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Comparing cotyledon, leaf and root resistance to downy mildew in radish (*Raphanus sativus* L.)

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Abstract Radish downy mildew (DM) caused by the oomycete *Hyaloperonospora brassicae* f. sp. *raphani* is a serious problem in radish crop, an edible root vegetable 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 under controlled conditions. Forty-four radish accessions were inoculated at cotyledons and true-leaves with *H. brassicae* isolate R10, collected in cotyledons of field plants. The roots were tested with isolates R10 and R6, this last one collected in roots of field radish. DM symptoms varied with the radish genotype and plant organ analysed. Twenty-seven resistant and partially resistant accessions were identified in all plant stages and are promising sources of resistance to DM, namely 16 commercial varieties, 10 breeding lines, and one landrace. A significant correlation was observed between cotyledon and leaf (1st and 2nd leaves) DM resistance, but low and no correlation was found between the resistance of true-leaves or cotyledons and roots, respectively. Cotyledon and leaf evaluation cannot be used to predict root resistance response in radish. 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. Interesting sources of DM resistance were identified that can be used in radish breeding programs.

Keywords Biotic resistance · Oomycete · Plant organ · *Raphanus sativus* L. · Screening methods

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Introduction

Radish (*Raphanus sativus* L., n = 9) is a root vegetable of the Brassicaceae family, which includes the small or Western radish (*Raphanus sativus* L. var. *sativus)* and the daikon radish (*Raphanus sativus* L. var. *longipinnatus* L.H. Bailey) varieties, 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 roots are much larger and the plant requires more time and space to grow. 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 *R. sativus* var. *sativus* produce much smaller roots and reach harvest stage in 3–4 weeks.

Radish downy mildew (DM) is an economically important disease in main production areas especially in autumn and spring under temperate and humid weather conditions (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 are usually very susceptible to DM and chemical control is not possible due to the short cultural cycle of radish 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 of 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 unsaleable. The protection of the foliage against the disease is important because the roots got infected by the conidia washed down from cotyledons and young 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 by avoiding overhead irrigation especially late in the day, removing plant debris after harvest, and also rotation with non-brassicas 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 radish production. Robust phenotyping data are fundamental for accurate germplasm selection and future use.

Breeding of vegetable horticultural crops, including radish, were essentially developed by private seed companies. Information on sources to DM resistance in radish is scarce and few radish genotypes resistant to DM are known (Bonnet and Blancard 1987; Jiang et al. 2012; Wang et al. 2014; Xu et al. 2014; Coelho and Monteiro 2018). 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.

The objectives of the present study were to develop screening methodologies for assessing DM resistance in different radish plant organs, to identify sources of resistance by screening a germplasm collection, and to compare the expression of resistance in cotyledons, true-leaves and roots in order to select the best evaluation methodology.

Material and methods

Plant material and plantlet production

A germplasm collection of radish with more than 200 accessions was screened for downy mildew resistance with *H. brassicae* isolate R10 at cotyledon stage. In the present study, a group of radish accessions with known cotyledon resistance to *H. brassicae* f. sp. *raphani* (37 resistant, 5 partially resistant, and 2 highly susceptible) was selected from that germplasm

collection 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 included in all the tests as a susceptible control and was also used to obtain fresh *H. brassicae* inoculum for the different experiments (Fig. 1c, f, and i).

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 6 days in trays of 3×3×5-cm cells, and for leaf and root evaluation the plants were grown during 14 days in larger cells of 4×4×5.5-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. The roots were tested with isolates R10 and R6 in 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 cleaned by some transfers onto fresh plant material under optimal conditions for DM growth (20 °C and high humidity). Clean isolates were stored at − 18 °C on infected cotyledons of the susceptible accession Rd197 (Table 1).

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 50–75 × 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 ± 1 °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 ± 1 °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 previously described procedures 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 with *H. brassicae* isolates R10 and R6, in separate trays, following the procedures described for leaf inoculation. The radish seeds were seeded superficially in alternate rows in order to facilitate root pulverization, and the resistance of radish roots was individually assessed at 12dpi. The isolates were tested in different experiments and a total of 24

Table 1 Forty-five radish accessions from breeding lines, commercial varieties, and genebanks tested for downy mildew resistance

