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Leaf position, leaf age and plant age affect the expression of downy mildew resistance in *Brassica oleracea*

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Abstract Downy mildew caused by the oomycete *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*) is a worldwide foliar disease of Brassica vegetables, which may cause seedling loss in the nurseries and damage to adult plants in the field. Disease symptoms start from the lower leaves and progress upwards. Three experiments were conducted under controlled environment conditions, using inoculated leaf discs, to determine the influence of leaf position, plant age, and leaf age on the expression of resistance to downy mildew in various *Brassica oleracea* genotypes. The upper leaves were more resistant than the lower leaves when 7–19 week-old plants of broccoli and Tronchuda cabbage were tested. Broccoli lines 'PCB21.32' and 'OL87123-2' were fully susceptible at the cotyledon stage, showed a clear resistance increase from lower to upper leaves at 6 weeks and 'PCB21.32' was fully resistant 16 weeks after sowing. Immature leaves were more resistant than adjacent fully expanded mature leaves. Susceptibility increased with leaf age when the same leaf was tested at two to 4-week intervals. Leaf age and upper-leaf position on the stem had opposite effects on disease score, since younger leaves collected from lower positions and older leaves collected from upper positions tended to score similarly in compatible interactions. The progression of downy mildew from the base of the plant upwards on *B. oleracea* in the field could be due to differences in leaf resistance in addition to environmental variation. To maximise the expression of a compatible reaction in adult plants lower leaves of Brassica plants that are at least 12 weeks-old should be used.

Keywords Disease resistance · Broccoli · Tronchuda cabbage · *Hyaloperonospora parasitica*

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Introduction

Downy mildew caused by the oomycete *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*) is an important foliar disease of Brassica vegetables worldwide, which may cause seedlings to be lost in nurseries and damage adult plants in the field, especially in temperate climates. The development of sporulating lesions of *H. parasitica* in adult plants induces defoliation and early leaf senescence, and reduces yield and head quality of broccoli and cauliflower (Niu et al. 1983; Jensen et al. 1999). Disease symptoms in the field start on the lower leaves and progress upwards (Natti et al. 1956; Coelho et al.

1998; Jensen et al. 1999). This disease pattern could be attributed to environmental differences, variation in inoculum availability and changes in leaf resistance.

Downy mildew resistance is affected by plant development in *Brassica oleracea*. The resistance of mature broccoli plants having eight or more leaves is independent of the resistance of young seedlings (Dickson and Petzoldt 1993) and the plants can be susceptible at the cotyledon stage and resistant at the adult stage (Coelho and Monteiro 2003b). In 'Couve Algarvia', resistance at cotyledon and adult-plant stages is under the control of two different genetic systems (Monteiro et al. 2005). Agnola et al. (2003) used leaf discs collected from 17 leaf positions in 10–18 week-old Brassica plants to show that incompatible reactions occur regardless of leaf position, but with compatible reactions, sporulation intensity varies with the position of the leaf on the stem.

The increasing resistance to pathogens with plant or leaf ageing is a form of resistance referred to as age-related resistance (ARR) (Kim et al. 1989). ARR in *Arabidopsis* is a developmentally regulated and environmentally sensitive defence response to *Pseudomonas syringae* (Kus et al. 2002). ARR is frequently reported in mature plants in different plant-pathogen interactions: potato-*Phytophthora infestans* (Warren et al. 1971), lettuce-*Bremia lactucae* (Dickinson and Crute 1974), tobacco-*Peronospora tabacina* (Reuveni et al. 1986; Cohen et al. 1987; Wyatt et al. 1991; Hugot et al. 2004) and pepper-*Phytophthora capsici* (Kim et al. 1989).

In order to clarify these questions, the use of leaf discs is adequate to assess disease resistance variation within adult plants because it is possible to compare, under the same environmental conditions, the resistance of leaves of different ages or leaves collected from different positions. The use of leaf-disc evaluation methods is more informative than field or greenhouse tests, and they can also be used to predict host response to natural epidemics in the field (Reuveni et al. 1986; Brown et al. 1999; Cohen et al. 2000; Olmstead et al. 2000; Visker et al. 2003).

The objective of this research was to produce a model to explain the variation of downy mildew resistance in *B. oleracea* by (1) determining the plant growth stage where adult-plant downy mildew resistance is clearly expressed, and (2) evaluating how resistance is expressed at different leaf ages and at different leaf positions. This model will also help to determine the best plant material to be inoculated when testing *B. oleracea* genotypes for downy mildew resistance.

Materials and methods

This research consisted of three experiments to evaluate host response to downy mildew in different *B. oleracea* genotypes by using different permutations of three variables: plant age; leaf age and leaf position on the stem.

