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1 Seed transmission of *Plectosphaerella cucumerina* of *Diplotaxis tenuifolia*

2

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9 Abstract Eight seed samples of wild rocket (Diplotaxis tenuifolia) obtained from commercial seed lots used for sowing by farms severely affected by Plectosphaerella cucumerina, were assayed for 10 11 the presence of the pathogen. Isolations were carried out on subsamples of seeds (400) unwashed or disinfected in 1% sodium hypochloride. The pathogenicity of the isolates of P. cucumerina obtained 12 13 was tested in two trials carried out on wild rocket. Four out of eight samples of rocket seeds were contaminated by P. cucumerina. Among the eleven isolates of P. cucumerina obtained from 14,400 14 disinfected or not disinfected seeds tested, none was isolated from disinfected seeds. All isolates 15 were pathogenic on wild rocket. The results obtained indicate that rocket seeds are a potential 16 source of inoculum for P. cucumerina. The possibility of isolating the pathogen from seeds, 17 although from a low percent of them, supports the hypothesis that the rapid spread of this new 18 disease of rocket recently observed in Italy is due to the use of infected propagation material. 19 Measures for prevention and control of the disease are discussed. 20

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22 Key words wild rocket, seed-borne pathogen, seed disinfection

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24 Introduction

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Wild rocket (Diplotaxis tenuifolia) is now widely cultivated and increasingly used in the 26 mediterranean cuisine both as a component of mixed salad and to decorate dishes. During spring 27 2012, symptoms of an unusual leaf spot disease were observed in several commercial greenhouses 28 near Salerno (southern Italy) and in northern Italy (near Bergamo) on plants of Diplotaxis tenuifolia 29 (cv. Selvatica). The first symptoms on leaves of affected plants consisted of small (1 mm) black-30 brown spots of irregular shape, later coalescing into larger spots, 1 cm diameter. Spots were 31 surrounded by a yellow-gray halo, and were mostly located on the foliar limb, rib and petiole. 32 Affected leaves were often distorted, appearing hook-like. The disease was severe under 75-90% 33

RH, at air temperature of 20-26 °C, and caused severe production losses. Particularly, affected tissues rotted quickly after packaging, during transit and commercialization of processed rocket (Garibaldi *et al.*, 2012). The same pathogen is associated with root and collar rots of horticultural crops in Italy (Matta and Garibaldi, 1980; Carlucci *et al.*, 2012) and has been very recently observed on endive (Garibaldi *et al.*, 2013). On wild rocket (*D. tenuifolia*), the disease was not yet reported in other countries.

40 Circumstantial evidence from surveys in the area interested by the disease suggested that the 41 sudden appearance of this disease was possibly due to the transmission of the pathogen by seeds.

The present study was undertaken to ascertain the extent of and the variation in occurrence of *P*.
 cucumerina in rocket seeds.

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45 Materials and methods

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47 Seed infection evaluation Two seed samples of Diplotaxis tenuifolia were obtained from the 48 commercial farm were the disease was first observed (Salerno) and six seed lots were obtained from 49 commercial farms located in Lombardy, where the disease was later observed (Gilardi *et al.*, 2012). 50 A total of eight seed samples were assayed for the presence of *Plectosphaerella cucumerina* (Table 51 1).

52 Subsamples represented by 400 seeds were tested on 90 mm diameter Petri plates (10 seeds/plate) containing potato dextrose agar (PDA) added with streptomycin sulphate at 25 mg L⁻¹, by following 53 54 the method described by Mathur and Kongsdal (2003). Isolations were carried out on seeds only washed in distilled water (not disinfected) or disinfected by soaking for 1 min in 1 % sodium 55 hypochloride and dried. Plates were incubated at 12 h/day of fluorescent light at 22 °C for ten days. 56 Forty plates/trial were prepared. Each sample was checked at least twice. Seeds infected by P. 57 cucumerina were surrounded by a whitish-orange mycelium. The identification of the colonies of P. 58 cucumerina were confirmed by microscopic observation (Palm et al., 1995) and by molecular 59 analysis (Garibaldi et al., 2012). 60

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Isolates used and their preservation The isolates obtained from seeds were coded as reported under tables 3 - 5. Two strains of *P. cucumerina* from Salerno (southern Italy) (coded RS-CC1, GenBank Accession No. AB469880) and PLC-27 from Bergamo (northern Italy) respectively, were used as controls. The different strains were maintained on PDA at 8 °C.