Code	Accession name	Accession state	Accession origin	Radish crop type	Cotyledon response
Rd001	PI 508495	Breeding line	Seed company	White daikon	R
Rd002	PI 483356	Breeding line	Seed company	Daikon	R
Rd003	PI 483357	Breeding line	Seed company	Daikon	R
Rd004	G30854	Breeding line	Seed company	Red daikon with white tip	R
Rd005	INRA 148	Breeding line	Seed company	Short cycle	R
Rd011	Java	Commercial variety	Seed company	Short cycle	R
Rd012	Bamba	Commercial variety	Seed company	Medium red with white tip	PR
Rd013	Lavergne	Commercial variety	Seed company	Ovate shape	R
Rd014	Pontvil	Commercial variety	Seed company	Short cycle	R
Rd015	05507 BS	Breeding line	Seed company	Short cycle	R
Rd017	Københavns Torve 9	Breeding line	NordGen (DK)	Round red with white tip	R
Rd020	Rovi	Breeding line	NordGen (DK)	Round red with white tip	R
Rd108	6412-Grazer Treib AS	Advanced cultivar	UKVGB (AUT)	Long red	R
Rd111	6595	Landrace	UKVGB (EGY)	Long red	R
Rd130	7220-Sassarese	Landrace	UKVGB (ITA)	–	R
Rd175	No. 3	Breeding line	Seed company	Long red with white tip	R
Rd176	No. 4	Breeding line	Seed company	–	R
Rd177	No. 5	Breeding line	Seed company	Short cycle	R
Rd180	No. 8	Breeding line	Seed company	Round red	R
Rd181	No. 9	Breeding line	Seed company	Long red	R
Rd182	No. 10	Breeding line	Seed company	Round red	R
Rd183	No. 11	Breeding line	Seed company	Long red with white tip	R
Rd184	No. 12	Breeding line	Seed company	Round red	R
Rd186	Rond Ecarlate Hatif	Commercial variety	Seed company	Round red	R
Rd187	Cerise	Commercial variety	Seed company	Round red	R
Rd188	Sezanne	Commercial variety	Seed company	Round red with white tip	R
Rd189	Gaudry 2	Commercial variety	Seed company	Small diameter	R
Rd190	National	Commercial variety	Seed company	Round red with white tip	R
Rd191	Alaric (Flamboyant 3)	Commercial variety	Seed company	Medium cycle	R
Rd192	Flambard (Flamboyant 5)	Commercial variety	Seed company	Medium cycle	R
Rd193	Flambo	Commercial variety	Seed company	Very long red with white tip	R
Rd194	Nelson	Commercial variety	Seed company	Half-long red with white tip	R
Rd195	Gandar	Commercial variety	Seed company	Long red with white tip	R
Rd196	Capitole	Commercial variety	Seed company	Long red with white tip	R
Rd197	SRR	Breeding line	Seed company	Round red	HS
Rd198	SFB	Breeding line	Seed company	Short cycle	R
Rd201	Treto	Commercial variety	Seed company	Long red with white tip	PR
Rd202	April Cross	Commercial variety	Seed company	White daikon	R
Rd203	Mino Summer Cross	Commercial variety	Seed company	White daikon	R
Rd204	Omny	Commercial variety	Seed company	White daikon	R
Rd205	Diablus	Commercial variety	Seed company	Long red with white tip	PR
Rd206	Expo	Commercial variety	Seed company	Long red with white tip	R
Rd207	Fluo	Commercial variety	Seed company	Long red with white tip	PR
Rd208	Tinto	Commercial variety	Seed company	Round red	HS

Table 1 (continued)

Code	Accession name	Accession state	Accession origin	Radish crop type	Cotyledon response
ER	Encarnado Redondo	Commercial variety	A.A. Dias, Lda (PT)	Round red	PR

NordGen Nordic Genetic Resource Center (Sweden); *UKVGB* UK Vegetable Genebank (Warwick, UK)

Phenotypic categories: R = Resistant, PR = Partially Resistant, S = Susceptible, and HS = Highly Susceptible