Experiment 1

A small-scale experiment was conducted using ten plants from broccoli line 'PCB17.11' (Table 1). After evaluating cotyledon resistance, as described below, the cotyledons were removed and the seedlings transferred to 16-cm pots filled with a peat-based substrate (Levington M2, Fisons, UK) and grown in a greenhouse for 12 weeks. Two leaf discs were collected from the third to tenth leaves on the stem counted from the base. We did not test the two oldest leaves at the bottom because they had dropped and the three youngest leaves at the apex because they were too small. The discs were inoculated and then incubated in a growth chamber using the leaf-disc protocol described below.

Table 1 *Brassica oleracea* hosts tested

Plant code	Sub species	Crop type	Description	Experiment
PCB17.11	*italica*	Broccoli	Inbred line (S_5)	1, 3
PCB21.32	*italica*	Broccoli	Inbred line (S_6)	2, 3
OL87123-2	*italica*	Broccoli	Inbred line (S_1)	2
PCM21.11	*tronchuda*	Couve Murciana	Inbred line (S_6)	3
PCA12.112	*tronchuda*	Couve Algarvia	Inbred line (S_3)	3
GK97362	–	Rapid cycling Brassica	Double-haploid line	3

Experiment 2

Twenty-five plants from each of the broccoli lines 'PCB21.32' and 'OL87123-2' (Table 1) were tested at the cotyledon stage. The cotyledons were removed after evaluation and the seedlings transplanted to 9-cm pots filled with a peat-based substrate (Levington M2, Fisons, UK) and maintained in a growth chamber at 20±1°C, 70±10%RH and a 20 h photoperiod under cool-white fluorescent light (Osram) at 250 μmol m^{-2} s^{-1}. All the plants were tested 6 weeks, 7 weeks, and 8 weeks after sowing by collecting one disc per leaf from leaves occupying different positions along the stem (between 3–13 nodes). Leaves were numbered upwards starting at the first node and the higher leaf evaluated at one date was repeated at the next date. The discs collected were inoculated using the leaf-disc protocol.

Experiment 3

Sixteen plants from each of four Brassica genotypes (Table 1) were tested at different plant ages by collecting discs from leaves of different ages and positions on the stem. Discs from the susceptible rapid-cycling genotype 'GK97362' were used as controls.

The seedlings were transplanted to 16-cm pots filled with a peat-based substrate (Levington M2, Fisons, UK) containing a slow-release fertiliser (Osmocote, Scotts International B.V.) and maintained in a growth chamber at 20±1°C, 70±10%RH and 20 h photoperiod under cool-white fluorescent light (Osram) at 250 μmol m^{-2} s^{-1} for 7 weeks. The plants were then transferred to a greenhouse (air temperature ranging from 10°C to 25°C). The plants were watered by hand during the experiment.

Two discs per leaf were collected from all the plants 7, 9, 11, 13, 16, and 19 weeks after sowing. A variable number of leaf ages and positions were tested for resistance on each collection date. The leaves were numbered upwards from nodes 1 to 18. The leaf positions not tested at each date were because leaves were either too small (leaves above the fourth position from the apex) or they had dropped. The leaf discs collected were inoculated using the leaf-disc protocol.

Inoculum preparation

The Portuguese *H. parasitica* isolate P523 (collected from a field of *B. oleracea* var. *tronchuda*, at Batalha, Portugal, in 1997) was used for all cotyledon and leaf-disc inoculations. The inoculum preparation followed the procedure described by Leckie et al. (1996). Freshly sporulating cotyledons of the susceptible maintenance plant stock were dipped in distilled water and gently agitated to dislodge conidia. The spore suspension was filtered through two layers of muslin to remove mycelial fragments and centrifuged at 370*g* for 3 min. The supernatant was discarded and the pellets re-suspended in distilled water. This was repeated twice and the pellets re-suspended in distilled water to reach a final concentration of 5×10^4 and 1×10^5 spores ml^{-1} for cotyledon and leaf-disc inoculation respectively.

Cotyledon testing

Seeds of the *B. oleracea* hosts were germinated in multi-cell trays (3×3×5 cm) filled with a peat-based substrate (Levington F2, Fisons, UK) and maintained in a growth chamber at 20±1°C, 70±10%RH and a 20 h photoperiod under cool-white fluorescent light (Osram) at 250 μmol m^{-2} s^{-1} for 1 week until the cotyledons were fully expanded. The seedlings were then inoculated by depositing two 10-μl droplets of conidial suspension on each cotyledon. The trays were enclosed in sealed polyethylene bags (RH= 100%) and incubated at 16±1°C in the dark for 24 h. The polyethylene bags were removed and the trays placed in the growth chamber under the conditions described for the production of the seedlings. Six days after inoculation, the trays were enclosed again in polyethylene bags and incubated at 16±1°C in the dark for 24 h to induce sporulation. The bags were removed and the interaction phenotype of each individual seedling was scored using a seven-class scale of increasing susceptibility (Table 2). Plants showing no symptoms (class 0) were assumed as escapes and discarded. The host resistance was classified according to the mean disease score (DS) of all the cotyledons tested: resistance ($DS<3.5$); intermediate resistant ($3.5\leq DS<4.5$) and susceptible ($DS\geq4.5$).

