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Evidence that a leaf-disk test allows assessment of isolate-specific resistance in *Brassica oleracea* crops against downy mildew (*Peronospora parasitica*)

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Abstract

A rapid resistance/susceptibility test for *Peronospora parasitica* (downy mildew) was established by inoculating leaf-disks of four *Brassica oleracea* accessions. Several conditions were tested: disk disinfection or not, agar medium with or without nutrients and with 50 or 100 ppm of benzimidazole. Using disinfected disks placed on agar (no nutrient and benzimidazole at 50 or 100 ppm), the responses of leaf-disks to four isolates were similar to those obtained using the classical cotyledon test, whereas undesired contaminations occurred in all other conditions. The possible effect of the particular leaf used for obtaining the disks was also studied. In each incompatible interaction tested, disks were resistant whatever the leaf used. In compatible interactions, susceptible phenotypes were observed on disks derived from the six lowest leaves, but disks from upper leaves were resistant. The genetic basis of resistance in a F1 hybrid broccoli was assessed, by testing six isolates on an F2 population derived from this hybrid. The cotyledon test only allows inoculation of two isolates per seedling, whereas many isolates can be tested on each plant by using leaf-disks. The segregation of the resistance to each of the six isolates was analysed: two dominant genes (tightly linked) control resistance to all isolates (one to five isolates; the other to only one isolate).

Introduction

Downy mildew of Brassicas caused by *Peronospora parasitica* is a worldwide disease, and under cool climates with frequent dew formation as in Brittany (France), the disease causes severe epidemics on different crucifers, including *Brassica* vegetables such as cauliflower (*Brassica oleracea* var. *botrytis*) and broccoli (*B. oleracea* var. *italica*). Although chemical control is possible (White et al., 1984; Brophy and Laing, 1992; Crute et al., 1994; Vishunavat et al., 1998), breeding for resistance remains the most relevant means for controlling the disease.

Both specific complete (also known as isolate-specific) and partial (non-isolate-specific) resistances have been described in *Brassica* crops (Jensen et al., 1999b; Mahajan et al., 1995; Moss and Lucas, 1991b). The first type provides a total resistance of the plant to a limited number of isolates, usually visible as a hypersensitive response (HR) at the infection sites. Hence, the identification of a collection of accessions, with the whole set of complete specific resistance genes efficient against all races of the pathogen identified so far, is of particular interest for breeding programmes, since complete protection of the crops against the disease can be considered.

Routine screening for sources of resistance to *Brassica* downy mildew can be carried out either in the field or in the laboratory. In the latter case, screening is performed by inoculating cotyledons of seedlings and by assessing the level of resistance 7 days later (Williams, 1985). This screening strategy seems to be efficient since several authors have shown that seedling and adult plant resistances are correlated (Coelho et al., 1998; Jensen et al., 1999a). Different resistance sources have been identified by means of

this test (Natti et al., 1967; Nashaat and Awasthi, 1995; Silué et al., 1996; Jensen et al., 1999a; Badul and Achar, 1998; Hoser-Krauze et al., 1991; Moss and Lucas, 1991a; Thomas and Jourdain, 1990; Wang et al., 2000).

Using the cotyledon stage test, two isolates can be tested on each seedling by inoculating each cotyledon with one isolate (Williams, 1985). Nashaat et al. (1997) have used this method to describe a new specific complete resistance gene efficient against two *P. parasitica* isolates. Two isolates can rarely be tested on each cotyledon (four per seedling) because the risk of mixing them is high. The results obtained in a test involving more than one isolate per seedling may thus be subject to caution. A recent study showed that an avirulent isolate induced resistance against a virulent one, even when a few hours separated both inoculations (Monot et al., 2002). Another problem of the classical cotyledon test is that susceptible seedlings die when infected with a virulent isolate and can therefore not be studied for other traits (useful in a genetic linkage analysis). Therefore, a leaf-disk assay would be useful because it would allow many isolates to be tested on a single plant and this plant would remain alive, even in the case of susceptibility. Leaf-disk pathogenicity tests have been developed and successfully used in several pathosystems including melon/*Sphaerotheca fuliginea* (Cohen, 1993), banana/*Mycosphaerella fujiensis* (El Hadrami et al., 1998), grape/*Plasmopara viticola* (Brown et al., 1999), cherry/*Podosphaera clandestine* (Olmstead et al., 2000), and cocoa/*Phytophthora palmivora* (Tahi et al., 2000).