66 Production of inoculum and pathogenicity test The different isolates of P. cucumerina were grown

in Petri plates on PDA added with 25 mg L^{-1} of streptomycin sulphate, incubated at 12 h/day of

fluorescent light at 23 °C for 7 days. Spore suspensions were prepared from the single isolates (Table 5). The concentration of spores was determined by hemacytometer and adjusted with deionized water to 1×10^6 CFU (colony forming units) ml⁻¹.

Seeds of *Diplotaxis tenuifolia* cv. Selvatica (Suba), previously disinfected by soaking for 1 min in 8 sodium hypochloride (disinfected) and washed in distilled water were sown in a steamed soil 8 mixture [with steamed mix soil of 50% Tecno2 (70% white peat and 30% clay) and 50% of Tiesse3 8 (60% white peat, 20% clay 20% perlite), Turco Silvestro terricci, Bastia d'Albenga, SV] in 2 L pots 8 and maintained at 25°C, with 12 hours/day of fluorescent light. Three replicates were used. Each 8 replicate consisted of 10-15 plants.

Thirty-day-old plants were artificially inoculated by spraying with a spore suspension $(1x10^6 \text{ CFU} \text{ ml}^{-1})$ of the different isolates. As comparison, the isolates of *P. cucumerina* coded RS-CC1 and PLC-27 obtained from wild rocket in Salerno and in Lombardy, respectively were used. Control plants were sprayed with water.

Typical symptoms of *P. cucumerina* started to be visible 8 days after artificial inoculation. Plants were checked for disease development and the percent of infected leaves was evaluated. The data are expressed as percent of infected leaves 15 days after the artificial inoculation (Table 5). *P. cucumerina* was consistently reisolated from the lesions. Data were statistically processed by means of variance analysis ANOVA and Tukey test (p<0.05).

86

87 **Results and discussion**

88

Four out of eight samples of wild rocket seeds, used for sowing in farms severely affected by *P. cucumerina*, were contaminated by the pathogen (Tables 3 and 4) and eleven isolates were obtained out of 7,200 not disinfected seeds. From disinfected seeds it was not possible to isolate any strain of *P. cucumerina*.

Eleven isolates of *P. cucumerina* obtained from the different seed lots, were coded (Tables 3 and 4), maintained in culture and tested in two trials for their pathogenicity on *D. tenuifolia*. The two trials provided consistent results. All eleven isolates obtained from seeds were pathogenic on the cultivar Selvatica (Table 5); inoculated plants showed typical symptoms. The virulence of the isolates obtained from seeds was similar to that of isolates obtained in the field from infected plants of *D. tenuifolia*.

99 The recent outbreak of *P. cucumerina* on wild rocket represents a potential threat to rocket 100 production in Italy. The disease has been detected on wild rocket, widely grown for processing. Identifying the primary source of inoculum is of critical importance for effective diseasemanagement.

103 This paper provides evidence that *P. cucumerina*, is frequently seed-transmitted (four 104 samples out of eight were contaminated), which suggests that seeds may be important in 105 disseminating the pathogen.

The results of this study do not provide information on the effects of *P. cucumerina* on the quality and germination ability of rocket seeds. The results of this study indicate that rocket seeds are a potential source of inoculum for development of *P. cucumerina*. The fast spreading of the disease that occurred first in southern Italy in 2012, fastly moving in a few months to northern Italy (Gilardi *et al.*, 2012) permits to hypothesize that the pathogen was introduced in Italy through infected seeds.