Leaf-disc protocol

Leaf resistance was evaluated following the leaf-disc method described by Monteiro et al. (2005). Leaf discs (2 cm diam) were cut from each leaf and placed

Table 2 Downy mildew interaction-phenotype classes used for cotyledon and leaf-disc evaluation

Class	Interaction phenotype
0	No host reaction, no sporulation
1	Light host necrosis localised on the upper cotyledon/leaf disc surface, no sporulation
2	Diffuse host necrosis localised on the upper cotyledon/ leaf disc surface, no sporulation
3	Host necrosis localised on the upper cotyledon/leaf disc surface, weak sporulation (five conidiophores) localised on the lower cotyledon/leaf disc surface confined to the point of infection
4	Host necrosis localised on the upper cotyledon/leaf disc surface, heavy sporulation localised on the lower cotyledon/leaf disc surface confined to point of infection
5	No necroses on the upper surface, sparse to moderate sporulation dispersed over the whole cotyledon/leaf disc surface
6	No necroses on the upper surface, abundant and dense sporulation dispersed over the whole cotyledon/leaf disc surface

over a plastic net in 10-cm diam plastic Petri dishes containing 1 g of medium-size perlite moistened with 10 ml of distilled water. The leaf discs were inoculated on the adaxial surface with two 10-µl droplets of the *H. parasitica* conidial suspension using a micropipette. The Petri dishes were covered and incubated for 24 h under the same conditions previously described for the cotyledons. After incubation, the Petri dishes were sealed with parafilm and placed randomly in a growth chamber under the conditions previously described for the production of the seedlings. Six days after inoculation, the leaf discs were lightly sprayed with distilled water and incubated at 16±1°C in the dark for 24 h to induce sporulation. Then the interaction phenotype of each leaf disc was individually scored according to the seven-class scale used for the cotyledons (Table 2). The differences in resistance were estimated by the mean DS of the observed leaf discs following the criteria used for the cotyledons.

Data analysis

Analysis of variance was performed on the data using Statistica version 6.0. Differences between means were separated by Tukey HSD test. Mean DS in the different cotyledons, leaf positions and ages was the mean value of all the cotyledons and leaf discs individually evaluated for each position and age.

Results and discussion

Effect of leaf position

The eight consecutive leaves of broccoli line 'PCB17.11' tested in Experiment 1 showed a significant increase in resistance to downy mildew from the bottom of the plant upwards in 12 week-old plants (Fig. 1). The lowest leaves were fully susceptible, the highest leaves resistant, and those in between expressed intermediate resistance responses. An increase in resistance from the lower to the upper leaves was also shown in Experiment 2 by 6 week-old plants, but the leaves of 7 week and 8 week-old plants above the six and nine positions were all resistant (Figs. 2 and 3). Experiment 3 also shows that the highest leaves were in general more resistant than the lowest leaves when 7–19 week-old plants were tested (Table 3). In 16 week-old plants, the greater resistance of the upper leaves was clear in line 'PCB17.11' but less evident in the three other hosts.

The gradual increases in leaf resistance from the bases to the apices of the plants in Experiments 1, 2 and 3 agrees with field observations of susceptible Brassica plants, where the lowest leaves are the first to show disease symptoms with larger lesions and heavier sporulation (Coelho et al. 1998; Jensen et al. 1999). Our results agree with those found by Agnola et al. (2003), who compared downy mildew susceptibility of leaf discs from fully-developed leaves occupying 17 leaf positions on 10–18 week-old Brassica plants and showed that discs collected below the sixth leaf are susceptible, and those collected above the sixth leaf are moderately susceptible to resistant.

Variable susceptibility according to the leaf position on the stem also exists in other host-pathogen systems. Potato late blight (*P. infestans*) shows a higher linear growth rate in basal leaves than in apical leaves independently of plant architecture, environmental conditions, plant age, and cultivar (Visker et al. 2003). It is therefore important to test leaves occupying the same position on the stem for a reliable disease resistance comparison between hosts.