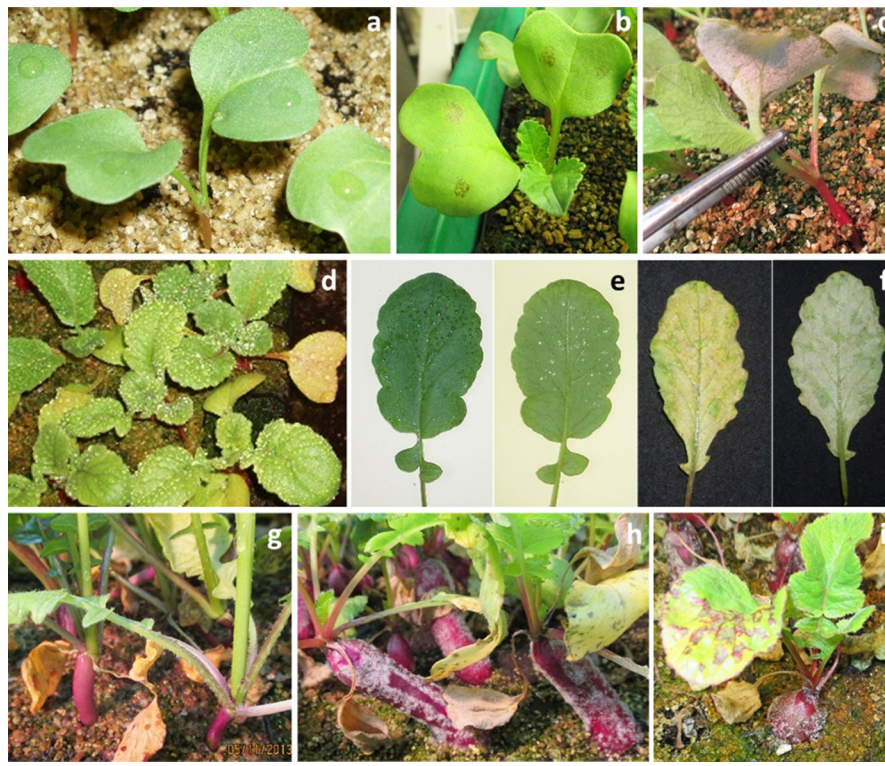

Fig. 1 Symptoms on plants inoculated with *H. brassicae* isolate R10 at seedling stage. Resistance: no host reaction and no sporulation, or small necrosis on the adaxial cotyledon/leaf surface and root. Susceptibility: sporulation dispersed over whole abaxial cotyledon/leaf surface and root, or abundant and dense sporulation dispersed over the whole cotyledon/leaf/root. **a** Drop inoculation of cotyledons with 6 days. **b** Resistant cotyledons of the accession Rd004 (class 1) 7dpi (days post-inoculation). **c** Susceptible cotyledons of the accession Rd197 (class 6) 7dpi. **d** Spraying inoculation of leaves with 14 days. **e** Adaxial and abaxial surface of a resistant leaf of the accession Rd198 with dark necrosis and no sporulation (class 1) 12dpi. **f** Adaxial and abaxial surface of a susceptible leaf of the accession Rd197 with dense sporulation (class 6) 12dpi. **g** Daikon long-red radish root of resistant accession Rd004 (class 1) 12dpi. **h** Long-red radish root of susceptible accession Rd201 (class 4) 12dpi. **i.** Round-red radish root of susceptible accession Rd197 (class 4) 12dpi

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, only showing necrosis restricted to the point of infection with no sporulation (Fig. 1b and e);

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 ($5.0 < DI \leq 6.0$) (Coelho and Monteiro 2018)

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 (Fig. 1c and f).

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 point of infection; and classes 3–4 were susceptible reactions with sparse to abundant sporulation respectively, dispersed over the whole radish surface (Fig. 1h and i).

A mean disease severity index (DI ± S.E.) 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 12. The correlation between R10 and R6 isolates were assessed via Pearson's coefficient, and between cotyledon, true-leaf and root DI values were assessed via Spearman'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 divided the accessions into four phenotypic categories

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 ($3.0 < DI \leq 4.0$)

(Table 4). At cotyledon 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.

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%) partially resistant, and 2 accessions (5%) 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 registered zero plants in classes 1–2, between 0 and 20% in classes 3–4, and between 80 and 100% in class 6 respectively (Table 4).

Root evaluation with two H. brassicae *isolates*

Unlike the cotyledons and true-leaves that were tested only with isolate R10, the roots were tested with two different isolates, R10 and R6, in independent experiments. The two isolates had different geographic origins. Isolate R10 was collected from cotyledons and isolate R6 from roots. There was a highly significant correlation ($r = 0.805$, $P = 0.000$) between the DI values of the two isolates (Fig. 2), which indicates the same general pattern of resistance across the accessions when inoculated with each isolate (Table 5).

Considering root inoculation with R10, 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), only Rd003 accession presented one plant 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. Accession Rd198, the only one classified as highly susceptible, registered 17% of the plants in class 1, 5% in class 2, and 78% in classes 3–4.

In root inoculation with R6, 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 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 two highly susceptible accessions, Rd194 and Rd198, registered 10 and 5% of the plants in class 1, 5% in class 2, and 85 and 90% in classes 3–4 respectively.

Isolate R6 was significantly more aggressive than R10 ($F_{isolate} = 26.72$, $P = 0.000$) and induced DI values equal or higher in all accessions, with the exception of the accessions Rd013, Rd175, and Rd189. However, no significant differences of virulence between isolates were observed on the same accession. Five accessions (Rd001, Rd002, Rd003, Rd004 and Rd130) were resistant and accession Rd198 was highly susceptible to both isolates (Fig. 2 and Table 5).

