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Downy mildew resistance and genetic variability in a wild rocket germplasm collection

Paula S. Coelho[1] | **João M. Reis**[2] | **Ana L. Pereira**[1] | **Aliana Vairinhos**[2] | **Violeta Lopes**[1] | **José M. Leitão**[2]

[1]INIAV – Instituto Nacional de Investigação Agrária e Veterinária, Av. da República, 2784-505 Oeiras, Portugal

[2]MED – Mediterranean Institute for Agriculture, Environment and Development & CHANGE – Global Change and Sustainability Institute, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Correspondence
Paula S. Coelho, INIAV – Instituto Nacional de Investigação Agrária e Veterinária, Av. da República, 2784-505 Oeiras, Portugal.
Email: paula.coelho@iniav.pt

José M. Leitão, MED – Mediterranean Institute for Agriculture, Environment and Development & CHANGE – Global Change and Sustainability Institute, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.
Email: jleitao@ualg.pt

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Abstract

One hundred accessions of a "core collection" of *Diplotaxis tenuifolia* (L.) DC. and *Eruca* spp. were screened at seedling stage for resistance to downy mildew. Accessions tested at the seedling stage were assigned to 0–6 interaction phenotypes. All cultivated rocket (*Eruca* spp.) accessions exhibited a resistant (R) response both in cotyledons and in young leaves. The wild rocket (*D. tenuifolia*) accessions exhibited higher susceptibility in cotyledons than in the 1st and 2nd leaves, with 16 and 47 accessions classified as resistant or partially resistant (PR) in the cotyledon and in leaves stages, respectively. Only three wild rocket accessions displayed an R phenotype in cotyledons and leaves. The most frequent response in cotyledons vs. leaves was the highly susceptible/susceptible (HS/S) combination (33 accessions), followed by the S/PR combination (18 accessions). A significant correlation ($r = 0.917$, $P < .000$) was observed between the disease index in cotyledons and leaves. The molecular markers analyses revealed a wide genetic distance between *Diplotaxis* and *Eruca*, which gather in two clearly separated species clusters. The molecular variability is accompanied by a wide diversity of interactions with the pathogen isolate. The closest similarities among *D. tenuifolia* accessions were found in accessions provided by the same breeding company. Future studies will be focused on two main objectives: (a) the assessment of the accessions behavior that have evidenced an R/R, S/PR, and HS/PR cotyledon and leaf response under greenhouse or field production and (b) the genome mapping of genetic features that provide downy mildew resistance.

Abbreviations: DI, disease severity index; HS, highly susceptible; ISSR, inter-simple sequence repeats; PCR, polymerase chain reaction; PR, partially resistant; R, resistant; RAPD, random amplified polymorphic DNA; S, susceptible; UPGMA, unweighted pair-group method with arithmetic averages.

1 | INTRODUCTION

The plants of genera *Diplotaxis* and *Eruca* of the Brassicaceae family are commonly known as "rocket." Among the four edible "rocket" species: *D. muralis* (L.) DC., *D. erucoides* (L.) DC., *D. tenuifolia* (L.) DC. (wild or perennial rocket), and *E. sativa* (L.) Cav. (salad or cultivated rocket), only the last two species are cultivated in large scale, boosted by the increasing demand for prepackaged and ready-to-eat salads (Bell & Wagstaff, 2014; Signore et al., 2020).

The most obvious morphologic traits that differentiate these two most cultivated rocket species are the yellow flowers and septate silique of *D. tenuifolia* vs. the white flowers and simple silique of the *Eruca* spp. (Figure 1). Nevertheless, these characteristics escape the observation of most growers due to the short cultural cycles until leaf production and commercialization.

Multiple aspects of the biology, crop management, nutritional properties, industrial processes, and uses of wild rocket (*D. tenuifolia*) can be consulted in a recently published review (Caruso et al., 2018).

Wild rocket leaves have a high content of fiber, iron, nutritionally important minerals, and antioxidant metabolites, such as ascorbic acid, flavonoids, and carotenoids (Cavaiuolo & Ferrante, 2014). Due to a high content of glucosinolates and release of volatile isothiocyanates, the wild rocket (*D. tenuifolia*) leaves have a specific spicy, strong, bitter taste, and a peculiar pungent aroma that determine the general consumer preference over the "cultivated" rocket [*E. sativa* (L.) Cav.] (Pasini et al., 2011). Additional comparative information on the phytochemical compounds and health-related metabolites produced by both species can be found in a recently published review article (Bell & Wagstaff, 2019).

Core Ideas

- 100 *D. tenuifolia* and *Eruca* spp. accessions were screened for downy mildew resistance at seedling stage.
- The wide genetic diversity of the collection was evidenced by RAPD and ISSR markers assessment.
- Closely clustered resistant accessions are useful for identification of resistance loci.
- The most resistant accessions were selected for further genomic studies.

The intensive greenhouse and field production of wild rocket promotes the emergence of downy mildew, an epidemic disease elicited by the oomycete H*yaloperonospora* spp. that badly affects the crop quality and often results in total production loss (Caruso et al., 2018).

This oomycete genus encompasses more than 100 species and affects economically important crops of the Brassicaceae family, particularly during the autumn and spring seasons when the infection occurs from wind-borne conidia, especially in the presence of high air humidity and mild temperature. Under the most favorable conditions for the development of the pathogen, high (90–98%) relative humidity and mild (15–20 ˚C) temperature, the parasite life cycle can be completed in 3–4 d. Under low humidity conditions, the disease can be difficult to recognize, but it becomes evident in the packaged product after an incubation period of 12 d. The whitish and dense conidiophores of *Hyaloperonospora* spp. usually grow on the abaxial side of leaves, but,

FIGURE 1 (a) Wild rocket (*Diplotaxis tenuifolia*) inflorescence (accession 7); (b) Cultivated rocket (*Eruca sativa*) inflorescence (accession 31)

in the most severe cases, they spread to both leaf surfaces (Garibaldi et al., 2004).