The aim of the present study was to develop a leaf-disk pathogenicity test allowing assessment of resistance/susceptibility to *P. parasitica* in *Brassica* vegetables. Therefore, it was necessary to identify the conditions which allowed to establish a correlation between results of the leaf-disk test and those of the classical (cotyledon) test. The leaf-disk test was used in a genetic analysis, by testing six different downy mildew isolates on each plant of an F2 population in which the resistance trait was known to segregate.

Materials and methods

Plant material

Four accessions were used: one cauliflower cv. Billabong, already described as highly susceptible to *P. parasitica* (Silué et al., 1995; 1996), and three F1 hybrid broccoli cultivars: BRP1, BRP2 and Milady. Their interaction phenotypes with several *P. parasitica* isolates have been previously determined in our laboratory by using the cotyledon test, and were confirmed here in the new experiments.

An F2 population was produced by selfing BRP1, in order to study the segregation of the resistance to several isolates (genetic analysis). Seedlings were produced in the greenhouse (Bécot et al., 2000). Before leaf-disk testing, they were first grown in $9 \times 9 \times 9\,cm^3$ plots and then in 20 cm diameter plots.

Peronospora parasitica *isolates*

For the development of the pathogenicity test, four *P. parasitica* isolates were used: FP03, FP06, FP15 and P503. Their characteristics and pathogenicity patterns were described by using the cotyledon test (Godard et al., 1999; Bécot et al., 2000). FP03 and FP06 were virulent on Billabong and avirulent on BRP1, BRP2 and Milady. Isolates FP15 and P503 were virulent on all accessions tested. Results obtained by replicating these experiments, as controls of our isolates and plants, are reported.

The genetic analysis of BRP1 resistance was carried out with six isolates (FP02, FP03, FP04, FP05, FP06 and FP12), whose characteristics were already described by using the cotyledon test (Godard et al., 1999; Bécot et al., 2000).

Inoculum preparation

Spores of each isolate were produced on 7-day-old seedlings of the susceptible variety Billabong. They were washed off by agitating sporulating cotyledons in sterile distilled water. The conidial suspension was adjusted to 2 or 5×10^4 spores ml^{-1}, for cotyledon and leaf-disk tests, respectively, after counting with a hemacytometer. In the cotyledon tests, 7-day-old seedlings were inoculated by depositing two 20 µl droplets on each cotyledon.

Disease assessment in the cotyledon test

In cotyledon tests, disease was assessed by using the six point (0, 1, 3, 5, 7, 9) scale developed by Williams (1985) in which 0 corresponds to an absence of visible symptoms and 9 to a heavy sporulation on both sides of the cotyledon. The other ratings correspond to intermediate classes of symptoms. A mean

disease index (DI) is then calculated using the formula:

$$DI = \sum_{i=0}^{9} \frac{(i*j)}{n}$$

where i is the interaction phenotype class, j the number of plants in each class and n the total number of plants tested. The accessions are then classified as resistant (R, DI $\leq$ 3.0), moderate resistant (MR, 3.0 $<$ DI $\leq$ 4.0), moderate susceptible (MS, 4.0 $<$ DI $\leq$ 5.0) and susceptible (S, DI $>$ 5.0), according to the respective mean DIs obtained.