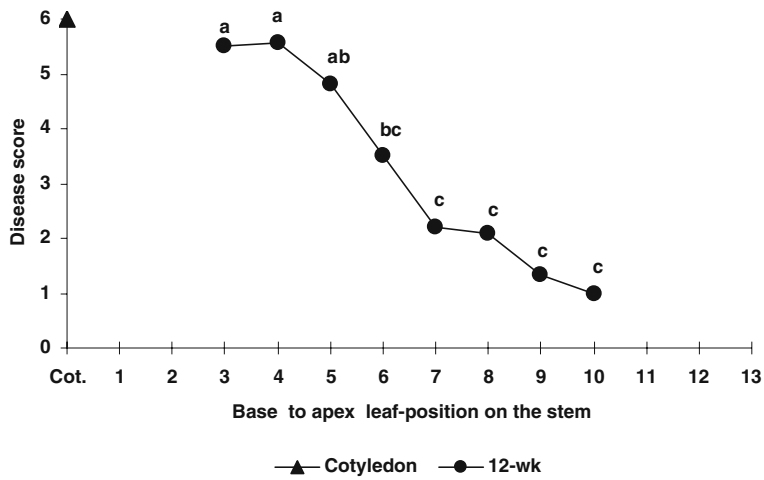

Fig. 1 Disease scores of cotyledons and leaves in various node positions in broccoli line 'PCB17.11' inoculated with *H. parasitica* at 1 week and 12 weeks after sowing (Experiment 1). Each value is the mean of 10 replicates. Values followed by different letters are significantly different ($P \leq 0.05$) according to Tukey HSD test

Effect of plant growth stage

Downy mildew resistance varied with plant growth stage. Broccoli lines 'PCB21.32' and 'OL87123-2' in Experiment 2 were susceptible at the cotyledon stage, showed a clear resistance increase from the lower to the upper leaves in 6 week-old plants, and all leaves tested above the sixth and ninth positions were resistant in 7 week and 8 week-old plants respectively (Figs. 2 and 3). These broccoli genotypes are also resistant as adult plants under field conditions (Coelho and Monteiro 2003b). Broccoli line 'PCB21.32' was also tested in Experiment 3 but resistance was expressed only above the eight-leaf position. The increase of resistance with plant age suggested that a plant susceptible at the cotyledon stage may express resistance 12 weeks or 13 weeks later. Older plants in Experiment 3 were more resistant than younger ones in lines 'PCB21.32', 'PCM21.11' and 'PCA12.112', but in line 'PCB17.11' the plants remained susceptible until they were 16 weeks-old (Table 3).

In Experiments 2 and 3 some hosts, susceptible as young plants, had a transition phase beyond which they expressed resistance to downy mildew (Figs. 2 and 3, and Table 3). However, a precise minimum age for the expression of adult plant resistance was not evident because the shift in expression of resistance occurred in plants that were between 7 weeks and 13 weeks-old, and was also influenced by the position of the leaf on the stem and the age of the leaf. ARR to oomycetes occurs in various species with older plants being more resistant than younger ones (Reuveni

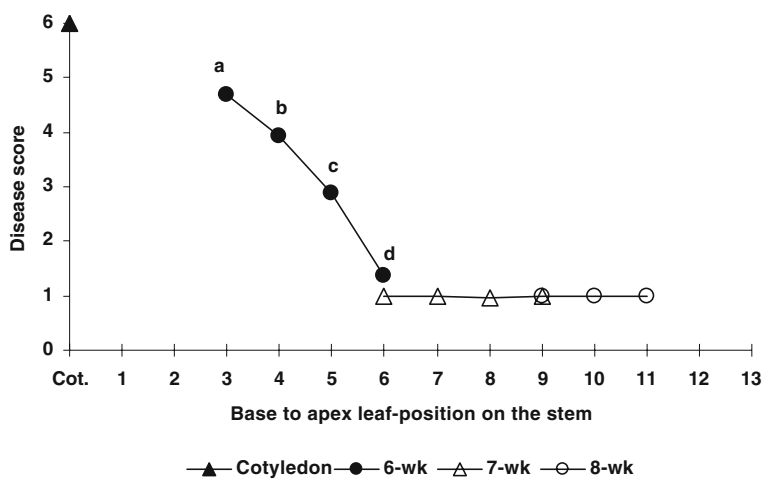

Fig. 2 Disease scores of cotyledons and leaves in various node positions in broccoli line 'PCB21.32' inoculated with *H. parasitica* at 1 week, 6 weeks, 7 weeks and 8 weeks after sowing (Experiment 2). Each value is the mean of 25 replicates. Values followed by different letters are significantly different ($P \leq 0.05$) according to Tukey HSD test

