Comparing Cotyledon, Leaf and Root Resistance To Downy Mildew in Radish (*Raphanus Sativus* L.)

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Abstract

Radish downy mildew (DM) is a disease caused by the oomycete *Hyaloperonospora brassicae* f. sp. *raphani* and it is a serious problem in radish production, an edible root vegetable crop of the Brassicaceae family. The objective of this research was to assess radish germplasm for DM resistance and to evaluate the response of different radish organs to the disease. Cotyledons, true-leaves and roots of 44 radish accessions were inoculated with *H. brassicae* isolates under controlled conditions. The cotyledons were individually evaluated 7dpi (days post-inoculation), and the leaves and roots 12dpi. DM symptoms varied with the radish genotype and plant organ analysed. Thirty-five resistant and partially resistant accessions were identified and are promising sources to DM. A significant correlation was observed between cotyledon and leaf (1st and 2nd leaves) DM resistance, but no correlation was found between the resistance of cotyledons or true-leaves and roots. Cotyledon and leaf response cannot be used to predict radish root resistance. However, cotyledon resistance has its own value because non-infected cotyledons will act as a barrier to slow disease progression to true-leaves and roots.

Introduction

Radish (*Raphanus sativus* L., n = 9) is a root vegetable of the Brassicaceae family, which includes the small or Western radish (*Raphanus sativus* var. *sativus*) and the daikon radish (*Raphanus sativus* var. *longipinnatus*), also known as Chinese oriental or Japanese radish traditionally used in East Asian cuisine. Daikon radish has similar growth requirements as Western radish, but the root has much larger and requires more space to growth. Depending on the cultivar, Longipinnatus radish group needs 50–80 days to harvest, requiring an early spring to mid-summer seeding date, because it is adversely affected by hot, dry weather and long days (APA 1988). On the other hand, Western radish varieties of *Raphanus sativus* var. *sativus* produce a much small root and reach harvest stage in 3–4 weeks.

Radish downy mildew (DM) is an economically important disease in main production areas worldwide (Glits 1977; Göker et al. 2009; Lee et al. 2017; Robles-Yerena et al. 2017; Wang et al. 2017; Coelho and Monteiro 2018). Commercial varieties of radish are usually very susceptible to DM and chemical control does not provide an effective crop protection due to the short cultural cycle that would require fungicide spraying too close to harvest.

The genus *Hyaloperonospora* (phylum Oomycota; family Peronosporaceae) is a group of biotrophic oomycetes responsible for DM disease in relevant crops from Brassicaceae family. DM in radish is caused by *Hyaloperonospora brassicae* f. sp. *raphani*, an airborne obligate pathogen strongly affected by temperature and air moisture. Favourable conditions for radish infection and disease dissemination are day and night moderate/cool temperatures of 20°C and 10–15°C, respectively, associated with high humidity (RH ≥ 80%) (Kofoet and Fink 2007). The first symptoms are yellow or brownish spots on the upper surfaces of radish cotyledons and mature leaves combined with a white sporulation on the corresponding abaxial epidermis. These spots eventually turn necrotic and the leaf dies. DM also infects radish roots that reveal a blackening area with *H. brassicae* sporulation, scarring and cracking, making them non-salable. Effective leaf disease control may prevent root damage because roots are infected by conidia that come from the leaves (Glits 1977).

The use of cotyledon evaluation to predict disease resistance in more advanced stages of plant development has the great advantage of being a faster and cheaper method requiring much less space, but for cotyledon evaluation to be effective, there must be a good correlation between the response of the cotyledons and the different organs with commercial value, which may not occur. However, cotyledon resistance in radish has commercial interest

because young plants are harvested with the cotyledons, that must be exempt of disease, and may be important to decrease the progression of the disease to adult leaves and roots.

Integrated Pest Management (IPM) strategies combine different measures focused on a long term prevention of pest damages, including the adjustment of cultural practices, such as weed control (increase air circulation), keeping leaves dry avoiding overhead irrigation especially late in the day, removing plant debris after harvest, and also rotation with non-brassicac crops. *H. brassicae* pathogen persists as oospores in soil on infected plant debris, so it is very important that cover crop plants are not susceptible and pathogen spores do not accumulate in the soil (Runno-Paurson et al. 2019).

In breeding programmes, together with agronomic and qualitative characteristics of the product it is important to include resistance to the main pests and diseases, thus providing healthier products with environmental and consumer benefits. The exploitation of new sources of DM disease resistance represents an important strategy in order to improve cultivated radish production. Robust phenotyping data are fundamental for accurate germplasm selection and future use, although few radish genotypes resistant to DM were identified so far (Jiang et al. 2012; Wang et al. 2014; Xu et al. 2014; Coelho and Monteiro 2018).

Due to the very short production cycle of radish a source of resistance to be effective must cover all the organs of the plant. The objectives of the present study were to develop a screening methodology for assessing DM resistance in different radish plant organs, to use this methodology to identify sources of resistance by screening a germplasm collection, and to compare the expression of resistance in cotyledons, true-leaves and roots.

