	*le*	
	Mp 3	2	1.0 b	1.7 a	1.0 b	1.5 a	2.0 a	1.8 a	1.5 A
		3	2.7	*le*	2.3	*le*	*le*	*le*	
	Pm 1	2	0.3 b	0.2 b	0.4 a	0.1 b	0.8 a	0.2 b	0.3 C
		3	0.8 b	0.7 b	0.7 b	0.9 b	1.4 b	3.0 a	
1000	Mp 1	2	1.9 a	1.8 a	1.5 b	1.1 c	2.0 a	2.0 a	1.7 B
		3	3.0 a	3.0 a	3.0 a	3.0 a	*le*	*le*	
	Mp2	2	2.0 a	1.4 b	2.0 a	2.0 a	2.0 a	2.0 a	1.9 A
		3	3.0 a	3.0 a	3.0 a	3.0 a	*le*	*le*	
	Mp 3	2	2.0 a	2.0 a	2.0 a	2.0 a	2.0 a	2.0 a	2.0 A
		3	3.0 a	*le*	3.0 a	*le*	*le*	*le*	
	Pm 1	2	1.0 bc	0.7 c	1.0 bc	0.9 bc	1.8 a	1.4 b	1.1 C
		3	1.1 c	1.2 c	1.1 c	2.2 b	2.8 a	*le*	

le = lesion expansion

For each spore concentration × fungal isolate combination (i.e., for each line of the table), lesion diameter means of genotypes showing the same lower case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

For each spore concentration, lesion diameter means over all genotypes (i.e., for the last column of the table) for each fungal isolate showing the same upper case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

($R^2 = 0.74$ for drops of 500 spores and $R^2 = 0.75$ for drops of 1000 spores).

Assessment of a scale combining both resistance components

To potentially simplify screening procedures, we additionally assessed the data with a scale including both resistance components. Mean AUDPC values based on this scale and assessments from 2 to 6 dai ranged from 9.8 to 15.8 with inoculum of 500 spores drop^{-1}, and from 12.0 to 17.5 with 1000 spores drop^{-1}, and displayed expected groupings among genotypes (Table 5B). Significant correlations were observed between AUDPC assessed on whole seedlings (data from Fig. 5), and AUDPC values from detached stipules inoculated with drops containing 500 spores ($R^2 = 0.81$) and with drops containing 1000 spores ($R^2 = 0.79$) after assessment with this modified scale.

Discussion

Expression of partial resistance

The results obtained in this study show that partial resistance of pea to *M. pinodes* is expressed and can be assessed on detached stipules in the form of two important epidemiological components: fleck coalescence and lesion expansion. In our experiments, the genotype DP reduced fleck coalescence, but showed lesion expansion similar to susceptible genotypes. This suggests that these parameters are under different genetic controls. With another legume fungus, *B. fabae*, the same phenomenon was observed with *Vicia narbonensis* which considerably delayed the initial establishment of infection, but was unable to limit spread in the leaflet tissue (Tivoli et al. 1986). This indicates that there are two different components in host resistance to disease, affected by spore concentration, age of the fungal colony from which

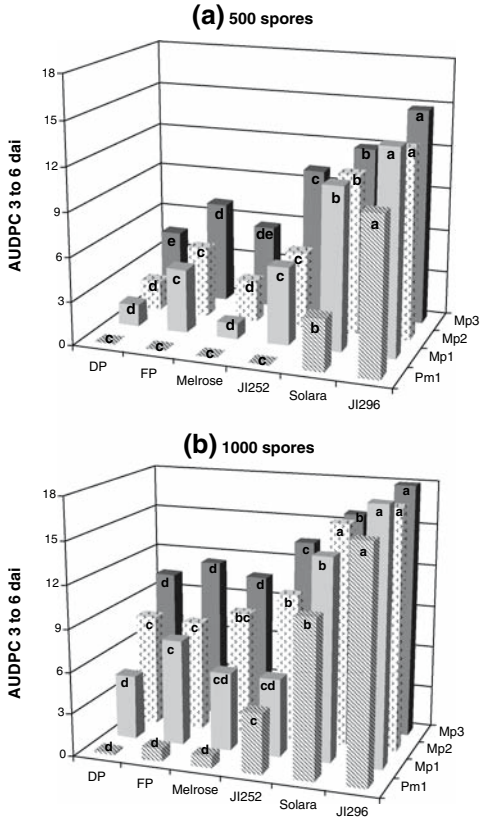

Fig. 4 Mean AUDPC calculated from lesion diameters from 3 to 6 days after inoculation on detached stipules of a set of six pea genotypes after point inoculation with spore suspensions of three isolates of *Mycosphaerella pinodes* (Mp1, Mp2 and Mp3) and one isolate of *Phoma medicaginis* var. *pinodella* (Pm1), at inoculum concentrations of (**a**) 500 spores and (**b**) 1000 spores drop^{-1}. For each fungal isolate, AUDPC means of genotypes showing the same letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