The *Hyaloperonospora* sp. that infects wild rocket (*D. tenuifolia*) is, apparently, specific to this species, and no cross-infection to other brassica species (including *Eruca* spp.) was observed so far in our assays (previous unpublished results, 2020, and this article).

General environmental concerns and stringent legislation regarding the use and registration of new pesticides, such as the one adopted in Europe (Erbach, 2012), imply the need for the development of new varieties resistant to pests and diseases that are less dependent on unfriendly environmental chemical products to ensure the production of healthy and safe food.

The assessment of the genetic diversity available for breeding of novel varieties, as well as the identification or the simple detection of genes conferring resistance to pests and diseases, is currently much more efficient due to the availability of modern molecular tools, including a large panoply of DNA markers and sequencing technologies.

Randomly amplified marker techniques, such as the inter-simple sequence repeats (ISSR) and random amplified polymorphic DNA (RAPD), have been largely used during the last three decades in multiple plant genetic studies for assessment of genetic relationships between individuals or populations and management of germplasm collections (Cabrita et al., 2014; Monte-Corvo et al., 2000; Rodrigues et al., 2014).

To the best of our knowledge, the use of molecular markers in *D. tenuifolia* studies is relatively scarce and restricted to the analysis of the relationships between this species and other species of the same or close genera.

Martín and Sánchez-Yélamo (2000) used ISSR markers to assess the genetic relationships among 10 *Diplotaxis* species, confirming the previously determined higher affinity between *D. tenuifolia*, *D. cretacea*, *D. simplex*, *D. viminea*, and *D. muralis* (Martínez-Laborde & Gómez-Campo, 1998), which was reconfirmed based on seed image analysis (Grillo et al., 2012). In combination with isozyme analysis, Eschmann-Grupe et al. (2003) used RAPD markers to study the genetic relationships among 19 *Diplotaxis* species, observing once again the closer clustering of the above mentioned five species, which supported the hypothesis of the maternal parent and the very likely paternal parent of the amphidiploid *D. muralis* (L.) DC. ($n = 21$) to be, respectively, *D. viminea* ($n = 10$) and *D. tenuifolia* (L.) DC. ($n = 11$).

As far as we know, no research data have been published regarding the assessment of the genetic diversity within wild rocket (*D. tenuifolia* L.) germplasm collections nor any evaluation of the accessions for their plant—*Hyaloperonospora* interaction.

During the last few years, a wild rocket germplasm collection has been established at the Instituto Nacional de Investigação Agrária e Veterinária (Portugal) and by gathering accessions from seed banks (Australia and Germany), breeding companies (from France, Italy, Netherlands, Spain, United States, and United Kingdom), local trade, and local collecting missions.

TABLE 1 Analyzed *Diplotaxis tenuifolia* (L.) DC. and *Eruca* spp. accessions

Accession origin	Country of origin	Number of accessions
Australian Grains Genebank	Australia	1
Leibniz Institute	Germany	4
Breeding companies	FR, IT, NL, SP, USA, UK	83
Local trade	Portugal	10
Collecting missions	Portugal	2
Total		100

The main specific objective of the present work is the evaluation of the host–pathogen interaction between 100 accessions of this novel wild rocket (*D. tenuifolia* L.) germplasm collection and the *Hyaloperonospora* sp. isolate D5, while assessing the molecular diversity and genetic relatedness of the evaluated accessions.

2 | MATERIALS AND METHODS

2.1 | Plant germplasm accessions

A set of 100 wild and cultivated rocket accessions was selected to test the resistance to downy mildew. The origin of the accessions is very diverse: seed banks, plant seed companies, local markets, and collecting missions in mainland Portugal (Table 1; Supplemental Table S1). An inventory was performed, reuniting the available information about the accessions and being coded to protect commercially sensitive information.

2.2 | Downy mildew screening methodology

2.2.1 | Plant growing conditions

The seeds were sown in plastic trays (3 × 3 × 5-cm cells) containing a peat-based compost (Gramoflor GmbH & Co. KG), covered with a layer of vermiculite and watered by capillary matting. The trays were placed in a growth chamber with a long-day lighting (19 h light, 5 h dark), 21 °C daytime and 19 °C nighttime temperatures, and 70 ± 10% relative humidity. The photoperiod was provided by LED lamps (LED ECOT814330F 14 W 4000K, ROBLAN) at an intensity of 250 μmol m^{-2} s^{-1}. Fourteen-day-old plants were tested in relation to the phenotypic response of the wild

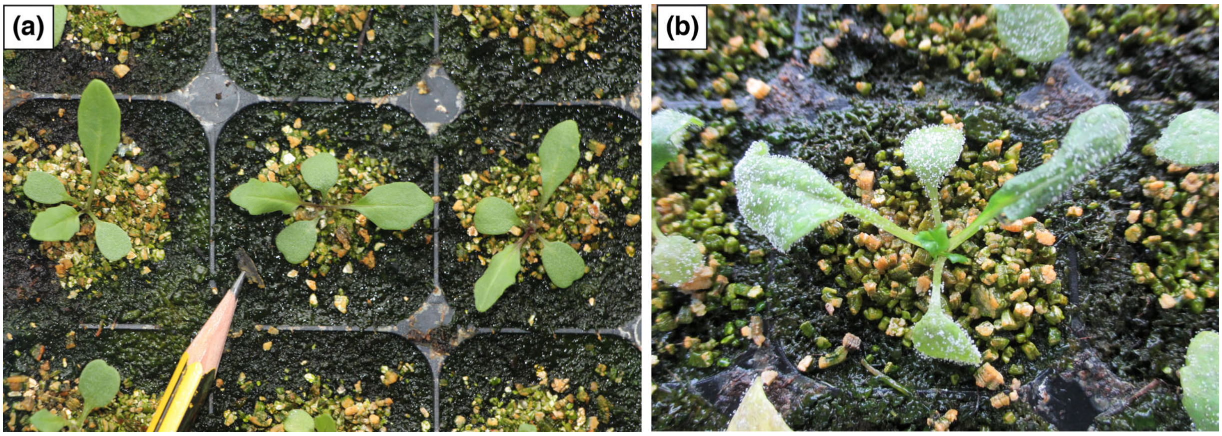

FIGURE 2 (a) A 14-d-old wild rocket (*Diplotaxis tenuifolia*) plant before being infected with *Hyaloperonospora* sp. pathogen; (b) A 21-d-old wild rocket (*Diplotaxis tenuifolia*) plant completely infected with *Hyaloperonospora* sp. isolate D5

rocket—*Hyaloperonospora* interaction following a standard protocol.