Material And Methods

Plant material and plantlet production

A group of radish accessions with known cotyledon resistance to *H. brassicae* f. sp. *raphani* was selected for testing seedling and root resistance (Table 1). The accessions had different origins, genetic backgrounds (breeding lines, commercial varieties and genebanks), and growing cycles. The radish accession Rd197 was used to obtain fresh *H. brassicae* inoculum for all experiments and was included in the tests as a susceptible control.

Table 1

Forty-five radish accessions from breeding lines, commercial varieties, and genebanks tested for downy mildew resistance.

Code	Accession name	Accession state	Seed donor (accession origin)	Radish crop type
Rd001	PI 508495	Breeding line	Vilmorin	White daikon
Rd002	PI 483356	Breeding line	Vilmorin	Daikon
Rd003	PI 483357	Breeding line	Vilmorin	Daikon
Rd004	G30854	Breeding line	Vilmorin	Red daikon with white tip
Rd005	INRA 148	Breeding line	Vilmorin	Short cycle
Rd011	Java	Commercial variety	Vilmorin	Short cycle
Rd012	Bamba	Commercial variety	Vilmorin	Medium red with white tip
Rd013	Lavergne	Commercial variety	Vilmorin	Ovate shape
Rd014	Pontvil	Commercial variety	Vilmorin	Short cycle
Rd015	05507 BS	Breeding line	Vilmorin	Short cycle
Rd017	Københavns Torve 9, Toftø	Breeding line	NordGen (DK)	Round red with white tip
Rd020	Rovi	Breeding line	NordGen (DK)	Round red with white tip
Rd108	6412-Grazer Treib AS	Advanced cultivar	UKVGB (AUT)	Long red
Rd111	6595	Landrace	UKVGB (EGY)	Long red
Rd130	7220-Sassarese	Landrace	UKVGB (ITA)	–
Rd175	No. 3	Breeding line	Syngenta Seeds	Long red with white tip
Rd176	No. 4	Breeding line	Syngenta Seeds	–
Rd177	No. 5	Breeding line	Syngenta Seeds	Short cycle
Rd180	No. 8	Breeding line	Syngenta Seeds	Round red
Rd181	No. 9	Breeding line	Syngenta Seeds	Long red
Rd182	No. 10	Breeding line	Syngenta Seeds	Round red
Rd183	No. 11	Breeding line	Syngenta Seeds	Long red with white tip
Rd184	No. 12	Breeding line	Syngenta Seeds	Round red
Rd186	Rond Ecarlate Hatif	Commercial variety	Gautier Seeds	Round red
Rd187	Cerise	Commercial variety	Gautier Seeds	Round red
Rd188	Sezanne	Commercial variety	Gautier Seeds	Round red with white tip
Rd189	Gaudry 2	Commercial variety	Gautier Seeds	Small diameter
Rd190	National	Commercial variety	Gautier Seeds	Round red with white tip

Code	Accession name	Accession state	Seed donor (accession origin)	Radish crop type
Rd191	Alaric (Flamboyant 3)	Commercial variety	Gautier Seeds	Medium cycle
Rd192	Flambard (Flamboyant 5)	Commercial variety	Gautier Seeds	Medium cycle
Rd193	Flambo	Commercial variety	Gautier Seeds	Very long red with white tip
Rd194	Nelson	Commercial variety	Gautier Seeds	Half-long red with white tip
Rd195	Gandar	Commercial variety	Gautier Seeds	Long red with white tip
Rd196	Capitole	Commercial variety	Gautier Seeds	Long red with white tip
Rd197	SRR	Breeding line	Vilmorin	Round red
Rd198	SFB	Breeding line	Vilmorin	Short cycle
Rd201	Treto	Commercial variety	Syngenta Seeds	Long red with white tip
Rd202	April Cross	Commercial variety	Gautier Seeds	White daikon
Rd203	Mino Summer Cross	Commercial variety	Gautier Seeds	White daikon
Rd204	Omny	Commercial variety	Gautier Seeds	White daikon
Rd205	Diablus	Commercial variety	Gautier Seeds	Half-long red with white tip
Rd206	Expo	Commercial variety	Vilmorin	Long red with white tip
Rd207	Fluo	Commercial variety	Vilmorin	Long red with white tip
Rd208	Tinto	Commercial variety	Vilmorin	Round red
ER	Encarnado Redondo	Commercial variety	A.A. Dias, Lda (PT)	Round red

Radish seeds were sown in plastic trays containing a peat-based compost (Gramoflor GmbH & Co. KG, Vechta, Germany), covered with a layer of vermiculite and watered by capillary matting. The trays were placed in controlled environment with a 19-h photoperiod, 21°C daytime and 19°C night-time temperatures, and 70 ± 10% relative humidity. The photoperiod was provided by cool-white fluorescent lamps and 250 µmol m⁻² s⁻¹ light intensity. For cotyledon screening the plants were grown during one week in trays of 3 x 3 x 5-cm cells, and for leaf and root evaluation the plants were grown during two weeks in larger trays of 4 x 4 x 5.5-cm. In root tests the tray cells were seeded in alternate rows, to ensure a good inoculation of the roots.