spores are harvested, and isolate pathogenicity. Furthermore, we have shown that partial resistance can collapse when factors are too favourable for disease development, in this case when aggressive spores from a 7 day-old culture were used, a highly aggressive isolate was chosen and/or detached stipules were inoculated at a high spore concentration. This phenomenon was mainly observed with the line DP during lesion expansion. The effect of spore age on infection processes was described for *B. fabae* (Harrison 1988). Here, it was shown that infection hyphae from only young conidia may be able to kill host cells before appreciable phytoalexin synthesis has occurred. This observation suggests than the same phenomenon could be involved in the case of *M. pinodes* and pea phytoalexins. The expression of partial resistance depends on parameters which are well defined, and its assessment is a compromise between disease expression and the expression of partial resistance. Our studies have also shown that each of the components of partial resistance assessed here was highly correlated with a seedling pathogenicity test.

Numerous factors may influence the expression of resistance. Biotic conditions that are best suited for pathogen development, high inoculum pressure and the use of highly aggressive strains are probably not suited for the identification of resistance components and partial resistance. We show that the best conditions to identify partial resistance are those with intermediate inoculum pressure, marginally favouring the pathogen. This idea was supported by Sakar et al. (1982) who showed that intermediate concentrations of *P. medicaginis* var. *pinodella* inoculum gave a better separation of mean foot-rot disease scores for three cultivars, compared to low or high concentrations. Results from our study suggest that high concentrations of inoculum make it more difficult to detect any differences among cultivars, whereas low concentrations can increase the variability in the data. Using similar approaches as described here, Wroth (1998a, b) studied resistance of host progenies and variation in pathogenicity among and within *M. pinodes* populations at two spore concentrations (500 and 1000 spores drop^{-1}). She observed a better discrimination among the breeding lines and a larger distribution pattern when leaves were inoculated with 500 spores, as well as a better characterisation of pathogen diversity at low inoculum pressure, mainly at day 10. Similar to results by Wroth (1998a, b), our results on the use of isolates with different levels of pathogenicity also lead to the following conclusions: to maximise the variation in host responses, it is better to use an aggressive isolate at low inoculum pressure (500 spores drop^{-1}) or a less aggressive isolate at high inoculum pressure (1000 spores drop^{-1}).

The observations we have made in this study are in agreement with the results obtained by Onfroy et al. (1999) and Prioul et al. (2003). The range between resistant and susceptible genotypes is the same as was observed by these authors. Based on 13 genotypes tested for the two components considered, this study

Table 5 Behaviour of a set of 13 pea genotypes after point inoculation of detached stipules with spore suspensions of *Mycosphaerella pinodes* isolate Mp1 at two concentrations expressed by; (A) Mean fleck coalescence scores at 2 and 3 dai and; (B) Mean AUDPC for lesion expansion assessed from 2 to 6 dai using a modified scale from Wroth (1998a)

No. spores drop^{-1}	dai	Genotypes													
		Champ	FP	GP	DP	Froidure	Melrose	935	JI252	940a	Solara	CP	JI96	JI296	Overall mean
(A)															
500	2	1.0 bc	0.5 d	0.9 c	1.0 bc	1.0 bc	1.0 bc	1.1 bc	1.4 b	1.1 bc	1.3 b	1.3 b	1.0 bc	1.9 a	1.1 B
	3	1.5 d	1.4 d	1.3 d	2.0 c	2.1 c	1.9 c	2.5 b	2.6 b	2.0 c	2.7 ab	2.9 ab	3.0 a	3.0 a	2.2 B
1000	2	1.6 d	1.0 c	1.0 e	1.9 cd	1.5 d	1.7 d	1.9 cd	1.9 cd	2.2 bc	2.3 b	1.9 cd	2.5 b	3.0 a	1.9 A
	3	2.4 b	2.1 c	2.0 c	2.9 a	2.8 a	2.7 a	3.0 a	3.0 a	3.0 a	3.0 a	3.0 a	3.0 a	3.0 a	2.8 A
(B)															
500		10.4 gh	9.8 h	10.6 fgh	11.5 efg	11.8 de	10.6 fgh	12.8 d	12.8 d	11.6 ef	13.9 c	15.3 ab	14.6 bc	15.8 a	12.4
1000		12.4 f	12.1 f	12.0 f	13.6 e	13.6 e	14.0 de	14.4 d	15.4 c	15.3 c	16.0 bc	16.0 bc	16.6 b	17.5 a	14.5

le = lesion expansion; Champ = cv. Champagne

For each spore concentration × dai combination (i.e., for each line of the table), lesion diameter means of genotypes showing the same lower case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