2.2.2 | *Hyaloperonospora* sp. isolate

The plants were tested using the *Hyaloperonospora* sp. isolate D5, collected in 2019 from field plants of a *D. tenuifolia*-specific commercial variety produced under organic farming conditions in Odemira (Vitacress Portugal).

In the laboratory, the initial field isolate was cleaned after some transfers into susceptible plants, and the noncontaminated isolate D5 was stored at −18 ˚C in infected wild rocket cotyledons.

2.2.3 | Preparation of the inoculum and plant inoculation

To produce the inoculum for screening experiments, recently sporulated cotyledons of the susceptible control infected with the isolate D5 were immersed and shaken into distilled water. The spore suspension was filtered through two layers of muslin to remove mycelial fragments, and the conidia were counted with a haemocytometer to a 50–75 × 10^3 conidia ml^{-1} final spore concentration.

Fourteen-d-old plants were inoculated using a handheld sprayer with the freshly prepared spore suspension. Following an incubation period of 24 h (dark, 16 ˚C, and 100% relative humidity), plants were placed in a growth chamber for 5 d for pathogen development under the above described conditions. Finally, the plants were incubated again for 24 h to induce *Hyaloperonospora* sporulation, and the 21-d-old plants were individually scored for downy mildew infection (Figure 2). The tests were carried out in three independent replicates.

TABLE 2 Interaction–phenotype (IP) classes used to evaluate downy mildew resistance of germplasm collection at 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 five 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

Note. The accessions were separated into four phenotypic categories: 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).

2.2.4 | Disease assessment and data analysis

The disease symptoms in cotyledons and in first two leaves of each plant were individually evaluated using a visual scale of 0–6 interaction–phenotype classes, taking into account the host response and the asexual sporulation of the pathogen, as detailed in Table 2.

The disease severity index (DI) was calculated for cotyledon and leaves in each accession, and the plant–pathogen phenotype was classified in four categories according to the DI values: 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). The correlation of the DI values in cotyledons and in leaves were assessed using Statistica version 12, and the relative significance of the *P*-values ($P < .05$) were determined.

2.3 | Molecular characterization of the accessions

2.3.1 | DNA extraction and evaluation

The genomic DNA extraction was performed as described in Farinhó et al. (2004) with minor modifications. One leaf from five plants of each accession was removed, washed with tap water, and wiped up with paper. The central nervures were removed using a scalpel, and the leaves were ground under liquid nitrogen in a mortar with a pestle. The obtained fine powder was transferred to a microfuge tube containing 500 µl of extraction buffer (250 mM Tris-HCL, pH 8.0, 25 mM EDTA, 1% SDS) until the final volume of the suspension reached approximately 750 µl. Then, RNase A (20 µg ml^{-1}) was added to the tube, and then transferred to a water bath at 65 ˚C for 15 min. Already at room temperature, one volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the tube, which after successive inversions for 1 min, was centrifuged at 13,000 rpm for 3 min. The upper phase was transferred to a new tube and extracted with 1 volume of chloroform/isoamyl alcohol (24:1), as described in the previous step. This second extraction was repeated once or twice, until the interphase appeared completely transparent. The final upper phase was transferred to another tube and precipitated with three volumes of cold absolute ethanol and kept at −20 ˚C. After centrifugation at 13,000 rpm, the DNA pellet was dried and resuspended in $TE_{0.1}$(10 mM Tris, 0.1 mM EDTA). The integrity of the extracted DNA and eventual contamination with RNA was assessed by agarose gel (1.4%) electrophoresis. The DNA concentration was determined by spectrophotometry (NanoDrop One, Thermofisher) after a previous comparison via agarose gel electrophoresis with different amounts of DNA extracted from *Pisum sativum* roots, which do not contain chlorophyll or other pigments that could bias the spectrophotometry results.

2.3.2 | RAPD-PCR analysis

The RAPD-polymerase chain reaction (PCR) amplifications were performed as described in Elisiário et al. (1999), with minor modifications, in 15 µl final volume reaction mixes containing: 10 ng genomic DNA, 1× gel load reaction buffer, 0.16 mM dNTPs, 0.6 U NZYTaq DNA polymerase, and 1.33 µM of each (single) primer. All RAPD primers used in this study were synthesized by Operon Technologies Inc. (Qiagen).

The PCR reactions were carried out in a Biometra UNO II (Thermoblock, Biotron) thermocycler according to the protocol: initial denaturation at 94 ˚C for 1 min and 30 sec, followed by 35 cycles of 30 sec at 94 ˚C, 30 sec at 36 ˚C, 1 min at 72 ˚C, and a final cycle of 10 min at 72 ˚C.

The amplification products were then analyzed by agarose gel (2%) electrophoresis. The gels were immersed into an ethidium bromide solution, transferred to water for diffusion of the stain from the gel, and photographed under ultraviolet transillumination with a digital camera (Canon EOS 1300D, Canon). The tested and selected RAPD primers are displayed in Table 3.

2.3.3 | ISSR analysis

The ISSR amplifications were performed in the same thermocycler using a protocol identical to that used for RAPD markers amplification, except for the annealing temperatures that varied from primer to primer depending on the estimated specific melting temperature.

The sequences of the tested and selected ISSR primers are registered in Table 4. All primers are degenerated by the combination of two primers. As commonly accepted, the annotation “R” stands for purines (A or G) and “Y” for pyrimidines (T or C).

2.3.4 | Data analysis

Only clear and distinct amplified bands were scored as 1 for presence and 0 for absence in the electrophoresis gels and included in the data binary matrix.