Origin of pathogen isolates and inoculum preparation

The resistance of cotyledons and true-leaves was tested with the *H. brassicae* isolate R10, and the roots were tested with isolates R10 and R6 in two independent experiments. The *H. brassicae* isolates were collected in field plants of *Raphanus sativus* var. *sativus* in different geographic origins. The isolate R10 was provided by Syngenta Seeds and was collected in cotyledons in the Netherlands (Venhuizen). The *H. brassicae* isolate R6 was provided by Gautier Seeds and was collected from roots in France (Bouches du Rhone). Field isolates were isolated, cleaned, and non-contaminated *H. brassicae* isolates were stored at -18°C in infected cotyledons of the susceptible accession Rd197.

Spore suspensions of the pathogen were prepared to produce inoculum to be used in the different experiments. Infected cotyledons of the susceptible control recently sporulated with *H. brassicae* were washed with distilled water, mycelial fragments were removed and the conidia were counted to a $50\text{--}75 \times 10^3$ conidia ml^{-1} final spore concentration using a haemocytometer.

DM screening methodology

Cotyledon inoculation

The cotyledons of six-day-old radish plants were inoculated by drop with a fresh conidial suspension of *H. brassicae* isolate R10, following the methodology described by Coelho and Monteiro (2018). Briefly, the fully expanded cotyledons were inoculated on the adaxial surface by depositing two 10- μl droplets of the inoculum on each lobe of the cotyledon using a micropipette (Fig. 1a). After inoculation, the plants were incubated at $16 \pm 1^\circ\text{C}$ in the dark, for 24-h, inside a propagator (RH = 100%) to support infection. Afterwards the plants were placed in a growth chamber during 5 days under the previously described conditions for seedlings production. Six days post-inoculation (dpi), the cotyledons were lightly sprayed with distilled water and re-incubated at $16 \pm 1^\circ\text{C}$ in the dark, for 24-h, to induce pathogen sporulation. A total of 24 plants per accession were evaluated at cotyledon stage in three independent replications.

Leaf inoculation

The first two leaves of 14-day-old radish plants were inoculated by pulverization using a handheld sprayer with a fresh conidial suspension of *H. brassicae* isolate R10 (Fig. 1d). The inoculated plants were submitted to the procedures previously described for cotyledon test, but a longer period for infection was necessary. Following an initial 24-h incubation period, plants were placed in a growth chamber during 10 days and individually scored for *H. brassicae* infection, after a 24-h incubation period. Two leaves per plant in a total of 10 plants per accession were tested in two independent replications.

Root inoculation

The roots of 14-day-old radish plants were inoculated by pulverization in separate trays with *H. brassicae* isolates R10 and R6, following the procedures described for leaf inoculation. The radish seeds were seeded superficially in alternate rows in order to facilitate the pulverization of the roots, and the radish root resistance was individually assessed at 12dpi. The isolates were tested in different experiments and a total of 24 plant roots per accession and isolate were evaluated in three independent replications.

Disease assessment and data analysis

The symptoms on cotyledons and on the first two true-leaves of each plant were evaluated using a visual scale of seven interaction-phenotype classes (IP classes), taking into account the host response and the relative amount of pathogen asexual sporulation (Table 2). Plants classified in class 0 indicated no symptoms (immune class); classes 1–2 were resistant responses, showed only necrosis restricted to the point of infection with no sporulation (Figs. 1b and 1e); classes 3–4 were intermediate responses characterized by pathogen sporulation confined to the point of infection; and classes 5–6 were susceptible reactions with sparse to abundant sporulation respectively, dispersed over the whole cotyledon or leaf surface (Figs. 1c and 1f).

Table 2

Interaction-phenotype (IP) classes used to evaluate downy mildew resistance of radish cotyledons and leaves.

IP classes	Host and pathogen response
0	No host reaction, no sporulation
1	Heavy necrotic flecking, no sporulation
2	Diffuse necrotic flecking, no sporulation
3	Necrotic flecking, rare sporulation confined to the point of infection (until 5 conidiophores)
4	Necrotic flecking, moderate to heavy sporulation confined to the point of infection
5	Any host response, sparse sporulation dispersed over whole cotyledon / leaf
6	Any host response, heavy sporulation dispersed over whole cotyledon / leaf
Accessions were separated in four phenotypic categories according to Disease Index (DI) values: R = Resistant ($DI \leq 2.5$), PR = Partially Resistant ($2.5 < DI \leq 4.0$), S = Susceptible ($4.0 < DI \leq 5.0$), and HS = Highly Susceptible ($5.0 < DI \leq 6.0$).	

Radish roots were evaluated using a visual scale of five IP classes (Table 3). Roots classified in class 0 indicate no symptoms (immune class) (Fig. 1g); class 1 was a resistant response, showed only necrosis restricted to the point of infection and no sporulation; class 2 was an intermediate response characterized by a rare *H. brassicae* sporulation confined to the infection point; and classes 3–4 were susceptible reactions with sparse to abundant sporulation respectively, dispersed over the whole radish surface (Figs. 1h and 1i).

Table 3

Interaction-phenotype (IP) classes used to evaluate downy mildew resistance of radish roots.