Comparing DM resistance in different organs

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 partially 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) also to isolate R10 (Fig. 1h). Even more contrasting was the accession Rd198 (breeding line), which was resistant in cotyledons and leaves (DI = 2.0 and 1.2 respectively) (Fig. 1e) and highly susceptible 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

Table 4 Number of plants per interaction-phenotype classes (IP classes), total number of plants evaluated and disease index (DI ± S.E.) values of forty-four radish accessions tested for downy mildew resistance at cotyledon and true-leaf stages with *H. brassicae* isolate R10

Code	Cotyledon - IP classes						Total	DI ± S.E		Leaves - IP classes						Total	DI ± S.E	
	1	2	3	4	5	6				1	2	3	4	5	6			
Rd001	14	3	2	1	0	0	20	1.5 ± 0.20	**R**	13	5	0	0	0	0	18	1.3 ± 0.11	**R**
Rd002	18	1	1	3	0	0	23	1.5 ± 0.23	**R**	15	5	0	0	0	0	20	1.3 ± 0.10	**R**
Rd003	15	1	4	3	0	1	24	2.0 ± 0.29	**R**	16	4	0	0	0	0	20	1.2 ± 0.09	**R**
Rd004	14	1	3	1	3	2	24	2.3 ± 0.37	**R**	17	1	0	0	0	0	18	1.1 ± 0.06	**R**
Rd005	16	2	6	0	0	0	24	1.6 ± 0.18	**R**	4	14	2	0	0	0	20	1.9 ± 0.12	**R**
Rd011	10	5	6	1	0	0	22	1.9 ± 0.21	**R**	5	8	1	3	2	1	20	2.6 ± 0.34	**PR**
Rd013	14	1	6	1	0	0	22	1.7 ± 0.22	**R**	2	13	5	0	0	0	20	2.2 ± 0.13	**R**
Rd014	13	2	4	4	0	1	24	2.1 ± 0.30	**R**	0	10	4	2	3	1	20	3.1 ± 0.29	**PR**
Rd015	13	0	7	3	0	0	23	2.0 ± 0.25	**R**	4	15	1	0	0	0	20	1.9 ± 0.11	**R**
Rd017	12	2	6	4	0	0	24	2.1 ± 0.25	**R**	14	6	0	0	0	0	20	1.3 ± 0.11	**R**
Rd020	10	0	9	3	0	1	23	2.4 ± 0.29	**R**	4	14	0	0	0	0	18	1.8 ± 0.10	**R**
Rd108	11	0	6	6	0	1	24	2.5 ± 0.31	**R**	10	0	0	3	1	5	19	3.0 ± 0.52	**PR**
Rd111	11	1	2	7	1	0	22	2.4 ± 0.32	**R**	13	6	1	0	0	0	20	1.4 ± 0.13	**R**
Rd175	17	0	3	3	0	0	23	1.7 ± 0.25	**R**	8	11	1	0	0	0	20	1.7 ± 0.13	**R**
Rd176	12	3	7	1	0	0	23	1.9 ± 0.21	**R**	10	8	0	0	0	2	20	1.9 ± 0.33	**R**
Rd177	15	1	7	1	0	0	24	1.8 ± 0.21	**R**	12	5	1	0	2	0	20	1.8 ± 0.28	**R**
Rd180	12	0	6	6	0	0	24	2.3 ± 0.27	**R**	16	3	0	0	1	0	20	1.4 ± 0.21	**R**
Rd181	11	1	4	7	0	1	24	2.5 ± 0.31	**R**	10	7	2	0	0	0	19	1.6 ± 0.16	**R**
Rd182	15	0	7	0	0	0	22	1.6 ± 0.20	**R**	19	0	1	0	0	0	20	1.1 ± 0.10	**R**
Rd183	15	2	5	2	0	0	24	1.8 ± 0.22	**R**	8	7	0	0	1	4	20	2.6 ± 0.44	**PR**
Rd184	16	0	4	1	0	1	22	1.7 ± 0.29	**R**	17	2	1	0	0	0	20	1.2 ± 0.12	**R**
Rd186	13	3	4	4	0	0	24	2.0 ± 0.24	**R**	6	14	0	0	0	0	20	1.7 ± 0.11	**R**
Rd187	15	2	2	2	0	1	22	1.8 ± 0.29	**R**	20	0	0	0	0	0	20	1.0 ± 0.00	**R**
Rd188	13	6	2	1	0	0	22	1.6 ± 0.18	**R**	10	9	1	0	0	0	20	1.6 ± 0.14	**R**
Rd189	16	3	4	1	0	0	24	1.6 ± 0.19	**R**	2	15	1	2	0	0	20	2.2 ± 0.17	**R**
Rd190	14	3	2	5	0	0	24	1.9 ± 0.25	**R**	4	14	1	1	0	0	20	2.0 ± 0.15	**R**
Rd191	11	6	0	3	0	0	20	1.8 ± 0.24	**R**	5	14	1	0	0	0	20	1.8 ± 0.12	**R**
Rd192	13	2	5	3	1	0	24	2.0 ± 0.27	**R**	2	18	0	0	0	0	20	1.9 ± 0.07	**R**
Rd193	18	2	1	0	0	0	21	1.2 ± 0.11	**R**	2	18	0	0	0	0	20	1.9 ± 0.07	**R**
Rd194	10	6	4	3	0	0	23	2.0 ± 0.23	**R**	4	14	2	0	0	0	20	1.9 ± 0.12	**R**
Rd195	13	5	0	5	1	0	24	2.0 ± 0.28	**R**	8	10	2	0	0	0	20	1.7 ± 0.15	**R**
Rd196	18	4	0	0	0	0	22	1.2 ± 0.08	**R**	19	0	0	0	0	0	19	1.0 ± 0.00	**R**
Rd198	13	3	3	3	1	0	23	2.0 ± 0.27	**R**	17	3	0	0	0	0	20	1.2 ± 0.08	**R**
Rd202	24	0	0	0	0	0	24	1.0 ± 0.00	**R**	11	8	1	0	0	0	20	1.5 ± 0.14	**R**
Rd203	22	0	2	0	0	0	24	1.2 ± 0.12	**R**	0	18	2	0	0	0	20	2.1 ± 0.07	**R**
Rd204	14	1	5	3	0	0	23	1.9 ± 0.25	**R**	8	9	2	0	0	0	19	1.7 ± 0.15	**R**
Rd206	9	0	2	4	0	0	15	2.1 ± 0.36	**R**	5	6	6	2	1	0	20	2.4 ± 0.26	**R**
Rd012	10	0	6	6	1	1	24	2.6 ± 0.32	**PR**	2	6	4	5	2	1	20	3.1 ± 0.31	**PR**
Rd201	4	0	5	5	1	0	15	2.9 ± 0.34	**PR**	1	3	7	4	3	2	20	3.6 ± 0.30	**PR**
Rd205	4	0	3	8	0	0	15	3.0 ± 0.34	**PR**	6	3	3	4	1	1	18	2.7 ± 0.37	**PR**
Rd207	5	0	2	8	0	0	15	2.9 ± 0.36	**PR**	1	2	5	9	3	0	20	3.6 ± 0.23	**PR**
ER	0	3	1	3	2	2	11	3.9 ± 0.46	**PR**	2	15	3	0	0	0	20	2.1 ± 0.11	**R**
Rd197	0	0	0	2	3	19	24	5.7 ± 0.13	**HS**	0	0	0	0	0	10	10	6.0 ± 0.00	**HS**