The Numerical Taxonomy System of Multivariable program (Rohlf, 2009) was used for cluster analysis. The genetic similarity between the accessions was reckoned by pairwise comparisons based on the percentage of common fragments using the coefficient DICE (Nei & Li, 1979), according to the following equation: similarity = $2N_{ab}/(N_a+N_b)$, where N_{ab} is the number of scored amplification products simultaneously present in accessions ‘a’ and ‘b’; N_a is the number of amplification products scored in accession ‘a’; and N_b is the number of scored fragments in accession ‘b’. The unweighted pair-group method with arithmetic averages (UPGMA) was used to calculate the cophenetic matrix used for the dendrogram construction. The cophenetic correlation coefficient (*r*) was calculated by comparison of the similarity matrix with the UPGMA-produced cophenetic matrix.

3 | RESULTS

3.1 | Downy mildew evaluation of germplasm collection

The resistance at the cotyledons and first two leaves was evaluated on 21-d-old plants (Figure 2). At the cotyledons, 11 accessions were classified as R, 13 PR, 24 S, and 52 HS. At

TABLE 3 Tested and selected random amplified polymorphic DNA (RAPD) primers

Primer	Sequence	Primer	Sequence	Primer	Sequence
OPA02	TGCCGAGCTG	OPAL05	GACTGCGCCA	**OPAN05**	GGGTGCAGTT
OPA03	AGTCAGCCAC	**OPAL07**	CCGTCCATCC	OPAN14	AGCCGGGTAA
OPB03	CATCCCCCTG	OPAL12	CCCAGGCTAC	**OPAN16**	GTGTCGAGTC
OPB09	TGGGGGACTC	OPAL15	AGGGGACACC	OPAN18	TGTCCTGCGT
OPB20	GGACCCTTAC	**OPAL17**	CCGCAAGTGT	OPAN20	GAGTCCTCAC
OPD14	CTTCCCCAAG	OPAL19	TCTGCCAGTG	OPAO01	AAGACGACGG
OPD19	CTGGGGACTT	OPAM03	CTTCCCTGTG	OPAO02	AATCCGCTGG
OPE13	CCCGATTCGG	OPAM04	GAGGGACCTC	OPAO08	ACTGGCTCTC
OPF15	CCAGTACTCC	OPAM08	ACCACGAGTG	OPAO10	GACATCGTCC
OPU01	ACGGACGTCA	**OPAM13**	CACGGCACAA	OPAO12	TCCCGGTCTC
OPAK06	TCACGTCCCT	OPAM14	TGGTTGCGGA	OPAO13	CCCACAGGTG
OPAK12	AGTGTAGCCC	OPAM16	TGGCGGTTTG	OPAO20	GGCTTGCCTG
OPAK14	CTGTCATGCC	OPAM19	CCAGGTCTTC		
OPAK20	TGATGGCGTC	**OPAM20**	ACCAACCAGG		

Note. The seven primers selected for further collection analysis are in bold.

TABLE 4 Tested and selected inter-simple sequence repeats (ISSR) primers

Primer	Sequence	Tł annealing	Primer	Sequence	Tł annealing
DiploArb_01	(CA)$_8$RG	–	**DiploArb_05**	(GA)$_8$YG	60 °C
DiploArb_02	(CA)$_8$RY	–	**DiploArb_06**	(AG)$_8$YT	58 °C
DiploArb_03	(GA)$_8$YT	58 °C	DiploArb_07	(AG)$_8$YC	–
DiploArb_04	(GA)$_8$YC	60 °C	**DiploArb_08**	(AC)$_8$YT	58 °C

Note. The five primers selected for further collection analysis are in bold.

first and second leaves, 19 accessions were classified as R, 36 PR, 38 S, and 7 HS (Table 5; Supplemental Table S2).

All eight (one-asterisk labelled) commercial and breeding lines of cultivated rocket (*Eruca* spp.) were resistant at the cotyledons and leaves to the *Hyaloperonospora* sp. isolate D5 collected on wild rocket plants. Only three (double-asterisk labelled) wild rocket (*D. tenuifolia* L.) accessions were resistant, at the cotyledons and first leaves, to the assayed isolate (Table 5; Figure 3).

Generally, with the exception of accession 156 (5.0/5.1), all the wild rocket accessions were more susceptible in the cotyledons than in the 1st and 2nd leaves. The 11 accessions resistant in cotyledons (eight *Eruca* spp. and three *D. tenuifolia*) exhibited the same resistant response in leaves, while the 13 accessions partially resistant in cotyledons displayed a partially resistant (5) or a resistant (8) phenotype in leaves.

The most frequent response in cotyledons vs. leaves was the HS/S combination displayed by 33 accessions, followed by the S/PR combination exhibited by 18 accessions. Thirteen accessions were highly susceptible in cotyledons and partially resistant in leaves, and six accessions were highly susceptible in both cotyledons and in leaves (Figure 3).

It is worth mentioning that no accessions were observed to be susceptible or highly susceptible in cotyledons and resistant in leaves. Twenty-four accessions showed a promising resistant or partially resistant response to downy mildew in cotyledons and in first leaves. A significant correlation (r = 0.917, $P < .000$, N = 100) was observed between the DI values in cotyledons and in leaves (Figure 4).

3.2 | Molecular diversity

The previous selection among 40 RAPD and eight ISSR primers, suitable for amplification of a higher number of clear polymorphic markers, resulted in the retainment of seven RAPD primers (OPU01, OPAL07, OPAL17, OPAM13, OPAM20, OPAN05, and OPAN16) and five ISSR primers (DiploArb_03, DiploArb_04, DiploArb_05, DiploArb_06, and DiploArb_08) for further molecular analysis (Tables 3 and 4).