Leaf-disk test

To study the correlation between the interaction phenotypes obtained with the leaf-disk test and in the classical cotyledon test, leaf-disks were removed from sufficiently developed leaves (at least 100 cm^2). Different conditions were tested: they were either disinfected in a 3% calcium hypochloride solution or simply rinsed in sterile distilled water, then placed in agar plates (14 cm diameter) with the upper side on the agar surface. The agar medium was completed with nutrient (MS, Murashige and Skoog, 1962) or not (no nutrient) and contained either 50 or 100 ppm of benzimidazole (SIGMA, France). Five droplets of 20 μl of a spore suspension (5×10^4 spores ml^{-1}) were deposited on each leaf-disk. Working with five droplets was a way to spread the isolate on the disk (a spraying process, as used for infecting whole plants, was not possible on leaf-disks placed in Petri dishes). After inoculation, Petri dishes were sealed and kept in a growth chamber in the dark for at least 8 h, then for 7 days under a 12 h photoperiod (16 °C night and 20 °C day, 30 μE light intensity). At least six leaf-disks per couple of accession/isolate were tested at least twice for each treatment.

In another experiment on the effect of the leaf position on the disk resistance/susceptibility response, each leaf from the bottom (number 1) to the top of the plant was tested (the highest number was 17). These 17 leaf positions were obtained on 10–18-week-old plants, in order to cut leaf-disks from sufficiently developed leaves (at least 100 cm^2). Ten disks per leaf position were tested for each genotype/isolate combination.

Disease assessment on inoculated leaf-disks

In most cases, the disk symptoms were scored as either susceptible (S, heavy sporulation) or resistant (R, no sporulation and/or HR reaction). Nevertheless, in a few cases, it was possible to observe intermediate levels of sporulation that were scored as either moderately resistant (MR) or moderately susceptible (MS).

Results

Responses of the four accessions tested at the seedling stage

Table 1 shows the responses of the four *Brassica* accessions inoculated with four *P. parasitica* isolates at the seedling stage. As expected, BRP1, BRP2 and Milady were resistant to two isolates (FP03, FP06), whereas Billabong was susceptible to all of them (DI $\geq$ 8.0). P503 and FP15 were virulent against all four accessions tested.

Development of a leaf-disk pathogenicity test

When the agar plate was completed with the MS medium (with benzimidazole at 50 or 100 ppm), leaf-disks, previously disinfected or not, were always contaminated with undesired microorganisms and these contaminants interfered with the development of downy mildew. A higher level of contamination was observed with 50 ppm benzimidazole compared to the 100 ppm, and induced a reduced *P. parasitica* sporulation on leaf-disks derived from susceptible cultivars. It was concluded that the MS medium was not appropriate for the disease susceptibility test.

By using 1% agar without any nutrient added, but containing 50 or 100 ppm of benzimidazole, and disks from disinfected leaves, contamination problems were solved. On leaf-disks simply rinsed in water (no disinfection), *P. parasitica* sporulation was difficult to score, mostly because of occasional contaminations.

Table 1. Responses of one cauliflower and three broccoli accessions to four *P. parasitica* isolates, using the cotyledon test. Numbers represent the DI calculated as described in the Material and methods section: a DI $>$ 5.0 is rated as susceptible; a DI $\leq$ 3.0 is rated as resistant

Isolates	Billabong Cauliflower	BRP1 Broccoli	BRP2 Broccoli	Milady Broccoli
FP03	9.0	1.0	1.2	1.1
FP06	8.8	2.0	2.1	1.5
P503	8.9	7.9	7.3	8.5
FP15	8.9	8.1	6.1	8.8

Table 2. Assessment of resistance/susceptibility of four *Brassica* accessions to four *Peronospora parasitica* isolates by means of a leaf-disk pathogenicity test. Leaf-disks were disinfected in a 3% hypochloride solution before being placed on 1% agar plates containing 50 or 100 ppm benzimidazole

Isolates	Benzimidazole					
	50 ppm				100 ppm	
	Billabong	BRP1	BRP2	Milady	Billabong	BRP1
FP03	S[1]	R[1]	R	R	nt	nt
FP06	S	R	R	R	S	R
P503	S	S	S	nt	S	S
FP15	nt	nt	nt	S	nt	nt

[1]: S = susceptible, R = resistant, nt: not tested.