IP classes	Host and pathogen response
0	No host reaction, no sporulation
1	Necrosis localized on pulverization area, no sporulation
2	Necrosis localized on pulverization area, rare sporulation in root surface (until 5 conidiophores)
3	Necrosis localized on pulverization area, sparse to moderate sporulation dispersed over whole root surface
4	Necrosis localized on pulverization area, heavy sporulation dispersed over whole root surface
Accessions were separated into four phenotypic categories according to Disease Index (DI) values: R = Resistant ($DI \leq 1.0$), PR = Partially Resistant ($1.0 < DI \leq 2.0$), S = Susceptible ($2.0 < DI \leq 3.0$), and HS = Highly Susceptible ($3.0 < DI \leq 4.0$).	

A mean disease severity index (DI) was calculated for each accession and organ. At cotyledon and true-leaf stages, the accessions were separated into four phenotypic categories according to DI value: R = Resistant ($DI \leq 2.5$), PR = Partially Resistant ($2.5 < DI \leq 4.0$), S = Susceptible ($4.0 < DI \leq 5.0$), and HS = Highly Susceptible ($5.0 < DI \leq 6.0$). The phenotypic categories at root stage were: Resistant ($DI \leq 1.0$), Partially Resistant ($1.0 < DI \leq 2.0$), Susceptible ($2.0 < DI \leq 3.0$), and Highly Susceptible ($3.0 < DI \leq 4.0$).

Analysis of variance was performed on the two *H. brassicae* isolates data at root stage and the significant differences between means were identified by Tukey HSD test ($P \leq 0.05$) using *Statistica* version 7. The correlations

between cotyledon, true-leaf and root DI values were assessed via Pearson's coefficients and the relative P -values significance ($P < 0.05$) were determined.

Results

Cotyledon and true-leaf pathogenicity tests

The disease index of 44 radish accessions screened at cotyledons and true-leaves for DM resistance separated the accessions into four phenotypic categories (Table 4). At cotyledon stage 37 accessions (84%) were resistant, 5 accessions (11%) partially resistant, and 2 accessions (5%) highly susceptible. In resistant accessions 43 to 100% of the plants were in classes 1–2, 0 to 52% in classes 3–4, and 0 to 21% in classes 5–6. Partially resistant accessions presented between 27 and 42% of plants in classes 1–2, 36 to 73% in classes 3–4, and 0 to 36% in classes 5–6. The two accessions Rd197 and Rd208 classified as highly susceptible did not register any plants in classes 1–3, 6 and 8% in classes 4, and 92 and 94% in classes 5–6 respectively.

Table 4

Number of plants per interaction-phenotype classes (IP classes), total number of plants evaluated and disease index (DI) values of forty-four radish accessions tested for downy mildew resistance at cotyledon and true-leaf stages with *H. brassicae* isolate R10.

Code	Cotyledon						Total	DI		Leaves						Total	DI	
	IP classes									IP classes								
	1	2	3	4	5	6				1	2	3	4	5	6			
Rd001	14	3	2	1	0	0	20	1.5	R	13	5	0	0	0	0	18	1.3	R
Rd002	18	1	1	3	0	0	23	1.5	R	15	5	0	0	0	0	20	1.3	R
Rd003	15	1	4	3	0	1	24	2.0	R	16	4	0	0	0	0	20	1.2	R
Rd004	14	1	3	1	3	2	24	2.3	R	17	1	0	0	0	0	18	1.1	R
Rd005	16	2	6	0	0	0	24	1.6	R	4	14	2	0	0	0	20	1.9	R
Rd011	10	5	6	1	0	0	22	1.9	R	5	8	1	3	2	1	20	2.6	PR
Rd012	10	0	6	6	1	1	24	2.6	PR	2	6	4	5	2	1	20	3.1	PR
Rd013	14	1	6	1	0	0	22	1.7	R	2	13	5	0	0	0	20	2.2	R
Rd014	13	2	4	4	0	1	24	2.1	R	0	10	4	2	3	1	20	3.1	PR
Rd015	13	0	7	3	0	0	23	2.0	R	4	15	1	0	0	0	20	1.9	R
Rd017	12	2	6	4	0	0	24	2.1	R	14	6	0	0	0	0	20	1.3	R
Rd020	10	0	9	3	0	1	23	2.4	R	4	14	0	0	0	0	18	1.8	R
Rd108	11	0	6	6	0	1	24	2.5	R	10	0	0	3	1	5	19	3.0	PR
Rd111	11	1	2	7	1	0	22	2.4	R	13	6	1	0	0	0	20	1.4	R
Rd175	17	0	3	3	0	0	23	1.7	R	8	11	1	0	0	0	20	1.7	R
Rd176	12	3	7	1	0	0	23	1.9	R	10	8	0	0	0	2	20	1.9	R
Rd177	15	1	7	1	0	0	24	1.8	R	12	5	1	0	2	0	20	1.8	R
Rd180	12	0	6	6	0	0	24	2.3	R	16	3	0	0	1	0	20	1.4	R
Rd181	11	1	4	7	0	1	24	2.5	R	10	7	2	0	0	0	19	1.6	R
Rd182	15	0	7	0	0	0	22	1.6	R	19	0	1	0	0	0	20	1.1	R
Rd183	15	2	5	2	0	0	24	1.8	R	8	7	0	0	1	4	20	2.6	PR
Rd184	16	0	4	1	0	1	22	1.7	R	17	2	1	0	0	0	20	1.2	R
Rd186	13	3	4	4	0	0	24	2.0	R	6	14	0	0	0	0	20	1.7	R

At cotyledon stage a total of 24 observations were made (8 observations x 3 replicates) per accession. At true-leaf stage a total of 20 observations were made (10 observations x 2 replicates) per accession.