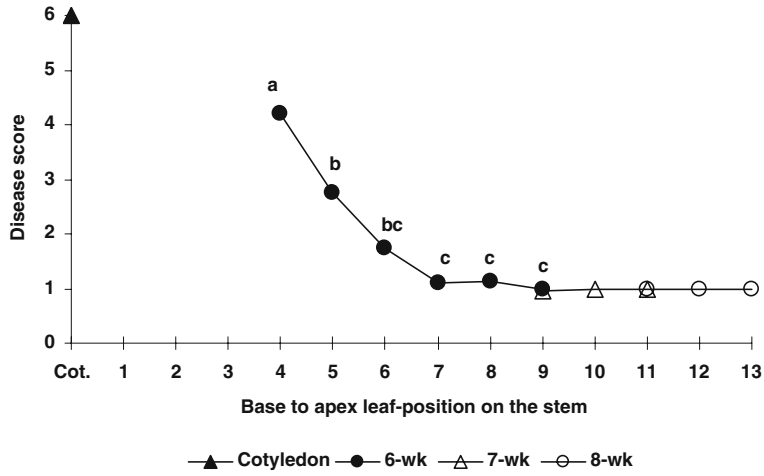

Fig. 3 Disease scores of cotyledons and leaves in various node positions in broccoli line 'OL87123-2' inoculated with *H. parasitica* at 1 week, 6 weeks, 7 weeks and 8 weeks after sowing (Experiment 2). Each value is the mean of 25 replicates. Values followed by different letters are significantly different ($P \leq 0.05$) according to Tukey HSD test

et al. 1986; Cohen et al. 1987; Wyatt et al. 1991; Hugot et al. 1999, 2004). Also, some broccoli lines show an evident transition from susceptibility to resistance between the sixth and seventh leaf stages (Dickson and Petzoldt 1993).

Monteiro et al. (2005) reported an independent F2 segregation between cotyledon and adult plant resistance to downy mildew in 'Couve Algarvia' with plants expressing each of the four possible combinations of susceptibility and resistance at cotyledon and adult-plant stages. The existence of plants that are susceptible through all growth stages and plants resistant at the cotyledon stage and susceptible as adult plants contradicts a permanent developmental-regulated resistance and supports the action of genes conferring resistance at each stage or at both stages of development. However, behind this qualitative genetic control there might be present a quantitative influence of ageing that decreases susceptibility in older plants. Experiment 3 supported this hypothesis because basal leaves from 19 week-old plants from line 'PCB17.11' tended to express a lower disease score than the basal leaves of younger 9–16 week-old susceptible plants (Table 3).

Effect of leaf age

To determine the effect of leaf age on downy mildew resistance we performed an analysis of variance separately for each individual leaf position between leaf ages. In Experiment 2, only two leaves were re-evaluated for two different dates, positions 6 and 9 on line 'PCB21.32' and positions 9 and 11 on line 'OL87123-2' (Figs. 2 and 3). With the 1-week interval, the downy mildew resistance of tested leaf positions did not change.

Experiment 3 showed that immature leaves did not become infected, which agreed with the hypothesis that upper leaves are more resistant. When the same leaves were tested repeatedly at two to 4-week intervals, older leaves were more susceptible than younger leaves. Susceptibility increased when the leaves were tested between 7 weeks and 9 weeks in all lines. Older leaves were significantly more susceptible on positions 2 to 9, 11, and 13 from line 'PCB17.11', on position 5 from line 'PCB21.32', on positions 3 to 7 from line 'PCM21.11', and on positions 4 to 6, and 11 from line 'PCA12.112' (Table 3).

Agnola et al. (2003) observed greater resistance to downy mildew in young leaves than in older leaves of Brassica hosts. Increased susceptibility to pathogens with leaf age has also been reported for other host-pathogen systems such as *Alternaria helianthi* on sunflower (Allen et al. 1983) or *P. tabacina* on tobacco leaves (Cohen et al. 1987). Leaf susceptibility does not increase continuously until leaf senescence because a state is reached when the leaf is no longer able to support pathogen growth. We have observed in susceptible broccoli that old, chlorotic leaves were difficult to infect with *H. parasitica* and did not sporulate (unpublished data). Therefore, for the best compatible reaction to be expressed, leaves should be inoculated when they are fully mature, but with enough time for the pathogen to develop before the leaves senesce naturally.

Table 3 Disease scores of leaves in various node positions in four *B. oleracea* hosts inoculated with *H. parasitica* at different plant and leaf ages (Experiment 3)