Once the contamination problem was solved, it was possible to compare the results of resistance/susceptibility as determined by our leaf-disks test (Table 2) to those obtained with the cotyledon test (Table 1). With either 50 or 100 ppm of benzimidazole, each variety/isolate combination showed results comparable to those at the seedling stage. Since no significant difference was observed between the two concentrations of benzimidazole (50 and 100 ppm), the lowest concentration was chosen for further experiments.

Based on the results presented above, it was concluded that the best conditions for the proposed leaf-disk test are as follows: leaf-disks are disinfected with 3% calcium hypochloride, then placed on 1% agar plates (no nutrient added) containing 50 ppm benzimidazole. Five droplets of 20 µl each of the *P. parasitica* spore suspension (5×10^4 spores ml^{-1}) are then deposited on each leaf-disk.

Responses of the leaf-disks according to leaf position

The aim of this experiment was to check whether plant age affected the results of the leaf-disk test. The results of leaf-disks obtained from 17 leaf positions (each position was identified by a number, as described in the Material and methods section) were compared. All leaves used were sufficiently developed, since they were cut from 10 to 18-week-old plants.

Six incompatible interactions (BRP1, BRP2 and Milady challenged with FP03 and FP06) were studied. In all cases, comparable responses (i.e. no sporulation) were observed on all leaf-disks. Figure 1A shows an example of this type of interaction.

For the compatible interactions (Billabong challenged with the three isolates FP03, FP06 and FP15, and the broccoli cultivars challenged with FP15), several sporulation intensities were observed depending on the position of the leaves. The six lowest leaves always provided disks with a susceptible phenotype. Above the sixth leaf, moderately susceptible to resistant phenotypes were observed. An example in this category is shown in Figure 1B. The leaf position at which the susceptible phenotype turns to the resistant one differs according to the accession/isolate interaction tested. However, in all cases, susceptibility was observed for the six lowest leaves.

Genetic analysis using the leaf-disk test

The segregation of the resistance trait was previously studied in an F2 population obtained from BRP1, by using the cotyledon test. For each of the two *P. parasitica* isolates FP05 and FP06, resistance in BRP1 appeared to be controlled by one dominant gene (unpublished data). Whether one gene only controls resistance in BRP1 to both isolates tested could not be determined, since two distinct sets of seedlings were studied for the two isolates. To answer this question, each plant has to be analysed individually with each isolate, in order to compare segregation of resistance to each isolate (genetic analysis). Therefore, the use of our new leaf-disk test was of great interest for testing several isolates per F2 plant, in order to further study the resistance of BRP1.

A new F2 population (240 plants) developed from selfing of BRP1 was produced, and each F2 plant was tested with six *P. parasitica* isolates. The segregation results obtained using the leaf-disk test are shown in Table 3 and showed that resistance to each of the six isolates was controlled by one dominant gene. Resistance to five isolates (FP03, FP04, FP05, FP06 and FP12) strictly co-segregated when comparing the phenotype interactions obtained for each F2 plant: an F2 plant resistant to one isolate was resistant to the four other ones, and it was similar for the susceptible plants (only a few data were missing; Table 3). This perfect co-segregation indicates that only a single dominant gene (designated *RP1*) controls resistance in BRP1 to these five isolates (as long as the few missing data do not hide any differences between these five segregation data sets). The segregation data to the sixth isolate FP02 indicated that this resistance was controlled by a second dominant gene (designated *RP2*). This second