The accessions were separated into four phenotypic categories at cotyledon and true-leaf stages: R = Resistant ($DI \leq 2.5$), PR = Partially Resistant ($2.5 < DI \leq 4.0$), S = Susceptible ($4.0 < DI \leq 5.0$), and HS = Highly Susceptible ($5.0 < DI \leq 6.0$).

Code	Cotyledon						Total	DI		Leaves						Total	DI		
Rd187	15	2	2	2	0	1	22	1.8	R	20	0	0	0	0	0	20	1.0	R	
Rd188	13	6	2	1	0	0	22	1.6	R	10	9	1	0	0	0	20	1.6	R	
Rd189	16	3	4	1	0	0	24	1.6	R	2	15	1	2	0	0	20	2.2	R	
Rd190	14	3	2	5	0	0	24	1.9	R	4	14	1	1	0	0	20	2.0	R	
Rd191	11	6	0	3	0	0	20	1.8	R	5	14	1	0	0	0	20	1.8	R	
Rd192	13	2	5	3	1	0	24	2.0	R	2	18	0	0	0	0	20	1.9	R	
Rd193	18	2	1	0	0	0	21	1.2	R	2	18	0	0	0	0	20	1.9	R	
Rd194	10	6	4	3	0	0	23	2.0	R	4	14	2	0	0	0	20	1.9	R	
Rd195	13	5	0	5	1	0	24	2.0	R	8	10	2	0	0	0	20	1.7	R	
Rd196	18	4	0	0	0	0	22	1.2	R	19	0	0	0	0	0	19	1.0	R	
Rd197	0	0	0	2	3	19	24	5.7	HS	0	0	0	0	0	10	10	6.0	HS	
Rd198	13	3	3	3	1	0	23	2.0	R	17	3	0	0	0	0	20	1.2	R	
Rd201	4	0	5	5	1	0	15	2.9	PR	1	3	7	4	3	2	20	3.6	PR	
Rd202	24	0	0	0	0	0	24	1.0	R	11	8	1	0	0	0	20	1.5	R	
Rd203	22	0	2	0	0	0	24	1.2	R	0	18	2	0	0	0	20	2.1	R	
Rd204	14	1	5	3	0	0	23	1.9	R	8	9	2	0	0	0	19	1.7	R	
Rd205	4	0	3	8	0	0	15	3.0	PR	6	3	3	4	1	1	18	2.7	PR	
Rd206	9	0	2	4	0	0	15	2.1	R	5	6	6	2	1	0	20	2.4	R	
Rd207	5	0	2	8	0	0	15	2.9	PR	1	2	5	9	3	0	20	3.6	PR	
Rd208	0	0	0	1	4	11	16	5.6	HS	0	0	1	3	0	16	20	5.6	HS	
ER	0	3	1	3	2	2	11	3.9	PR	2	15	3	0	0	0	20	2.1	R	
Total							964										858		
At cotyledon stage a total of 24 observations were made (8 observations x 3 replicates) per accession. At true-leaf stage a total of 20 observations were made (10 observations x 2 replicates) per accession.																			
The accessions were separated into four phenotypic categories at cotyledon and true-leaf stages: R = Resistant (DI ≤ 2.5), PR = Partially Resistant (2.5 < DI ≤ 4.0), S = Susceptible (4.0 < DI ≤ 5.0), and HS = Highly Susceptible (5.0 < DI ≤ 6.0).																			

A similar response was observed on the plants inoculated on the 1st and 2nd leaves, once 34 accessions (77%) were classified as resistant, 8 accessions (18%) as partially resistant, and 2 accessions (5%) as highly susceptible. In resistant accessions 55 to 100% of the plants were in classes 1–2, 0 to 40% in classes 3–4, and between 0 and 10% in classes 5–6. Partially resistant accessions showed 15 to 75% of the plants in classes 1–2, 0 to 70% in classes 3–4, and between 11 and 32% in classes 5–6. The two highly susceptible accessions Rd197 and Rd208 did not register plants in classes 1–2, 0 and 20 % in classes 3–4, and 100 and 80% in class 6 respectively (Table 4).

Root evaluation with two **H. brassicae** isolates

Unlike the cotyledons and true-leaves that were tested with isolate R10 only, roots were tested with isolates R10 and R6, in two independent experiments. There was a highly significant correlation ($r = 0.81$, $P < 0.000$) between DI induced by the two isolates (Fig. 2) and most of the accessions showed the same general pattern of resistance when inoculated with each isolate (Table 5).