Table 4 (continued)

Code	Cotyledon - IP classes						Total	DI ± S.E		Leaves - IP classes						Total	DI ± S.E	
	1	2	3	4	5	6				1	2	3	4	5	6			
Rd208	0	0	0	1	4	11	16	5.6 ± 0.15	**HS**	0	0	1	3	0	16	20	5.6 ± 0.21	**HS**
Total							**964**									**858**		

At cotyledon stage means and standard errors were calculated from a total of 24 observations (8 observations × 3 replicates) for each accession, and at true-leaf stage were calculated from a total of 20 observations (10 observations × 2 replicates) for each 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)

true-leaves (DI between 1.7 and 2.0) and susceptible in roots to both isolates (DI between 2.2 and 3.5).

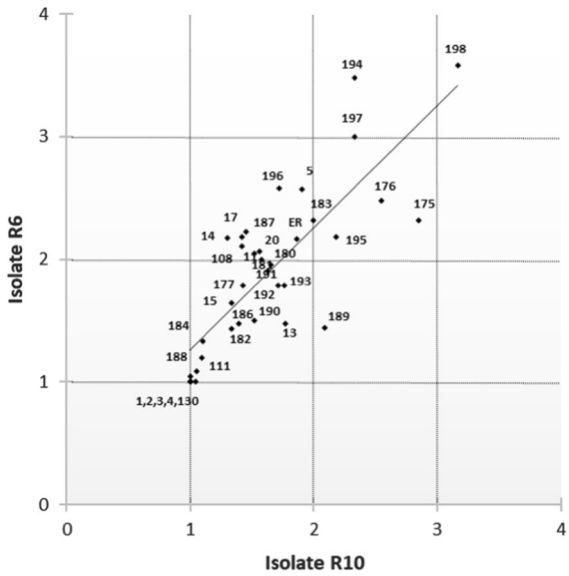

Fig. 2 Correlation between Disease Index (DI) values of thirty-six radish accessions inoculated with *H. brassicae* isolates R10 and R6 on the roots (r = 0.805, *P* = 0.000)

A significant moderate positive correlation (r = 0.443, *P* = 0.003, N = 44) was recorded between DI values of cotyledons and true-leaves (Fig. 3a), a low correlation was observed between leaves and roots (r = 0.354, *P* = 0.018, N = 44), and between cotyledons and roots (r = 0.187, *P* = 0.224, N = 44) the correlation was not significant (Fig. 3b and 3c).