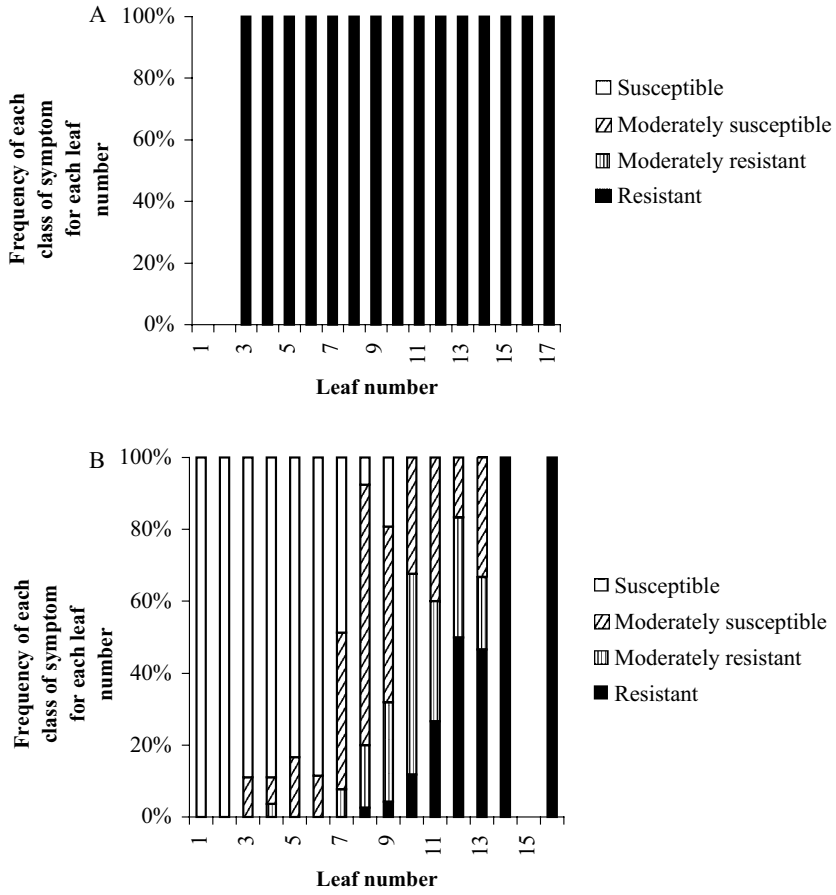

Figure 1. Leaf-disk responses in the case of (i) an incompatible interaction: BRP2/FP06 (A), and (ii) a compatible interaction: Billabong/FP06 (B). For each leaf position, ten leaf-disks were inoculated with the isolate FP06 and sporulation on each disk was scored according to a quantitative scale with four classes of symptoms. Results are expressed in the frequency of each class of symptom per leaf number.

gene seems closely linked to *RP1*, since only two F2 plants resistant to FP02 were susceptible to all the other isolates. For these two plants, pathogenicity tests were repeated 4 times and their phenotype was always confirmed.

Discussion

The same responses were obtained on leaf-disks inoculated with *P. parasitica* as those obtained on cotyledons by using the classical cotyledon test.

Other studies using a leaf-disk assay were established in several other plant species. In most of them, leaf-disks are not disinfected before the inoculation. Brown et al. (1999) used non-disinfected grape leaf-disks, and the responses obtained with regard to the pathogen *Plasmopara viticola* were well correlated with those observed in fields. Similar results were obtained in other interactions, such as melon/*Sphaerotheca fuliginea* (Cohen, 1993) and cocoa/*Phytophthora palmivora* (Tahi et al., 2000) with no previous disinfection of leaf-disks. This study has shown that in *B. oleracea*, leaf-disks had to be disinfected in order to obtain results comparable to those observed on seedlings by using the classical cotyledon test. Olmstead et al. (2000) also disinfected the leaves before placing them in Petri dishes in the pathosystem cherry/*Podosphaera clandestina*.