Leaf position[x]	Plant age at inoculation (weeks after sowing)												Leaf age
	7-week		9-week		11-week		13-week		16-week		19-week		
	Broccoli 'PCB17.11'												Significance
18	…		…		…		…		…		2.7a		–
17	…		…		…		…		…		1.9a		–
16	…		…		…		…		…		2.5a		–
15	…		…		…		…		…		2.2a		–
14	…		…		…		…		…		2.9a		–
13	…		…		…		…		1.6d	B	3.2a	A	***
12	…		…		…		…		2.5cd		3.0a		NS
11	…		…		…		…		2.9bcd	B	4.2a	A	*
10	…		…		…		3.4d		3.1bc		4.0a		NS
9	…		…		2.3d	B	3.8cd	A	3.9ab	A	…		***
8	…		…		3.8bc	B	4.1cd	AB	4.6a	A	…		*
7	…		…		3.4cd	B	4.4bc	A	5.7a	A	…		***
6	…		3.6c	B	4.6ab	A	4.9ab	A	6.0a	A	…		***
5	…		4.4b	B	5.2a	AB	5.6a	A	…		…		**
4	2.2	C	4.6b	B	5.5a	A	…		…		…		***
3	2.1	B	5.5a	A	…		…		…		…		***
2	1.8	B	5.7a	A	…		…		…		…		***
Significance	NS		***		***		***		***		**		
	Broccoli 'PCB21.32'												
18	…		…		…		…		…		1.4		–
17	…		…		…		…		…		1.4		–
16	…		…		…		…		1.1		1.4		NS
15	…		…		…		…		1.6		2.2		NS
14	…		…		…		…		1.4		…		–
13	…		…		…		1.8c		1.6		…		NS
12	…		…		…		1.8c		2.0		…		NS
11	…		…		2.6b		2.4bc		…		…		NS
10	…		…		2.4b		2.5bc		…		…		NS
9	…		…		2.5b		2.2bc		…		…		NS
8	…		3.7		3.4ab		3.5ab		…		…		NS
7	…		3.8		3.9a		4.2a		…		…		NS
6	…		4.1		4.7a		4.0a		…		…		NS
5	1.6	B	4.6	A	5.0a	A	…		…		…		***
Significance	–		NS		***		***		NS		NS		
	Couve Murciana 'PCM21.11'												
17	…		…		…		…		…		1.6		–
16	…		…		…		…		…		1.7		–
15	…		…		…		…		…		1.8		–
14	…		…		…		…		1.0		1.8		NS

Table 3 (continued)

Leaf position[x]	Plant age at inoculation (weeks after sowing)												Leaf age
	7-week		9-week		11-week		13-week		16-week		19-week		
13	…		…		…		…		1.2		2.3		NS
12	…		…		…		…		1.2		2.5		NS
11	…		…		…		…		1.3		3.0		NS
10	…		…		…		1.9d		1.6		…		NS
9	…		…		2.6c		3.1c		1.7		…		NS
8	…		…		3.3bc		3.7bc		…		…		NS
7	…		…		3.2bc	B	4.4ab	A	…		…		***
6	…		3.7c	B	4.2ab	B	5.2a	A	…		…		***
5	2.1b	B	4.0bc	A	4.1ab	A	5.5a	A	…		…		***
4	2.8ab	B	4.8ab	A	4.8a	A	…		…		…		***
3	3.5a	B	5.8a	A	5.5a	AB	…		…		…		***
Significance	*		***		***		***		NS		NS		
	Couve Algarvia ‘PCA12.112’												
15	…		…		…		…		…		1.0b		–
14	…		…		…		…		…		1.0b		–
13	…		…		…		…		1.0		1.0b		NS
12	…		…		…		…		1.0		1.0b		NS
11	…		…		…		…		1.0	B	2.0a	A	**
10	…		…		…		1.0c		1.0		…		NS
9	…		…		1.2b		1.0c		1.0		…		NS
8	…		…		1.6b		1.7c		…		…		NS
7	…		1.9b		2.2b		3.0b		…		…		NS
6	…		2.4b	B	3.7a	A	4.5a	A	…		…		***
5	…		3.4a	B	4.9a	A	…		…		…		**
4	1.6	B	3.2ab	A	4.8a	A	…		…		…		***
Significance	–		***		***		***		NS		***		

Each value is the mean of 16 replicates

(…) Leaves not tested because they had dropped or were too small. Leaves above the fourth position from the apex were not tested

Small letters refer to mean separation for leaf position (columns) and capital letters refer to mean separation for leaf age (lines). Mean separation was by Tukey’s HSD test. Values in columns and in lines followed by different letters are statistically significant at $P \leq 0.05$. NS, *, **, *** Non-significant or significant at $P \leq 0.05$, 0.01 or 0.001, respectively

[x] Leaf positions were counted from plant node 1 upwards

Combined effects

Brassica genotypes tested in Experiment 3 expressed different patterns of adult plant resistance to downy mildew that corroborate previous field and greenhouse tests (Coelho and Monteiro 2003a, b; Monteiro et al. 2005). Line ‘PCB17.11’ was susceptible, lines ‘PCB21.32’ and ‘PCM21.11’ were resistant, and line ‘PCA12.112’ was extremely resistant, which may have influenced the effects of leaf position and age on the disease score as explained below.