Discussion

The present research shows some interesting sources of resistance to downy mildew previously identified at cotyledon stage by our research team (unpublished results). Seven daikons (*R. sativus* var. *longipinnatus*), four breeding lines (Rd001, Rd002, Rd003, Rd004) and three commercial varieties (Rd202, Rd203, Rd204) respectively, were resistant in cotyledons, leaves and roots to *H. brassicae* isolate R10. The four breeding lines were also resistant to isolate R6 when tested on the roots.

In the group of Western radish (*R. sativus* var. *sativus*) twenty-eight accessions showed potential resistance to *H. brassicae* isolate R10 at cotyledon, leaves and roots with a resistant/partially resistant response (DI ≤ 4.0 for cotyledon and leaves; and DI ≤ 2.0 for roots) (Tables 1, 4 and 5). Twenty-four accessions were also tested with the isolate R6 at roots, and fifteen accessions (8 commercial varieties, 6 breeding lines, and one landrace) maintained the resistant/partially resistant response observed at cotyledons and leaves. The landrace Rd111, a long red radish from UKVGB (UK Vegetable Genebank), was resistant at cotyledons and leaves, and partially resistant at roots to the two isolates. Three accessions from NordGen and UKVGB germplasm banks, two breeding lines (Rd017, Rd020) and one advanced cultivar (Rd108) respectively, resistant at cotyledon and leaves showed some susceptibility to isolate R6 in the roots. Accession Rd130 showed a resistant response in roots to both isolates.

The commercial varieties Rd191 and Rd192 (Alaric-Flamboyant 3 and Flambard-Flamboyant 5 respectively) presented a resistant response at cotyledons and true-leaves and a partial resistant response at roots. This result confirms Bonnet and

Table 5 Number of plants per interaction-phenotype classes (IP classes), total number of plants evaluated and disease index (DI ± S.E.) values of radish accessions tested for downy mildew resistance on the roots with *H. brassicae* isolates R10 and R6