TABLE 5 Results of cotyledon and first two leaves (cotyledon/leaves) downy mildew assessment. Accession origin, disease index (DI), and phenotipic category of plant–pathogen interaction of the *Diplotaxis tenuifolia* and *Eruca* spp. accessions

		Cotyledon/leaves				Cotyledon/leaves	
Collection code	**Accession origin**	**Disease index**	**Phenotypic category**	**Collection code**	**Accession origin**	**Disease index**	**Phenotypic category**
1	BC	4.2/3.6	S/PR	59	BC	5.3/4.2	HS/S
3	BC	5.3/4.0	HS/PR	60	BC	4.8/3.5	S/PR
4	BC	5.4/4.6	HS/S	61	BC	5.3/4.1	HS/S
5	BC	5.1/3.9	HS/PR	62	BC	5.2/4.3	HS/S
6	BC	5.9/4.4	HS/S	63	BC	5.8/4.9	HS/S
7	BC	5.8/3.7	HS/PR	64	BC	5.1/4.4	HS/S
8	BC	5.4/3.3	HS/PR	65	BC	6.0/5.8	HS/HS
9	BC	5.6/4.4	HS/S	68*	BC	1.2/1.1	R/R
10	BC	5.3/3.2	HS/PR	69	BC	5.6/5.0	HS/S
12	BC	5.7/4.2	HS/S	71*	BC	1.3/1.0	R/R
15	BC	5.7/4.3	HS/S	72	BC	5.9/5.3	HS/HS
16	BC	4.1/2.8	S/PR	73	BC	5.5/5.0	HS/S
17	BC	5.2/3.6	HS/PR	74	BC	5.8/5.7	HS/HS
18	BC	3.3/2.4	PR/R	81	BC	4.9/3.9	S/PR
19	BC	4.8/3.5	S/PR	84	BC	4.8/3.0	S/PR
20	BC	5.2/5.2	HS/HS	91	IPK	5.4/3.8	HS/PR
21	BC	4.0/2.9	PR/PR	92	IPK	3.5/2.3	PR/R
22	BC	3.7/3.1	PR/PR	93	IPK	5.6/3.4	HS/PR
23	BC	3.1/2.7	PR/PR	97	IPK	4.8/2.6	S/PR
24	BC	4.8/3.8	S/PR	98	BC	5.4/4.3	HS/S
26	BC	4.0/3.4	PR/PR	99	BC	5.3/4.0	HS/PR
27	LT	5.5/5.0	HS/S	100	BC	5.4/4.4	HS/S
29*	LT	1.3/1.0	R/R	101	BC	5.8/5.0	HS/S
30*	LT	1.2/1.0	R/R	102	BC	5.1/3.9	HS/PR
31*	LT	1.1/1.0	R/R	103	BC	4.6/3.8	S/PR
32	LT	4.7/3.9	S/PR	104	LT	4.6/3.6	S/PR
33	BC	5.5/4.6	HS/S	105*	LT	1.3/1.1	R/R
35	BC	5.5/4.2	HS/S	106*	LT	1.1/1.0	R/R
36	BC	5.0/4.0	S/PR	134	CM	4.3/3.4	S/PR
37	BC	5.8/4.3	HS/S	146	AGG	6.0/5.8	HS/HS
38	BC	3.0/2.3	PR/R	151	CM	3.6/2.7	PR/PR
39	BC	3.4/2.3	PR/R	152	LT	5.0/5.0	S/S
40	BC	5.2/3.1	HS/PR	153*	LT	1.2/1.0	R/R
41	BC	5.0/4.1	S/S	154	BC	4.4/3.9	S/PR
42	BC	5.7/4.5	HS/S	155	BC	4.9/4.1	S/S
43	BC	5.4/4.2	HS/S	156	BC	5.0/5.1	S/HS
44	BC	5.4/3.5	HS/PR	157	BC	4.3/4.0	S/PR
45	BC	5.6/4.7	HS/S	158	BC	2.9/1.9	PR/R
46	BC	4.5/3.6	S/PR	159	BC	4.6/4.4	S/S
47	BC	5.4/4.6	HS/S	160	BC	4.2/3.1	S/PR
48	BC	5.6/4.8	HS/S	161	BC	5.2/4.9	HS/S
49	BC	5.6/4.8	HS/S	162	BC	3.5/2.4	PR/R
50	BC	5.2/4.3	HS/S	163	BC	3.2/1.8	PR/R

(Continues)

TABLE 5 (Continued)

Collection code	Accession origin	Cotyledon/leaves Disease index	Phenotypic category	Collection code	Accession origin	Cotyledon/leaves Disease index	Phenotypic category
51	BC	5.6/4.4	HS/S	164**	BC	2.2/1.7	R/R
52	BC	5.8/5.0	HS/S	165**	BC	2.0/1.6	R/R
54	BC	5.7/4.9	HS/S	166	BC	4.8/4.4	S/S
55	BC	5.7/5.3	HS/HS	167	BC	5.1/3.9	HS/PR
56**	BC	2.1/1.5	R/R	168	BC	4.4/3.1	S/PR
57	BC	5.1/4.4	HS/S	169	BC	5.1/5.0	HS/S
58	BC	5.5/4.3	HS/S	170	BC	3.3/2.3	PR/R

Note. Accession origin: BC = Breeding company, LT = Local trade, IPK = Leibniz Institute, CM = Collecting mission, AGG = Australian Grains Genebank. Accessions marked with asterisk indicate a resistant response in cotyledons and leaves for *Eruca* spp. with one asterisk and *Diplotaxis tenuifolia* with two asterisks, respectively. The accessions were classified in four phenotypic categories at cotyledon and 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).