In this study, we tested whether the leaf used in the test (identified by its position on the plant) influenced the response of the leaf-disks. Although in the present

Table 3. Segregation in resistant (R) and susceptible (S) plants in an F2 population (240 plants) obtained by selfing BRP1 (a F1 hybrid broccoli). Six *Peronospora parasitica* isolates were tested using the established pathogenicity leaf-disk test

Isolates	Segregation			Hypothesis tested (3 : 1) $\chi^{2(2)}$
	R[(1)]	S[(1)]	Missing data	
FP02	187[(3)]	52	1	1.34
FP03	185[(3)]	54	1	0.74
FP04	185[(3)]	53	2	0.95
FP05	185[(3)]	52	3	1.18
FP06	185[(3)]	54	1	0.74
FP12	185[(3)]	55	0	0.55

[(1)]R = resistant, S = susceptible.
[(2)]Each χ^2 value was calculated on the basis of the phenotypes obtained (missing data, if any, were obviously not taken into account). To accept the hypothesis of the segregation tested (3 R : 1 S, segregation of a dominant resistance gene), the χ^2 should not exceed 3.84 ($P \leq 0.05$).
[(3)]185 F2 plants were resistant to all 6 isolates. Of the remaining F2 plants, 2 were only resistant to the FP02 isolate but susceptible to the 5 other isolates (most of the others were susceptible to all six isolates, or to most of these isolates since a few phenotype data are missing).

work, no differences were observed for disks cut from leaf one to six, the disks from the leaves above the sixth one on a susceptible cultivar (as determined by the conventional cotyledon test) showed more resistant phenotypes (MS, MR and R). In this latter case, with leaves above the sixth one, susceptibility symptoms (sporulation intensity) decreased with increasing leaf number. Dickson and Petzoldt (1993) also showed that susceptibility to downy mildew can turn to resistance in broccoli grown under field conditions. The problem of fluctuating results related to the leaf considered was also described by Cohen (1993) who showed in the pathosystem melon/*Sphaerotheca fuliginea* that only the third leaf response in an *in vitro* test was in agreement with the one of the whole plant. Brown et al. (1999) showed that bottom grapevine leaves were more resistant to *Plasmopara viticola* than those from the top. In that work, only fully expanded leaves between node four and six were used. The present study was useful since it revealed limitations in the use of the test, which can be used to accurately predict the response of young *Brassica* plants to downy mildew, as long as leaves under the sixth one are used for producing the leaf-disks.

A segregation analysis of the resistance trait in an F2 population was carried out using this test. Results obtained with the developed *in vitro* pathogenicity test showed that the resistance to each of the six isolates (FP02, FP03, FP04, FP05, FP06 and FP12) is controlled by one dominant gene (3 R : 1 S segregation). For two isolates (FP05 and FP06), this segregation was in agreement with results obtained previously using the cotyledon test on another set of F2 plants. This result showed that the present leaf-disk test gives reliable responses.

A single dominant gene (*RP1*) confers the resistance to five isolates (FP03, FP04, FP05, FP06 and FP12). Resistance to the sixth isolate, FP02, seemed to be controlled by a second dominant gene, designated *RP2*, closely linked to *RP1* (only two F2 plants provided a different response with FP02 than with the five other isolates). Recent genetic and molecular studies have revealed a particular genomic organization of resistance genes. Indeed, in many plant species, these genes are organized in clusters that can confer resistance to several pathogens (for example, in tomato, Yuan et al., 2002; in soybean, Graham et al., 2002; in *Medicago truncatula*, Zhu et al., 2002; in lettuce, Shen et al., 2002; in potato, Van der Vossen et al., 2000; in *Arabidopsis*, Richly et al., 2002; and for a review, Michelmore, 2000). Many clusters of resistance genes were reported in *A. thaliana* (Botella et al., 1997; Speulman et al., 1998; Cooley et al., 2000), and in particular, clusters of downy mildew resistance genes (Parker et al., 1997; Botella et al., 1998; Van der Biezen et al., 2002). Therefore, the close genetic linkage we observed between *RP1* and *RP2* is in accordance with the presence of a cluster of *P. parasitica* resistance genes in brocoli.

Now further work aims at finding molecular markers linked to the resistance genes reported in this study. The leaf-disk test presented here will be very useful for screening *B. oleracea* genotypes in order to develop new cultivars with high level of resistance to *P. parasitica*.

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