Further research should be carried out to determine the epidemiological significance of seed-112 113 borne inoculum as well as efficient methods to eliminate this threat to rocket production. The use of P. cucumerina-free certified propagation material will become an essential qualification to 114 115 worldwide distribution of this crop. Seed dressing with registered and effective fungicides should also represent one more option for disease management. Such treatments should also take into 116 consideration the possible contamination of rocket seeds, as already reported, by Fusarium wilt 117 agents (Garibaldi et al., 2004). The fact that no isolates were obtained from disinfected seeds allows 118 to speculate that the pathogen is an external contaminant of seeds. In such a case, seed disinfection 119 should help reducing the dissemination of the pathogen. Beside the use of chemicals, also other 120 control methods should be exploited: a method based on the use of aerated steam, which proved 121 effective in the control of seed-borne diseases of cereals (Forsberg et al., 2005) and of legumes will 122 be tested, as well as the use of biocontrol agents and natural products (Tinivella et al., 2009). 123

Since the conventional pathogen detection techniques may lack the sensitivity required to 124 detect seed-borne pathogens, the detection treshold of P. cucumerina in rocket seeds could be 125 increased by using molecular techniques, such as PCR and RAPD as already shown in the case of 126 Fusarium wilt of basil (Chiocchetti et al., 2001), lettuce (Pasquali et al., 2007; Mbofung and Pryor, 127 128 2010) and other vegetables (Lievens et al., 2012) and in the case of Phoma valerianellae in lamb's lettuce seeds (Pellegrino et al., 2010). Interestingly, it should be noticed that PCR and Real-Time 129 130 PCR methods have been already developed for the detection and quantification of P. cucumerina, 131 when used as biocontrol agent of potato cyst nematodes (*Globodera* spp.) (Atkins *et al.*, 2003).

132

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140 **References**

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Table 1 List of seed samples of Diplotax	tis tenuifolia tested
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0040/1	lot	Cultivar	Location of the farm	n Seed Company	
1A		Rucola selvatica	Salerno (NA)	Anseme (Cesen	a, FC)
2S		Rucola selvatica	Salerno (NA)	Suba (Longiano	o, FC)
71/CB		Winter	Bergamo (BG)	Orosem (Azzan	o S. Paolo, BG)
1387/2	2805PP	Charisma	Bergamo (BG)	Cora Seeds (Ma	rtorano, FO)
R 1020	033	Giove	Bergamo (BG)	T&T (Sant'Ann	a Di Chioggia, VE)
43/FR	C-7	Extra	Bergamo (BG)	Franchi Sement	i (Grassobbio, BG)
B1014	48	Venere	Bergamo (BG)	T&T (Sant'Ann	a Di Chioggia, VE)
31CM	-1	Summer	Bergamo (BG)	Orosem (Azzan	o S. Paolo, BG)
Table	2 List an	d layout of the trials	carried out		
Table Trial	2 List an Seed sam	d layout of the trials	carried out	Number of seeds	Date
Table Trial N.	2 List an Seed sam	d layout of the trials on the trials of the	carried out	Number of seeds evaluated,	Date
Table Trial N.	2 List an Seed sam	d layout of the trials on the trials of the	carried out	Number of seeds evaluated, disinfected (D)	Date
Table Trial N.	2 List an Seed sam	d layout of the trials on the trials of the	carried out	Number of seeds evaluated, disinfected (D) and not	Date
Table Trial N.	2 List an Seed sam	d layout of the trials on the trials of the	carried out	Number of seeds evaluated, disinfected (D) and not disinfected (ND)	Date
Table Trial N.	2 List an Seed sam	d layout of the trials on the trials of the	carried out	Number of seeds evaluated, disinfected (D) and not disinfected (ND) 400 D /400 ND	Date 13/06/2012
Table Trial N. 1 2	2 List an Seed sam 1A; 2S 1A; 2S	d layout of the trials on the trials of the	carried out	Number of seeds evaluated, disinfected (D) and not disinfected (ND) 400 D /400 ND 400 D /400 ND	Date 13/06/2012 24/07/2012
Table Trial N. 1 2 3	2 List an Seed sam 1A; 2S 1A; 2S 1A; 2S	d layout of the trials on the trials of the	carried out	Number of seeds evaluated, disinfected (D) and not disinfected (ND) 400 D /400 ND 400 D /400 ND 400 D /400 ND	Date 13/06/2012 24/07/2012 11/10/2012
Table Trial N. 1 2 3 4	2 List an Seed sam 1A; 2S 1A; 2S 1A; 2S Winter, 0	d layout of the trials on the trials of the	carried out	Number of seeds evaluated, disinfected (D) and not disinfected (ND) 400 D /400 ND 400 D /400 ND 400 D /400 ND 400 D /400 ND	Date 13/06/2012 24/07/2012 11/10/2012 25-29/07/2012