Code	Isolate R10 - IP classes					Total	DI ± S.E	Isolate R6 - IP classes					Total	DI ± S.E
	1	2	3	4				1	2	3	4			
Rd001	24	0	0	0	24	1.0 ± 0.00	**R**	19	0	0	0	19	1.0 ± 0.00	**R**
Rd002	24	0	0	0	24	1.0 ± 0.00	**R**	23	1	0	0	24	1.0 ± 0.04	**R**
Rd003	21	1	0	0	22	1.0 ± 0.05	**R**	23	0	0	0	23	1.0 ± 0.00	**R**
Rd004	24	0	0	0	24	1.0 ± 0.00	**R**	19	0	0	0	19	1.0 ± 0.00	**R**
Rd130	22	0	0	0	22	1.0 ± 0.00	**R**	11	0	0	0	11	1.0 ± 0.00	**R**
Rd202	24	0	0	0	24	1.0 ± 0.00	**R**	–	–	–	–	0	nt	
Rd203	23	0	0	0	23	1.0 ± 0.00	**R**	–	–	–	–	0	nt	
Rd204	19	0	0	0	19	1.0 ± 0.00	**R**	–	–	–	–	0	nt	
Rd005	10	4	6	1	21	1.9 ± 0.22	**PR**	4	5	11	3	23	2.6 ± 0.20	**S**
Rd011	17	2	2	2	23	1.5 ± 0.21	**PR**	10	2	7	2	21	2.0 ± 0.24	**PR**
Rd012	4	2	2	1	9	2.0 ± 0.37	**PR**	–	–	–	–	0	nt	
Rd013	13	3	4	2	22	1.8 ± 0.23	**PR**	16	4	2	1	23	1.5 ± 0.18	**PR**
Rd014	15	4	1	0	20	1.3 ± 0.13	**PR**	11	1	7	4	23	2.2 ± 0.26	**S**
Rd015	17	2	1	1	21	1.3 ± 0.17	**PR**	9	6	1	1	17	1.6 ± 0.21	**PR**
Rd017	18	3	2	1	24	1.4 ± 0.17	**PR**	9	4	5	4	22	2.2 ± 0.25	**S**
Rd020	12	10	0	1	23	1.6 ± 0.15	**PR**	6	3	5	1	15	2.1 ± 0.27	**S**
Rd108	13	5	0	1	19	1.4 ± 0.18	**PR**	7	5	5	2	19	2.1 ± 0.24	**S**
Rd111	19	1	0	0	20	1.1 ± 0.05	**PR**	20	2	0	0	22	1.1 ± 0.06	**PR**
Rd177	16	2	2	1	21	1.4 ± 0.19	**PR**	12	2	2	3	19	1.8 ± 0.27	**PR**
Rd180	14	5	2	2	23	1.7 ± 0.20	**PR**	10	5	5	2	22	2.0 ± 0.22	**PR**
Rd181	13	3	1	2	19	1.6 ± 0.23	**PR**	9	6	3	3	21	2.0 ± 0.24	**PR**
Rd182	17	2	1	1	21	1.3 ± 0.17	**PR**	16	2	2	1	21	1.4 ± 0.19	**PR**
Rd183	13	2	3	5	23	2.0 ± 0.27	**PR**	7	2	7	3	19	2.3 ± 0.27	**S**
Rd184	17	2	0	0	19	1.1 ± 0.07	**PR**	17	2	1	1	21	1.3 ± 0.17	**PR**
Rd186	18	2	2	1	23	1.4 ± 0.17	**PR**	15	5	3	0	23	1.5 ± 0.15	**PR**
Rd187	17	1	3	1	22	1.5 ± 0.19	**PR**	9	0	5	4	18	2.2 ± 0.31	**S**
Rd188	20	0	1	0	21	1.1 ± 0.10	**PR**	16	4	0	0	20	1.2 ± 0.09	**PR**
Rd190	16	4	1	2	23	1.5 ± 0.20	**PR**	13	5	1	1	20	1.5 ± 0.18	**PR**
Rd191	14	1	1	3	19	1.6 ± 0.27	**PR**	10	4	6	1	21	1.9 ± 0.22	**PR**
Rd192	13	2	5	1	21	1.7 ± 0.22	**PR**	10	4	4	1	19	1.8 ± 0.22	**PR**
Rd193	12	4	3	2	21	1.8 ± 0.23	**PR**	11	3	3	2	19	1.8 ± 0.25	**PR**
Rd196	12	2	1	3	18	1.7 ± 0.28	**PR**	3	5	8	3	19	2.6 ± 0.22	**S**
Rd205	17	1	0	0	18	1.1 ± 0.06	**PR**	–	–	–	–	0	nt	
Rd206	18	1	1	2	22	1.4 ± 0.20	**PR**	–	–	–	–	0	nt	
Rd207	13	3	2	5	23	2.0 ± 0.26	**PR**	–	–	–	–	0	nt	
Rd208	16	4	3	0	23	1.4 ± 0.15	**PR**	–	–	–	–	0	nt	
ER	9	2	1	3	15	1.9 ± 0.32	**PR**	4	3	4	1	12	2.2 ± 0.30	**S**
Rd175	7	0	2	11	20	2.9 ± 0.32	**S**	12	0	1	9	22	2.3 ± 0.32	**S**
Rd176	9	1	3	9	22	2.5 ± 0.30	**S**	9	1	3	8	21	2.5 ± 0.31	**S**
Rd189	9	6	3	4	22	2.1 ± 0.25	**S**	12	1	3	0	16	1.4 ± 0.20	**PR**
Rd194	6	6	5	4	21	2.3 ± 0.24	**S**	2	1	3	15	21	3.5 ± 0.21	**HS**
Rd195	7	3	4	3	17	2.2 ± 0.29	**S**	9	4	5	4	22	2.2 ± 0.25	**S**
Rd197	11	1	5	6	23	2.3 ± 0.28	**S**	1	1	1	3	6	3.0 ± 0.52	**S**
Rd201	8	3	4	6	21	2.4 ± 0.28	**S**	–	–	–	–	0	nt	

Table 5 (continued)

Code	Isolate R10 - IP classes					Total	DI ± S.E	Isolate R6 - IP classes					Total	DI ± S.E
	1	2	3	4				1	2	3	4			
Rd198	3	1	4	10	18	3.2 ± 0.27	**HS**	1	1	3	14	19	3.6 ± 0.19	**HS**
Total					**943**							**702**		

nt – not tested. At root stage means and standard errors were calculated from a total of 24 observations (8 observations × 3 replicates) for each accession and isolate. F accession = 14.05 ($P = 0.000$), F isolate = 26.72 ($P = 0.000$), F accession x isolate = 1.75 ($P = 0.005$). The accessions were separated into four phenotypic categories: 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)

Blancard (1987) who reported that the Flamboyant variety was relatively resistant.

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

In the current research we inoculated 14-day-old seedlings showing the two first leaves full expanded and mature. The correlation observed between DM resistance at cotyledons and 1st and 2nd true-leaves ($r = 0.443$, $P = 0.003$) was positive with a moderate coefficient, which means that screening for resistance can be done by testing either cotyledons or young leaves.