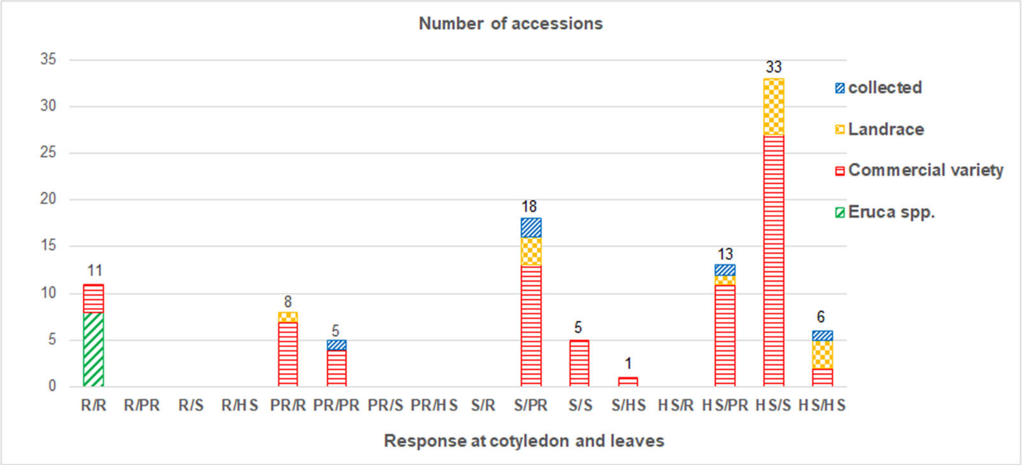

FIGURE 3 Distribution of the 100 accessions inoculated with *Hyaloperonospora* sp. isolate D5, according to their downy mildew response at cotyledon and leaves (R, Resistant; PR, Partially resistant; S, Susceptible; HS, Highly susceptible) and type of accession

The molecular analysis of the 100 accessions with these 12 primers allowed the amplification of clear and easy-to-score 62 RAPD and 48 ISSR markers. The RAPD primers amplified an average number of ∼8.9 scored markers per primer, which was relatively less than the ∼9.6 average markers amplified by the ISSR primers (Figures 5 and 6).

The UPGMA analysis of the genetic similarity data resulted in a cophenetic matrix graphically displayed as a dendrogram (Figure 7), which exhibits a strong correlation (r = 0.99598) with the initial genetic similarity matrix (Supplemental Table S3). The first clear result of these analyses is the presence in the dendrogram of two main clusters: a major cluster (A) that gathers all *D. tenuifolia* accessions, and a second major cluster (B) that reunites all *Eruca* spp. accessions. This main discrimination confirms the rightness of information about the supplied seed accessions, while highlighting the rightness of the obtained host–pathogen interaction results and the accuracy of the performed DNA markers analyses.

As expected, the lowest similarity value (0.126) was found between a *D. tenuifolia* accession (no. 156) and an *E. sativa* accession (no. 30). The lowest similarity value among the *Diplotaxis* accessions (0.697) was observed between accessions 23 and 92. Together with accession 93, these three accessions showed the lowest similarity with several accessions of the same species.

The closest similarities among *D. tenuifolia* accessions were found among accessions 44 and 42 (0.994) and 44 and 43 (0.994). These three accessions are gathered in a tight cluster that also includes accessions 45 and 46, closely linked to accessions 6, 40, and 41. It is worth mentioning that all the referred accessions were provided by the same breeding company. Another example of closer accessions clustering

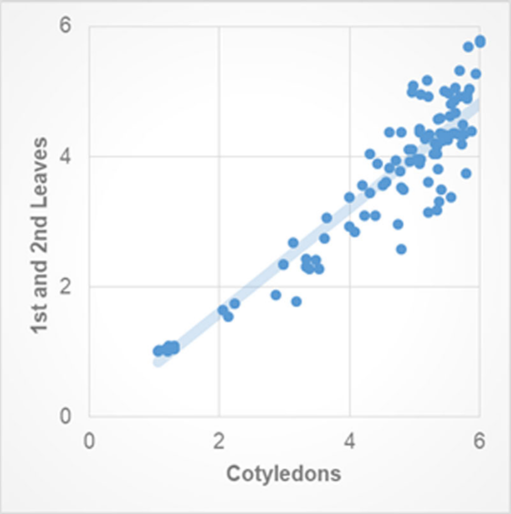

FIGURE 4 Correlation between the disease index of cotyledons and first two leaves in the 100 accessions inoculated with *Hyaloperonospora* sp. isolate D5 (r = 0.917, $P < .000$, N = 100)

provided by the same donor is the one of accessions 8, 10, 17, and 19, which, unlike other two accessions (no. 20 and 57) in the same cluster, are partially resistant to downy mildew.

The *Eruca* spp. accessions exhibit a wide molecular diversity with genetic similarity values that vary from 0.914 (no. 30 and 31) to 0.605 (no. 31 and 68). The low genetic similarity among some *Eruca* accessions needs to be further scrutinized in more detail.

4 | DISCUSSION

With the exception of the *Eruca* accessions, which were all resistant, there was no clear relationship between the downy mildew resistance of the accessions and their origin. Commercial varieties and landraces of *D. tenuifolia* showed different resistance behaviors in cotyledons and in leaves. Regarding the five accessions from germplasm banks, the accession 146 from Australian Grains Genebank was highly susceptible in cotyledons and leaves. The four accessions from Leibniz Institute were partially resistant (no. 92), susceptible (no. 97), and highly susceptible (no. 91 and 93) in the cotyledons and resistant (no. 92) and partially resistant (no. 91, 93, and 97) in the leaves. Accessions 134 and 151 collected in mainland Portugal showed a susceptible (no. 134) and partially resistant (no. 151) response in the cotyledons and a partially resistant reaction in the leaves.

The molecular markers analyses have evidenced a large genetic variability among the germplasm accessions tested for plant–pathogen interaction. The accessions 42, 43, and 44 were the most similar and very closely gathered to accessions 45 and 46 and slightly more apart from accessions 40 and 41. The fact that these accessions were provided by the same company confirm the accuracy of the performed molecular analyses. Although most of these accessions exhibited a susceptible phenotypic interaction with the *Hyaloperonospora* isolate D5, the accessions 40, 44, and 46 displayed a partially resistant phenotype. The accessions 8, 10, 17, and 19 constitute a second case of close clustered partially resistant accessions that are provided by the same breeding company.

In the above two cases of close clustering of partially resistant accessions provided by the same donor, we can establish as a working hypothesis the possible sharing of the same resistance locus among the partially resistant accessions provided by the same company. This hypothesis will be taken into consideration in our further attempt of mapping and identification of the downy mildew resistance genes.