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Trial N.	Seed samples evaluated	Number of <i>P. cucumerina</i> colonies (isolate code) detected out of 400 seeds tested/sample		
		Not disinfected (ND)	Disinfected (D)	
1	28	3 (17RS2-ND; 18RS2-ND; 19RS2- ND);	0	
	1A	0	0	
2	28	2 (22RS2-ND; 15RS2-ND)	0	
	1A	1(6RS1-ND)	0	
3	28	2 (3RS2-ND; 4RS2-ND)	0	
	1A	0	0	
Total number of <i>P</i> . <i>cucumerina</i> colonies out of 4,800	28	7 (0.29%)	0 (0%)	
	1A	1 (0.04%)	0 (0%)	

Table 3 Evaluation of the presence of *P. cucumerina* from seed samples of wild rocket obtained

trom a farm at Salerno, the area of first detection of the d	disease
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Table 4 Evaluation of the presence of *P. cucumerina* from seed samples of wild rocket from the

207 Lombardy area

Trial N.	Seed sample	Number of <i>P. cucumerina</i> colonies (isolate code) detected out of 400 seeds tested/sample		
		Not disinfected (ND)	Disinfected (D)	
1	Summer Charisma,	0	0	
	Extra, Venere,			
	Giove,	1(6G-ND)	0	
	Winter	1 (27W-ND)	0	
2	Summer Charisma,	0	0	
	Extra, Venere,			
	Giove,	0	0	
	Winter	1(3W-ND)	0	
Total number of		3 (0.03%)	0 (0%)	
P. cucumerina				
colonies out of				
9,600				

Isolate code (from trial number)	Seed lot	Cv (Seed company)	% of infected leaves	
18 RS 2-ND (1)	2S	Selvatica (Suba)	40.0	bc ^y
19 RS 2-ND (1)	2S	Selvatica (Suba)	53.3	bc
20 RS 2-ND (1)	2S	Selvatica (Suba)	46.7	bc
22RS2-ND (2)	2S	Selvatica (Suba)	33.3	b
15RS2-ND (2)	2S	Selvatica (Suba)	63.3	c
6RS1-ND (2)	1A	Selvatica (Anseme)	43.3	bc
3RS2-ND (3)	2S	Selvatica (Suba)	56.7	bc
4RS2-ND (3)	2S	Selvatica (Suba)	33.3	b
6G-ND (4)	R 102033	Giove (T&T)	40.0	bc
27W-ND (4)	71/CB	Winter (Orosem)	56.7	bc
3W-ND (5)	71/CB	Winter (Orosem)	56.7	bc
RS-CC1 ^x	-	-	60.0	c
PLC-27 ^x	-	-	53.3	bc
Not inoculated control	-	_	0.0	a

Table 5 Virulence of different isolates of *P. cucumerina* from infected seeds, expressed as
 percentage of infected leaves 15 days after the artificial inoculation

^x Control strains, RS-CC1 and PL-C27, obtained from infected leaves of wild rocket

^y Means in the same column, followed by a common letter, do not differ significantly according to

214 Tukey's test (P<0.05).