Table 5

Number of plants per interaction-phenotype classes (IP classes), total number of plants evaluated and disease index (DI) value of radish accessions tested for downy mildew resistance on the roots with *H. brassicae* isolates R10 and R6.

Code	Isolate R10				Total	DI		Isolate R6				Total	DI	
	IP classes							IP classes						
	1	2	3	4				1	2	3	4			
Rd001	22	0	0	0	22	1.0	R	19	0	0	0	19	1.0	R
Rd002	23	0	0	0	23	1.0	R	23	1	0	0	24	1.0	R
Rd003	21	1	0	0	22	1.0	R	23	0	0	0	23	1.0	R
Rd004	21	0	0	0	21	1.0	R	19	0	0	0	19	1.0	R
Rd005	10	4	6	1	21	1.9	PR	4	5	11	3	23	2.6	S
Rd011	17	2	2	2	23	1.5	PR	10	2	7	2	21	2.0	PR
Rd012	4	2	2	1	9	2.0	PR	-	-	-	-	0	nt	
Rd013	13	3	4	2	22	1.8	PR	16	4	2	1	23	1.5	PR
Rd014	15	4	1	0	20	1.3	PR	11	1	7	4	23	2.2	S
Rd015	17	2	1	1	21	1.3	PR	9	6	1	1	17	1.6	PR
Rd017	18	3	2	1	24	1.4	PR	9	4	5	4	22	2.2	S
Rd020	12	10	0	1	23	1.6	PR	6	3	5	1	15	2.1	S
Rd108	13	5	0	1	19	1.4	PR	7	5	5	2	19	2.1	S
Rd111	19	1	0	0	20	1.1	PR	20	2	0	0	22	1.1	PR
Rd130	22	0	0	0	22	1.0	R	11	0	0	0	11	1.0	R
Rd175	7	0	2	11	20	2.9	S	12	0	1	9	22	2.3	S
Rd176	9	1	3	9	22	2.5	S	9	1	3	8	21	2.5	S
Rd177	16	2	2	1	21	1.4	PR	12	2	2	3	19	1.8	PR
Rd180	14	5	2	2	23	1.7	PR	10	5	5	2	22	2.0	PR
Rd181	13	3	1	2	19	1.6	PR	9	6	3	3	21	2.0	PR

nt – not tested. At root stage a total of 24 observations were made (8 observations x 3 replicates) per accession and isolate. F accession = 13.78 (p = 0.000), F isolate = 25.39 (p = 0.000), F accession x isolate = 1.73 (p = 0.005). Accessions were separated into four phenotypic categories based on DI values: R = Resistant ($DI \leq 1.0$), PR = Partially Resistant ($1.0 < DI \leq 2.0$), S = Susceptible ($2.0 < DI \leq 3.0$), and HS = Highly Susceptible ($3.0 < DI \leq 4.0$).

Code	Isolate R10				Total	DI		Isolate R6				Total	DI	
Rd182	17	2	1	1	21	1.3	PR	16	2	2	1	21	1.4	PR
Rd183	13	2	3	5	23	2.0	PR	7	2	7	3	19	2.3	S
Rd184	17	2	0	0	19	1.1	PR	17	2	1	1	21	1.3	PR
Rd186	18	2	2	1	23	1.4	PR	15	5	3	0	23	1.5	PR
Rd187	17	1	3	1	22	1.5	PR	9	0	5	4	18	2.2	S
Rd188	20	0	1	0	21	1.1	PR	16	4	0	0	20	1.2	PR
Rd189	9	6	3	4	22	2.1	S	12	1	3	0	16	1.4	PR
Rd190	16	4	1	2	23	1.5	PR	13	5	1	1	20	1.5	PR
Rd191	14	1	1	3	19	1.6	PR	10	4	6	1	21	1.9	PR
Rd192	13	2	5	1	21	1.7	PR	10	4	4	1	19	1.8	PR
Rd193	12	4	3	2	21	1.8	PR	11	3	3	2	19	1.8	PR
Rd194	6	6	5	4	21	2.3	S	2	1	3	15	21	3.5	HS
Rd195	7	3	4	3	17	2.2	S	9	4	5	4	22	2.2	S
Rd196	12	2	1	3	18	1.7	PR	3	5	8	3	19	2.6	S
Rd197	3	0	1	2	6	2.3	S	1	1	1	3	6	3.0	S
Rd198	3	1	4	10	18	3.2	HS	1	1	3	14	19	3.6	HS
Rd201	8	3	4	6	21	2.4	S	-	-	-	-	0	nt	
Rd202	24	0	0	0	24	1.0	R	-	-	-	-	0	nt	
Rd203	23	0	0	0	23	1.0	R	-	-	-	-	0	nt	
Rd204	19	0	0	0	19	1.0	R	-	-	-	-	0	nt	
Rd205	17	1	0	0	18	1.1	PR	-	-	-	-	0	nt	
Rd206	18	1	1	2	22	1.4	PR	-	-	-	-	0	nt	
Rd207	13	3	2	5	23	2.0	PR	-	-	-	-	0	nt	
Rd208	16	4	3	0	23	1.4	PR	-	-	-	-	0	nt	
ER	9	2	1	3	15	1.9	PR	4	3	4	1	12	2.2	S
Total					920			702						
nt – not tested. At root stage a total of 24 observations were made (8 observations x 3 replicates) per accession and isolate. F accession = 13.78 (p = 0.000), F isolate = 25.39 (p = 0.000), F accession x isolate = 1.73 (p = 0.005). Accessions were separated into four phenotypic categories based on DI values: R = Resistant (DI ≤ 1.0), PR = Partially Resistant (1.0 < DI ≤ 2.0), S = Susceptible (2.0 < DI ≤ 3.0), and HS = Highly Susceptible (3.0 < DI ≤ 4.0).														