The lowest similarity value among the *Diplotaxis* accessions (0.697) was observed between accessions 23 and 92. Together with accession 93, these three accessions showed the lowest similarity with several *Diplotaxis* accessions. An additional comparative analysis focusing on these three accessions needs to be carried out for a more detailed morphological and molecular characterization, in the last case using different types of Sequence Tagged Site markers and genotyping by sequencing.

The *Eruca* spp. accessions exhibit a wide diversity with genetic similarity values that vary from 0.914 (accession 30 vs. 31) to 0.605 (accession 31 vs. 68). The low genetic similarity among some *Eruca* accessions, particularly the low similarity showed by accession 68 and the remaining accessions of the same genus, needs also to be further scrutinized in more detail. However, this is out of the scope of the present study.

All above described trials for establishment of the host–pathogen interactions among 100 wild and cultivated rocket (*D. tenuifolia* and *Eruca* spp.) accessions and the pathogenic *Hyaloperonospora* sp. isolate D5 were performed in growth chambers. However, with regard to the accessions that have evidenced more auspicious cotyledon and leaves responses: R/R, S/PR, and HS/PR, it would be interesting to evaluate the behavior of these accessions under common uncontrolled greenhouse or field farmers' production conditions.

In a so far carried out preliminary study, 19 accessions have been already tested under natural infection in field production conditions. Significant correlations were found between the downy mildew response in growth chambers and the behavior of the adult plants of the same accessions in field trials. The field trials need to be repeated with more accessions and the populations of pathogens that infected the plants in the different tests need to be compared to establish their genetic similarity.

In a previous study with *B. oleracea*, in which the response to downy mildew in cotyledons and adult plants was

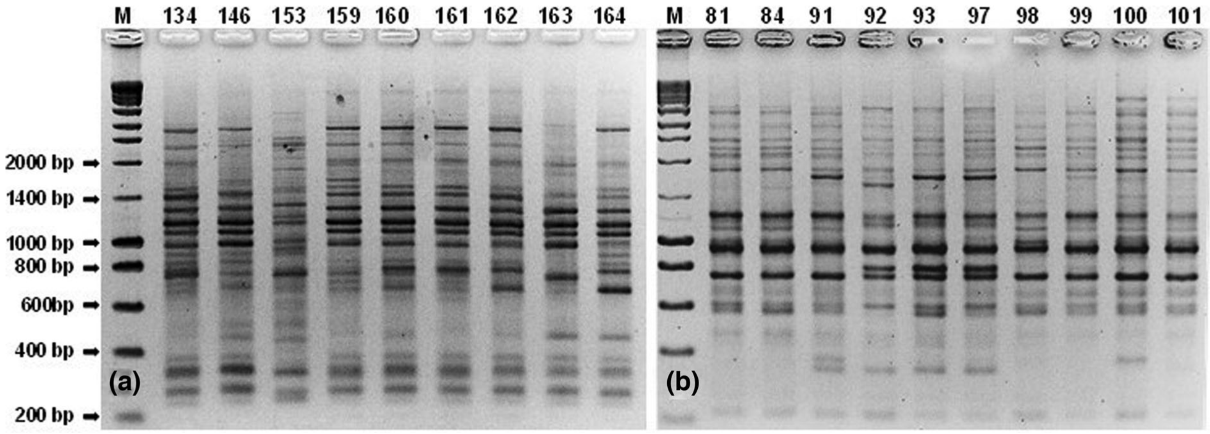

FIGURE 5 Random amplified polymorphic DNA (RAPD) amplification of *Diplotaxis tenuifolia and Eruca* spp. accessions with Operon Technologies primers (a) OPU01 and (b) OPAN16. Notice the very different amplification pattern of the *Eruca* sp. accession 153

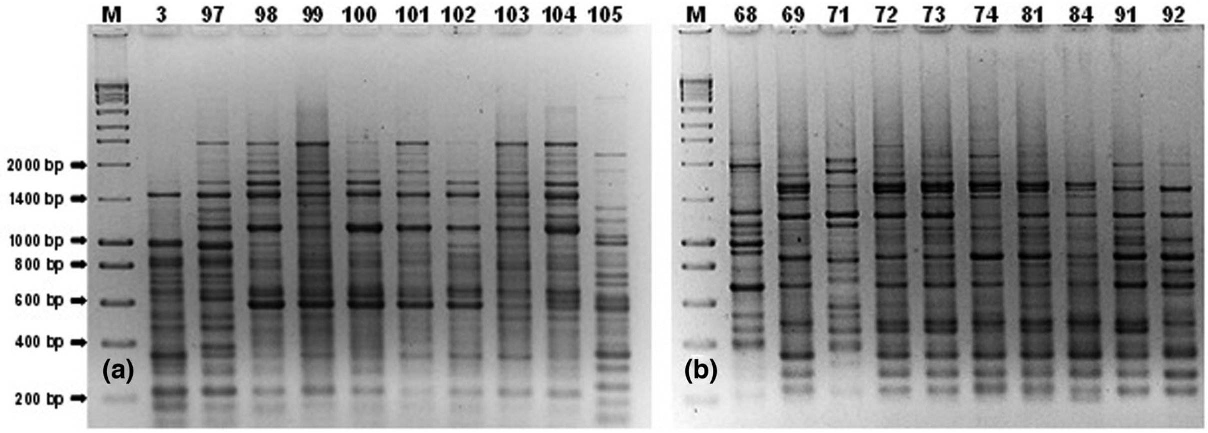

FIGURE 6 Inter-simple sequence repeats (ISSR) amplification of *Diplotaxis tenuifolia* and *Eruca* spp. accessions with primers (a) DiploArb_04 and (b) DiploArb_03. Notice the very different amplification patterns of the *Eruca* spp. accessions 68, 71, and 105

compared, the resistance increased with plant growth and some accessions, even, very susceptible at cotyledon stage exhibited moderate or high resistance as field adult plants (Coelho et al., 1998). In fact, in *Brassica oleracea* the downy mildew resistance at cotyledon and adult-plant stage in Couve Algarvia cabbage was found to be under the control of two different genetic systems (Monteiro et al., 2005).