In Experiment 3 the influences of leaf age and upper-leaf position had apparent reverse effects on disease scores. Older leaves collected from upper positions tended to have similar scores to younger leaves collected from lower positions. For instance, in line ‘PCB17.11’ leaf position 2 on 9 week-old, position 4 on 11 week-old, position 5 on 13 week-

old and position 7 on 16 week-old plants showed similar disease scores (Table 3). The pattern of resistance variation with leaf age and position described for the susceptible line 'PCB17.11' was not evident in the resistant lines 'PCB21.32' and 'PCM21.11' or in the extremely resistant line 'PCA12.112', because after a certain plant developmental stage all leaves were resistant independently of their age or position on the stem. For instance, disease scores on 16 week-old plants varied between leaves from 1.6 to 6.0 on line 'PCB17.11', from 1.1 to 2.0 on line 'PCB21.32' and from 1.0 to 1.7 on line 'PCM21.11'. The disease score was a uniform 1.0 for all leaves tested on line 'PCA12.112'.

Resistant hosts in Experiment 3 showed increased susceptibility on old, lower leaves, e.g. position 5 on line 'PCB21.32', positions 3 to 6 on line 'PCM21.11' and positions 4 and 5 on line 'PCA12.112'. This is not supported by Agnola et al. (2003), who verified that in resistant hosts the discs are resistant whatever the leaf position. Other species react differently. Considering the resistance to potato blight (*P. infestans*), the effect of leaf position is more important than the effect of plant age and leaf age, and the degree of resistance of a specific leaf remains constant during its entire lifetime (Visker et al. 2003). ARR is a common response in several species. Resistance of tobacco plants to blue mould (*P. tabacina*) increases with plant age, but not with leaf age or leaf position, which suggests the presence of a plant-age related disease inhibitor (Reuveni et al. 1986).

The progress of downy mildew in the plants under field conditions is from the bottom-up, with basal leaves being the first to exhibit disease symptoms and showing the largest lesions and greatest sporulation. It is therefore appropriate in field scoring to use an index that takes into account the number of infected leaves and the size of the lesions (Coelho and Monteiro 2003b). Such an index is an efficient tool to estimate disease progression in the plant and to identify genotypes that hinder disease development.

It could be argued from field data that the more severe disease symptoms on basal leaves are induced by the presence of inoculum and better infection conditions because these leaves are closer to the ground, are less well ventilated and retain moisture for longer periods. However the evaluation by the leaf-disc method, where all leaves were tested under the same experimental conditions, clarified that the differences in disease score between leaves occupying various positions on the plant can be explained by the variation in leaf susceptibility in addition to environmental differences.

Testing disease resistance in the field is expensive, labour-intensive and only produces reliable results if the environmental conditions are favourable for the development of downy mildew. Laboratory testing using the leaf-disc method is more efficient because it can be repeated, done at any time and the environment does not influence the results. Since the test uses a limited number of leaves collected only once, the disease score may vary with plant age and with the kind of leaves sampled for testing. To maximise the expression of a compatible reaction in adult plants lower leaves should be used of plants that are at least 12 weeks-old. However lower leaves may not provide a fair discrimination between resistant and susceptible adult plants because these leaves may show compatible reactions in putative resistant plants. The best discrimination is achieved by testing leaves collected from various positions along the plant.

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References

Agnola, B., Boury, S., Monot, C., Quillévéré, A., Hervé, Y., & Silué, D. (2003). Evidence that a leaf-disk test allows assessment of isolate-specific resistance in *Brassica oleracea* crops against downy mildew (*Peronospora parasitica*). *European Journal of Plant Pathology, 109*, 471–478. doi:10.1023/A:1024217223829.

Allen, S. J., Brown, J. F., & Kochman, J. K. (1983). The effects of leaf age, host growth stage, leaf injury, and pollen on the infection of sunflower *Alternaria helianthi*. *Phytopathology, 73*, 896–898.

Brown, M. V., Moore, J. N., Fenn, P., & McNew, R. W. (1999). Comparison of leaf disk, greenhouse, and field screening procedures for evaluation of grape seedlings for downy mildew resistance. *HortScience, 34*(2), 331–333.

Coelho, P. S., & Monteiro, A. A. (2003a). Expression of resistance to downy mildew at cotyledon and adult plant stages in *Brassica oleracea* L. *Euphytica, 133*, 279–284. doi:10.1023/A:1025787608919.

Coelho, P. S., & Monteiro, A. A. (2003b). Inheritance of downy mildew resistance in mature broccoli plants. *Euphytica, 131*, 65–69. doi:10.1023/A:1023008619400.

Coelho, P., Leckie, D., Bahcevandziev, K., Valério, L., Astley, D., Boukema, I., et al. (1998). The relationship between cotyledon and adult plant resistance to downy mildew (*Peronospora parasitica*) in *Brassica oleracea*. *Acta Horticulturae, 459*, 335–342.