Isolate R6 was more aggressive than R10 inducing DI values equal or higher in all accessions, with the exception of the accessions Rd013, Rd175, and Rd189. However, no significant differences were observed in the virulence

between isolates in the same accession. Five accessions (Rd001, Rd002, Rd003, Rd004 and Rd130) were resistant at the roots to both isolates and accession Rd198 was highly susceptible to both (Fig. 2).

In R10 inoculation, 8 accessions (18%) were resistant, 29 accessions (64%) partially resistant, 7 accessions (16%) susceptible, and only one accession (2%) highly susceptible. In resistant accessions all the plants were in class 1 (appearance of necrosis without sporulation), with the exception of Rd003 accession which presented one plant (5%) in class 2. Partially resistant accessions included between 44 and 95% of the plants in class 1, 0 to 43% in class 2, and 0 to 35% in classes 3–4. Susceptible accessions registered between 29 and 50% of plants in class 1, 0 to 29% in class 2, and 32 to 65% in classes 3–4. The only accession Rd198 classified as highly susceptible registered 17% of the plants in class 1, 5% in class 2, and 78% in classes 3–4.

In R6 inoculation, 5 accessions (14%) were resistant, 16 accessions (44%) partially resistant, 13 accessions (36%) susceptible, and two accessions (6%) highly susceptible. In resistant accessions all the plants were in class 1 (appearance of necrosis without sporulation), with the exception of Rd002 accession which presented one plant (4%) in class 2. Partially resistant accessions included between 43 and 91% of the plants in class 1, 6 to 35% in class 2, and 0 to 43% in classes 3–4. Susceptible accessions registered between 16 and 54% of plants in class 1, 0 to 26% in class 2, and 37 to 66% in classes 3–4. The accessions Rd194 and Rd198 classified as highly susceptible registered 10 and 5% of the plants in class 1, 5% in class 2, and 85 and 90% in classes 3–4 respectively.

Comparing DM resistance in different organs

A highly significant positive correlation ($r = 0.81$, $P < 0.000$) was recorded between DI values of cotyledons and true-leaves (Fig. 3a), but between cotyledons and roots ($r = 0.17$, $P \geq 0.05$) and leaves and roots ($r = 0.23$, $P \geq 0.05$) the correlation was not significant (Figs. 3b and 3c).

Responses were significantly different between accession and plant organ concerning resistance/susceptibility to DM. For instance, Rd208 was very susceptible in cotyledons and leaves (DI = 5.6 in both) and showed an interesting partial resistance in the roots (DI = 1.4) to isolate R10. On the contrary, Rd201 was partially resistant in cotyledons and true-leaves (DI = 2.9 and 3.6 respectively) and was susceptible in roots (DI = 2.4). Even more contrasting was the accession Rd198 (breeding line) resistance in cotyledons and leaves (DI = 2.0 and 1.2 respectively) and high susceptibility in roots to isolates R10 and R6 (DI = 3.2 and 3.6 respectively). Likewise, four accessions Rd175, Rd176, Rd194, and Rd195 were resistant in cotyledons and true-leaves (DI between 1.7 and 2.0) and susceptible in roots to both isolates (DI between 2.2 and 3.5).

Discussion

In this study we identified 35 radish accessions showing potential resistance to DM at cotyledon, leaves and roots ($DI \leq 4.0$ for cotyledon and leaves; and $DI \leq 2.0$ for roots) from different sources (19 commercial varieties, 14 breeding lines, one landrace and one advanced cultivar). The resistant group includes landrace Rd111 and the advanced cultivar Rd108 from UKVGB (UK Vegetable Genebank), which were resistant in the cotyledons, and the breeding lines Rd017 and Rd020 from the NordGen (Nordic Genetic Resource Center), which were resistant in the cotyledons and 1st and 2nd leaves producing no sporulation.

Information on sources to DM resistance in radish is scarce. Bonnet and Blanchard (1987) referred the Flamboyant variety as being relatively resistant. We also observed that the commercial varieties Rd191 and Rd192 (Alaric-Flamboyant 3 and Flamboyant 5 respectively) presented a resistant response at cotyledons and true-leaves and a partial resistant response at roots. Xu et al. (2014), using a bulked segregant analysis, identified a

radish line resistant to DM at the seedling stage controlled by a single dominant locus, and three molecular markers were recognised closely linked to the resistant locus within a 10.0 centiMorgans (cM) distance.