For each spore concentration, lesion diameter means over all genotypes (i.e., for the last column of the table) showing the same upper case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

For each spore concentration (i.e., for each line of the table), AUDPC means of genotypes showing the same lower case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

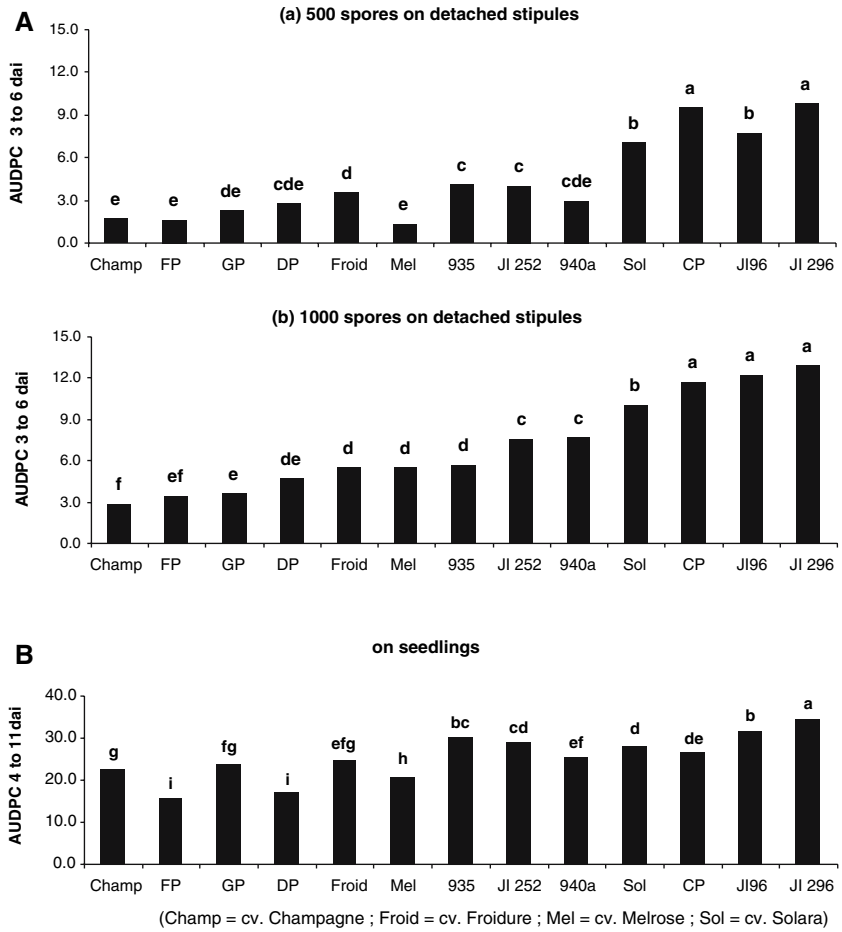

Fig. 5 Behaviour of a set of 13 pea genotypes with spore suspensions of *Mycosphaerella pinodes* isolate Mp1; (**A**) after point inoculation on detached stipules at inoculum concentrations of (a) 500 spores and (b) 1000 spores drop^{-1}, expressed by mean AUDPC calculated from lesion diameters from 3 to 6 dai; and (**B**) after spraying on seedlings at 10^5 spores ml^{-1}. Expressed by mean AUDPC calculated from disease severity assessed from 4 to 11 dai. AUDPC means of genotypes showing the same letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

has demonstrated that the difference between resistant and susceptible genotypes is best determined using fleck coalescence rather than on the rate of subsequent lesion expansion, which is the same for resistant or susceptible genotypes. In addition, we confirmed that in spite of the weak pathogenicity of *P. medicaginis* var. *pinodella*, the range of resistance expression is the same for *M. pinodes* and *P. medicaginis* var. *pinodella*. Partial resistance does not appear to be species-specific between these two very close species of the ascochyta complex. The mechanisms of resistance to both pathogens could therefore be the same.