Cohen, Y., Pe'er, S., Balass, O., & Coffey, M. D. (1987). A fluorescent technique for studying growth of *Peronospora tabacina* on leaf surfaces. *Phytopathology, 77*(2), 201–204. doi:10.1094/Phyto-77-201.

Cohen, Y., Petrov, L., & Baider, A. (2000). A leaf-disc bioassay for screening cucumbers for resistance to downy mildew. *Acta Horticulturae, 510*, 277–282.

Dickinson, C. H., & Crute, I. R. (1974). The influence of seedling age and development on the infection of lettuce by *Bremia lactucae*. *The Annals of Applied Biology, 76*, 49–61. doi:10.1111/j.1744-7348.1974.tb01356.x.

Dickson, M. H., & Petzoldt, R. (1993). Plant age and isolate source affect expression of downy mildew resistance in broccoli. *HortScience, 28*(7), 730–731.

Hugot, K., Aimé, S., Conrod, S., Poupet, A., & Galiana, E. (1999). Developmental regulated mechanisms affect the ability of a fungal pathogen to infect and colonize tobacco leaves. *The Plant Journal, 20*(2), 163–170. doi:10.1046/j.1365-313x.1999.00587.x.

Hugot, K., Rivière, M.-P., Moreilhon, C., Dayem, M. A., Cozzitorto, J., Arbiol, G., et al. (2004). Coordinated regulation of genes for secretion in tobacco at late developmental stages: association with resistance against oomycetes. *Plant Physiology, 134*, 858–870. doi:10.1104/pp.103.034173.

Jensen, B. D., Hockenhull, J., & Munk, L. (1999). Seedling and adult plant resistance to downy mildew (*Peronospora parasitica*) in cauliflower (*Brassica oleracea* convar. *botrytis* var. *botrytis*). *Plant Pathology, 48*, 604–612. doi:10.1046/j.1365-3059.1999.00388.x.

Kim, Y. J., Hwang, B. K., & Park, K. W. (1989). Expression of age-related resistance in pepper plants infected with *Phytophthora capsici*. *Plant Disease, 73*, 745–747. doi:10.1094/PD-73-0745.

Kus, J. V., Zaton, K., Sarkar, R., & Cameron, R. K. (2002). Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. *The Plant Cell, 14*, 479–490. doi:10.1105/tpc.010481.

Leckie, D., Astley, D., Crute, I. R., Ellis, P. R., Pink, D. A. C., Boukema, I., et al. (1996). The location and exploitation of genes for pest and disease resistance in European gene bank collections of horticultural brassicas. *Acta Horticulturae, 407*, 95–101.

Monteiro, A. A., Coelho, P. S., Bahcevandziev, K., & Valério, L. (2005). Inheritance of downy mildew resistance at cotyledon and adult-plant stages in 'Couve Algarvia' (*Brassica oleracea* var. *tronchuda*). *Euphytica, 141*(1–2), 85–92. doi:10.1007/s10681-005-5696-8.

Natti, J. J., Hervey, G. E. R., & Sayre, C. B. (1956). Factors contributing to the increase of downy mildew of broccoli in New York State and its control with fungicides and Agrimycin. *Plant Disease Reporter, 40*(2), 118–124.

Niu, X., Leung, H., & Williams, P. H. (1983). Sources and nature of resistance to downy mildew and turnip mosaic in Chinese cabbage. *Journal of the American Society for Horticultural Science, 108*(5), 775–778.

Olmstead, J. W., Lang, G. A., & Grove, G. G. (2000). A leaf disk assay for screening sweet cherry genotypes for susceptibility to powdery mildew. *HortScience, 35*(2), 274–277.

Reuveni, M., Tuzun, S., Cole, J. S., Siegel, M. R., & Kúc, J. (1986). The effects of plant age and leaf position on the susceptibility of tobacco to blue mold caused by *Peronospora tabacina*. *Phytopathology, 76*(4), 455–458.

Visker, M. H. P. W., Keizer, L. C. P., Budding, D. J., Van Loon, L. C., Colon, L. T., & Struik, P. C. (2003). Leaf position prevails over plant age and leaf age in reflecting resistance to late blight in potato. *Phytopathology, 93*(6), 666–674. doi:10.1094/PHYTO.2003.93.6.666.

Warren, R. C., King, J. E., & Colhoun, J. (1971). Reaction of potato leaves to infection by *Phytophthora infestans* in relation to position on the plant. *Transactions of the British Mycological Society, 57*, 501–514.

Wyatt, S. E., Pan, S. Q., & Kúc, J. (1991). β-1, 3-glucanase, chitinase, and peroxidase activities in tobacco tissues resistant and susceptible to blue mould as related to flowering, age and sucker development. *Physiological and Molecular Plant Pathology, 39*, 433–440. doi:10.1016/0885-5765(91)90009-7.