Cotyledon DM resistance observed in accessions Rd001 and Rd004 (daikon type) was a dominant inherited trait controlled by a single dominant gene, and in accession Rd193 (long red root) the resistance might be conferred by two dominant genes with complementary action (Coelho and Monteiro 2018). These accessions showed also promising resistant responses at leaves and roots, but no information was available about the genetic control of resistance at these stages.

In the current research we inoculated 14 day-old seedlings showing the two first leaves full expanded and mature. The good correlation between DM resistance at cotyledons and 1st and 2nd true-leaves allows the use of cotyledon resistance to predict adult-plant resistance. The estimate of leaf resistance based on cotyledon resistance would save time and work. Also, cotyledon resistance allowed to assay a large number of plants and observed interaction phenotype (IP) are stable since tests are conducted under controlled environmental conditions. However, in the case of radish cotyledon resistance has its own value because non-infected cotyledons will act as a barrier to slow disease progression to true-leaves and roots.

Differently from radish, in broccoli and cabbage (*Brassica oleracea*) DM susceptibility may increase with leaf ageing because there are reported examples of resistant plants at cotyledon stage that became infected on the true-leaves (Agnola et al. 2003; Coelho et al. 2009). In such a case cotyledon resistance cannot be used to predict adult-plant resistance since the two types of resistance were very poorly correlated.

Different studies showed that the resistance of adult brassicas having eight or more leaves is independent from the resistance at seedling stage because plants can be susceptible at the cotyledon stage and resistant at the adult stage or to express cotyledon resistance that continues until full plant maturity (Dickson and Petzoldt 1993; Coelho et al. 1998; Jensen et al. 1999; Coelho and Monteiro 2003; Coelho et al. 2009). 'Couve Algarvia', a Portuguese Tronchuda kale (*B. oleracea*), is a particular case where resistance at cotyledon and adult-plant stages is under the control of two independent genetic systems and so all combinations between cotyledon and mature plant resistance may occur (Monteiro et al. 2005).

To clarify the genetic control responsible for cotyledon, true-leaf and root resistance in radish, genetic studies must be done in the different organs of the plant. However, cotyledon and young true-leaf resistance in radish have higher horticultural relevance because radish has a very short and quick growing cycle. The disease starts on the cotyledons and then progresses to the leaves and roots. Cotyledon resistance may act as a protective barrier to slow the spread of the disease to the crop. Root damage in radish is important because root is the edible part, but the resistance of canopy is also important since it can protect root infection. DM disease attacks throughout the plant cycle and may kill plants or delay their development leading to a huge crop reduction. A good disease control on the leaves is a key issue for high productivity and quality in radish crop.

Root inoculation by spraying is more difficult than applying the same method to cotyledons and leaves, and may be less effective. Part of the root is covered by soil, which promotes some protection against infection, and root infection may be hampered by the higher difficulty of retaining inoculum drops on root surface, in comparison with cotyledons and leaves that have horizontal surfaces. However, the consistency of the results of the two independent root inoculations with isolate R10 and R6 isolates shows that the method we used to test the roots was reliable.

The seven Japanese radish daikon accessions (*Raphanus sativus* L. var. *longipinnatus* L.H. Bailey) evaluated in this study have a longer vegetative cycle than the conventional radish varieties, need 50–80 days to harvest (APA 1988), may grow up to 75-cm long with a diameter of up to 25-cm and weigh several kilograms. The roots of these plant were tested at an earlier stage of development in comparison with standard radishes. To confirm whether the stage of development may affect the expression of root resistance, daikon radish should be tested later on during the cycle or ideally under field conditions.

Declarations

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Figures

Figure 1

Symptoms on plants inoculated at seedling stage. Resistance: no host reaction and no sporulation, or small necrosis on the adaxial cotyledon/leaf surface. Susceptibility: sporulation dispersed over whole abaxial cotyledon/leaf surface, or abundant and dense sporulation dispersed over the whole cotyledon/leaf. 1a. Cotyledon inoculation by drop. 1b. Resistant cotyledons (class 1). 1c. Susceptible cotyledons (class 6). 1d. Leaf inoculation by pulverization. 1e. Adaxial and abaxial surface of a resistant leaf with dark necroses and no sporulation (class 1). 1f. Adaxial and abaxial surface of a susceptible leaf with dense sporulation (class 6). 1g. Resistant long-red radish root (class 1). 1h. Susceptible long-red radish root (class 4). 1i. Susceptible round-red radish root (class 4).

Figure 2

Correlation between Disease Index (DI) values of thirty-six radish accessions inoculated with *H. brassicae* isolates R10 and R6 on the roots ($r=0.81$, $P < 0.000$).

Figure 3

a. Correlation between Disease Index (DI) values of cotyledon and true-leaf inoculation ($r=0.81$, $P < 0.000$). b. Correlation between DI values of cotyledon and root inoculation ($r=0.17$, $P \geq 0.05$). c. Correlation between DI values of true-leaf and root inoculation ($r=0.23$, $P \geq 0.05$).