The present work opens the way towards two main lines of study. The first line aims to confirm, under field production conditions, the downy mildew resistance identified in some accessions under controlled environmental conditions. The second line of study aims to the genome mapping and further identification of the genetic features that provide the downy mildew resistance exhibited by some accessions. It would also be interesting to test the resistance of *Eruca* spp. to *Hyaloperonospora* isolates from other hosts, namely with isolates from the genus *Eruca* spp.

Based on our previous experience in the genome localization of a downy mildew resistance gene in *Brassica oleracea* (Carlier et al., 2004, 2011, 2012, 2016; Farinhó et al., 2004, 2007), the first crosses between highly resistant and highly susceptible wild rocket accessions have been already performed to obtain the necessary mapping populations. The F_1 plants have been already characterized for their interaction with the *Hyaloperonospora* isolate D5 and self-pollinated to generate the F_2 progenies. Because the accessions selected for initial progenitors are relatively heterozygous, both generations (F_1 and F_2) may be used for the identification of markers linked to the molecular features, most likely quantitative trait loci that determine the exhibited resistances.

In conclusion, the molecular analyses revealed a wide genetic diversity within accessions of both *Diplotaxis* and *Eruca* and the absence of repeated genotypes. The genetic diversity was accompanied by a wide diversity of interactions

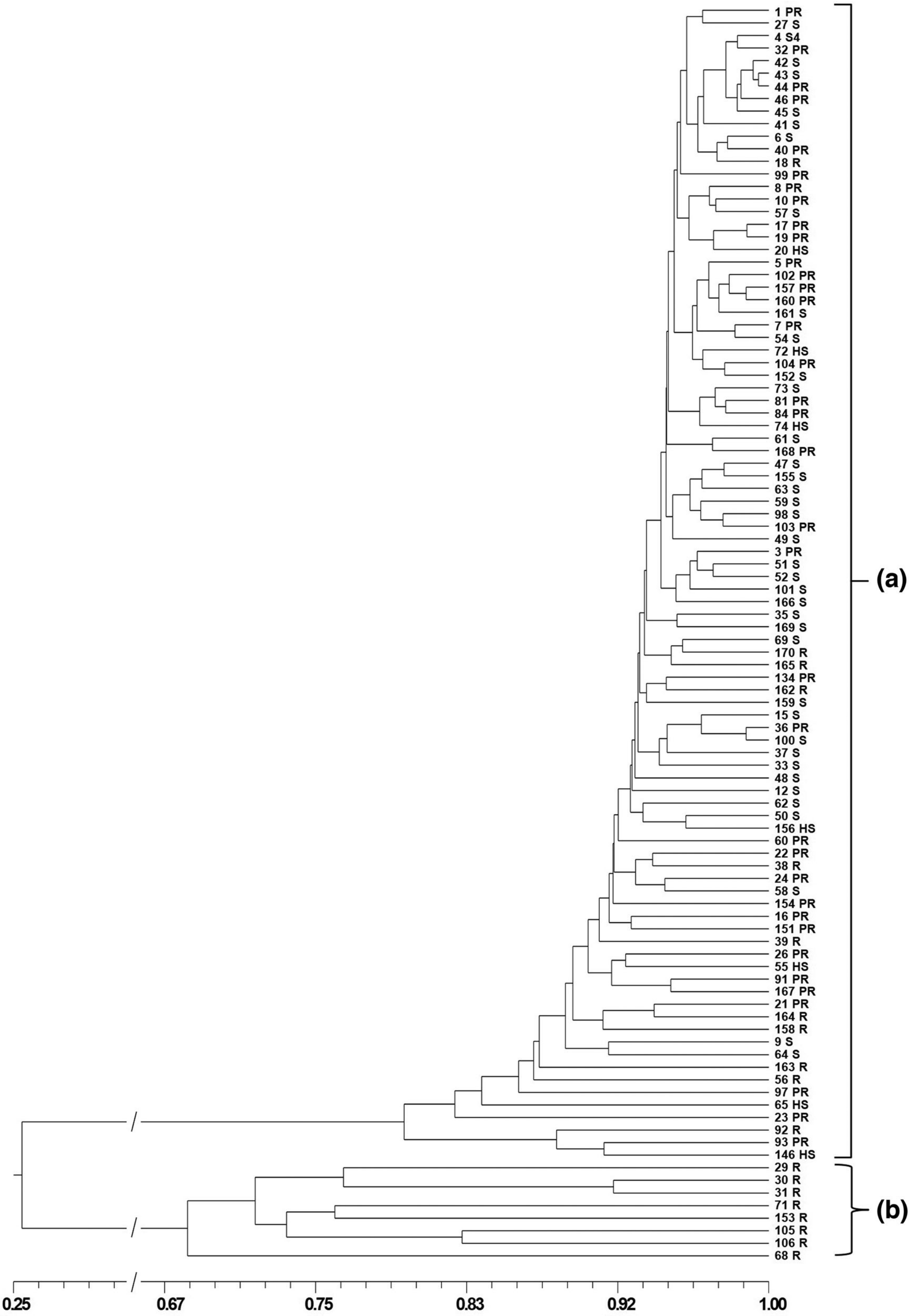

FIGURE 7 Dendrogram resulting from the unweighted pair-group method with arithmetic averages (UPGMA) analysis of the genetic relationships assessed by random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers, using the coefficient of similarity DICE, of 100 *Diplotaxis tenuifolia* and *Eruca* spp. accessions evaluated for their interaction with the pathogenic *Hyaloperonospora* sp. isolate D5. The letters, following the accession number, refer to the phenotypic classification of resistance assessed on the first two leaves

with the *Hyaloperonospora* sp. isolate D5, which showed to be nonpathogenic to the *Eruca* spp. accessions.

AUTHOR CONTRIBUTIONS

Paula Coelho: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing. João Reis: Formal analysis, Investigation. Ana Pereira: Investigation. Aliana Vairinhos: Investigation. Violeta Lopes: Data curation. José Leitão: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. All authors have read and agreed with the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

ORCID

Paula S. Coelho https://orcid.org/0000-0002-7133-998X

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SUPPORTING INFORMATION

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