Methodology of screening

An understanding of the parameters that determine ideal conditions for the precise assessment of partial resistance among host genotypes is of crucial importance for the establishment of standardised environmental and inoculation conditions. Under such conditions, specific methodologies can be developed to assess the disease. Inoculum concentration, inoculum age, growth conditions of plants and plant phenology should be taken into account when determining components of resistance (Parlevliet 1979) and studying the conditions under which resistance is expressed. In our environmental conditions, the best conditions we have established to display partial resistance to *M. pinodes* on detached stipules of pea are: stipule or leaflet from node 3 or 4, drop size of 10 µl with 500 or 1000 spores harvested from a colony of 10–14 days, and use of a moderately aggressive isolate. The disease scale based on that by Wroth (1998a), which takes into account both components of resistance together (fleck coalescence and disease expansion), simplifies disease assessment and permits studies of a large number of host

genotypes. The strong correlation we obtained between the seedling test and the test on detached organs, which has also been observed by Dolar et al. (1994) on chickpea and Hwang et al. (2006) on pea inoculated with the respective ascochyta blight pathogens, strongly supports the feasibility of using detached leaf methods for resistance screening or other purposes. Both methodologies (seedling and detached stipule), address different resistance reactions. Spray inoculation of intact seedlings with spore suspensions, gives information on the overall behaviour of a genotype for its level of resistance whereas the detached stipule methodology is better suited for giving information on different components of resistance. Point inoculations of leaves have already been used for several objectives: to study resistance and/or components of resistance (Dolar et al. 1994; Bouhassan et al. 2003) and factors acting on phases of epidemic cycles (Heath and Wood 1969; Carisse and Peyrachon 1999), to characterise isolates for their pathogenicity/virulence (Nasir et al. 1992; Wroth 1998b) and to screen genotypes/lines for their resistance (Wroth 1999; Warkentin et al. 1995; Kohpina et al. 2000; Zhang et al. 2006).

The choice of method for scoring disease progress depends upon the objectives of the work. If the objective is to dissect partial resistance on a few host genotypes, both components of resistance, fleck coalescence and lesion diameter, can be used in routine screening, which were well correlated with a seedling test. A simplification of the method could be envisaged, consisting of an assessment of fleck coalescence at 2–3 dai, and lesion diameter at 5–6 dai (respectively for inoculum 1000 and 500 spores per 10 µl drop^{-1}). However, in some situations, earlier assessments better aligned to differentiate between different incubation times, may be more appropriate. For screening tests using hundreds of lines, it is likely to be more suitable to use the more comprehensive scale as described here, and modified from Wroth (1998a) as a first step, before dissecting specific components of resistance. Assessing disease with this scale at two dates will implicitly take into account both components of resistance, fleck coalescence and lesion expansion beyond the inoculation drop.

As shown by Bretag and Brouwer (1995) and Wroth and Khan (1999), it is difficult to evaluate partial resistance to ascochyta blight in the field, due to factors interacting with disease severity assessments: agronomic traits (such as plant maturity, lodging, plant height and canopy architecture) or environmental conditions (such as climatic conditions and disease pressure levels). To obtain clearer insight into the main genetic effects involved in resistance, Prioul et al. (2003) and Hwang et al. (2006) tried to minimize these interactions by assessing resistance under controlled conditions. Fondevilla et al. (2005) and Hwang et al. (2006) have shown that cultivar rankings fluctuated across methodologies, but that ranking tended to be stable at the extremes (most resistant, most susceptible) between field and controlled conditions assessments. Likewise in most field trials, we observed significant differences between extreme genotypes DP and JI296 for their resistance to *M. pinodes* (data not shown). This methodology of detached stipules was used by Baranger et al. (2006) to develop further studies on genetic knowledge of resistance and QTL or gene identification. These authors have identified six QTL specifically involved in reducing *M. pinodes* fleck coalescence and lesion expansion.

We conclude that quantitative resistance can be expressed on detached pea stipules only under certain conditions, by expression on fleck coalescence and on lesion expansion. Other resistance components, mainly the reproduction of the pathogen (latent period, pycnidia/pseudothecial formation, number of spores), need to be studied. Reports show that often experimental conditions are the same to display different components of resistance. Vijanen-Rollinson et al. (1998) for instance, used the same conditions to study diverse components of quantitative resistance to powdery mildew in pea (conidial germination, infection efficiency, latent period and conidial production). Bouhassan et al. (2003) also analysed various components of partial resistance to chocolate spot in faba bean (incubation period, number of spots, lesion diameter, latency period and sporulation) under environmental conditions common to all components. The optimal experimental conditions we have defined for the expression of pea resistance to *M. pinodes* on fleck coalescence and lesion expansion might therefore be adapted to the study of other components of resistance. Further studies are needed to confirm this or show that some component evaluation would need specific environmental conditions. Furthermore, how these

components affect epidemic development on resistant genotypes in the field remains to be determined.

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