The prediction 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) is stable since tests are conducted under controlled environmental conditions. However, cotyledon resistance in radish has its own value because non-infected cotyledons will act as a barrier to slow disease progression to true-leaves and roots.

The absence of high correlations between the resistance of the roots, and the cotyledons and leaves, implies the need to test DM resistance in the foliage and in the roots. A total of seven accessions resistant in cotyledons and leaves to *H. brassicae* isolate R10 were observed, which showed a susceptible (Rd175, Rd176, Rd189, Rd194, Rd195, and Rd201) and highly susceptible response (Rd198) in the roots. On the contrary two accessions Rd208 and Rd197, highly susceptible in the cotyledons and leaves to the isolate R10, registered a partially resistant and susceptible response, respectively, in the roots.

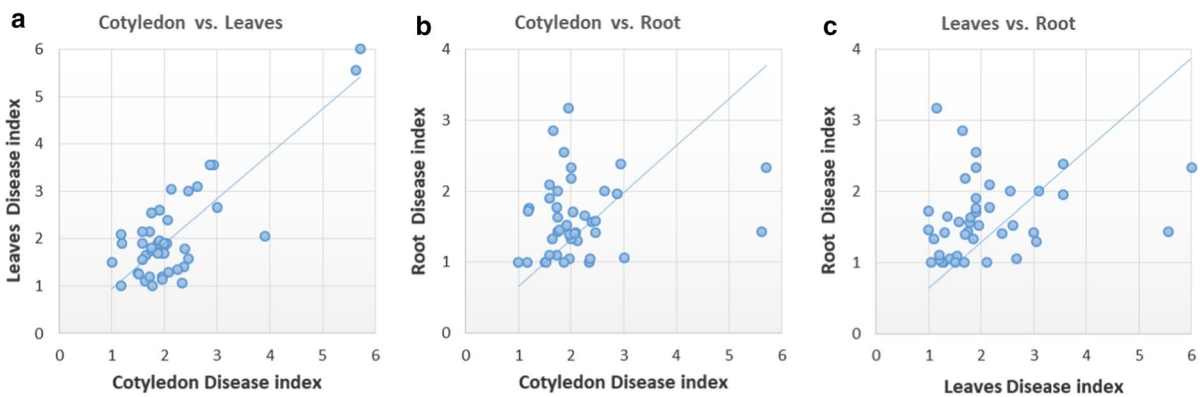

Fig. 3 **a** Correlation between Disease Index (DI) values of cotyledon and true-leaf inoculation ($r = 0.443$, $P = 0.003$, $N = 44$). **b** Correlation between DI values of cotyledon and root inoculation ($r = 0.187$, $P = 0.224$, $N = 44$). **3c.** Correlation between DI values of true-leaf and root inoculation ($r = 0.354$, $P = 0.018$, $N = 44$) inoculated with *H. brassicae* isolate R10

In broccoli and cabbage (*Brassica oleracea*) DM resistance response usually increase with plant ageing. There are reported examples of susceptible plants at cotyledon stage that became resistant 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. However, no resistant accessions at cotyledons and susceptible at adult plant were found.

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 may express cotyledon resistance that continues until full plant maturity (Dickson and Petzoldt 1993; Coelho et al. 1998, 2009; Jensen et al. 1999; Coelho and Monteiro 2003). 'Couve Algarvia', a Portuguese Tronchuda kale (*B. oleracea*), is a particular case in which 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 considering the different organs of the plant. However, cotyledon and young true-leaf resistance in radish has higher horticultural relevance than in cabbage 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 could 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 greater 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 isolates R10 and R6 shows that the method we used to test the roots is reliable.

The root evaluation with two different *H. brassicae* isolates showed that despite some differences in the aggressiveness of the isolates, isolate R6 being more aggressive, the two isolates do not significantly differ in their virulence on the hosts. We are not aware of studies on the variability of *H. brassicae* isolates collected in radish, which is important for the selection of the best genotypes to use in breeding. Similarly to what was done for *Brassica oleracea* (Coelho et al. 2012), it would be interesting to test radish resistant accessions with isolates from different geographic locations, to inform about how effective the host resistance would be in the eventual presence of different *H. brassicae* races.

The seven Japanese radish daikon accessions evaluated in this study have a longer vegetative cycle than the conventional radish varieties, require 50–80 days from seed 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. In order to confirm whether the resistant response observed at 26 days is maintained throughout the entire vegetative cycle, it would be interesting to test daikon radish at a later stage of the growing cycle or ideally under field conditions.

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Declarations

Conflict of interest 'Not applicable'.

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