Seed transmission of Plectosphaerella cucumerina, causal agent of leaf spot of Diplotaxis tenuifolia in Italy.

Seed transmission of *Plectosphaerella cucumerina*, causal agent of leaf spot of *Diplotaxis tenuifolia*

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Abstract *Plectosphaerella cucumerina* has been recently described as the causal agent of a leaf spot on wild rocket (*Diplotaxis tenuifolia*). Eight seed samples of wild rocket obtained from commercial seed lots used for sowing by farms severely affected by *P. cucumerina*, were assayed for 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 was tested in two trials carried out on wild rocket. Four out of eight samples of rocket seeds were contaminated by *P. cucumerina*. Eleven isolates of *P. cucumerina* were obtained from 7,200 disinfected seeds tested, while none was isolated from an equal number of disinfected seeds**.** All isolates were pathogenic on wild rocket. The results obtained indicate that rocket seeds are a potential source of inoculum for *P. cucumerina*. The possibility of isolating the pathogen from seeds, although from a low percent of them, supports the hypothesis that the rapid spread of this new disease of rocket recently observed in Italy is due to the use of infected propagation material. Measures for prevention and control of the disease are discussed.

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

Introduction

Wild rocket (*Diplotaxis tenuifolia*) is now widely cultivated and increasingly used in the mediterranean cuisine both as a component of mixed salad and to decorate dishes. During spring 2012, symptoms of an unusual leaf spot disease were observed in several commercial greenhouses near Salerno (southern Italy) and in northern Italy (near Bergamo) on plants of *Diplotaxis tenuifolia* (cv. Selvatica). The causal agent of the disease has been identified as *Plectosphaerella cucumerina* (Garibaldi *et al*. 2012). The first symptoms on leaves of affected plants consisted of small (1 mm)

black-brown spots of irregular shape, later coalescing into larger spots, 1 cm diameter. Spots were surrounded by a yellow–gray halo, and were mostly located on the foliar limb, rib and petiole. Affected leaves were often distorted, appearing hook-like. The disease was severe under 75-90% 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.

Circumstantial evidence from surveys in the area interested by the disease suggested that the 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.

Materials and methods

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

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 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 hypochloride and dried. Plates were incubated at 12 h/day of fluorescent light at 22 °C for ten days. Forty plates/trial were prepared. Each sample was checked at least twice. Seeds infected by *P. cucumerina* were surrounded by a whitish-orange mycelium. The identification of the colonies of *P. cucumerina* were confirmed by microscopic observation of hyaline elliptical and ovoid conidia born on phialides developed from a whitish-orange mycelium produced on PDA (Palm *et al*., 1995) and by analysis of internal transcribed spacer (ITS) region (Garibaldi *et al*., 2012).

Isolates used and their preservation The isolates obtained from seeds were coded as reported under tables 3 - 5. Two strains of *P. cucumerina,* obtained from infected leaves, 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.

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 $1x10^6$ CFU (colony forming units) ml^{-1}.

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

Thirty-day-old plants were artificially inoculated by spraying with a spore suspension ($1x10^6$ CFU 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 ($p<0.05$).

Results and discussion

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. Seed disinfection with sodium hypochlorite reduced seed infection to below detection level so that 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*.

The recent outbreak of *P. cucumerina* on wild rocket represents a potential threat to rocket 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 disease management.

This paper provides evidence that *P. cucumerina*, is frequently seed-transmitted (four samples out of eight were contaminated), which suggests that seeds may be important in 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-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 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 consideration the possible contamination of rocket seeds, as already reported, by Fusarium wilt agents (Garibaldi *et al*., 2004). The fact that no isolates were obtained from disinfected seeds allows to speculate that the pathogen is an external contaminant of seeds. In such a case, seed disinfection should help reducing the dissemination of the pathogen. Beside the use of chemicals, also other control methods should be exploited: a method based on the use of aerated steam, which proved effective in the control of seed-borne diseases of cereals (Forsberg *et al*., 2005) and of legumes will be tested, as well as the use of biocontrol agents and natural products (Tinivella *et al*., 2009).

Since the conventional pathogen detection techniques may lack the sensitivity required to detect seed-borne pathogens, the detection treshold of *P. cucumerina* in rocket seeds could be increased by using molecular techniques, such as PCR and RAPD as already shown in the case of Fusarium wilt of basil (Chiocchetti *et al*., 2001), lettuce (Pasquali *et al*., 2007; Mbofung and Pryor, 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

PCR methods have been already developed for the detection and quantification of *P. cucumerina*, when used as biocontrol agent of potato cyst nematodes (*Globodera* spp.) (Atkins *et al*., 2003).

ACKNOWLEDGEMENTS

Work carried out in the framework of the projects "Seed health: development of seed treatment methods, evidence for seed transmission and assessment of seed health (TESTA)", funded by the European Union Seventh Framework Programme (FP7/2007-2013) under Grant Agreement 311875 and "Pant and food biosecurity (PLANT FOOD SEC)", funded by the European Union Seventh Framework Programme (FP7/2007-2013) under Grant Agreement 261752.

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197 **Table 1** List of seed samples of *Diplotaxis tenuifolia* tested
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Code/lot	Cultivar	Location of the farm	Seed Company
1A	Rucola selvatica	Salerno	Anseme (Cesena)
2S	Rucola selvatica	Salerno	Suba (Longiano)
71/CB	Winter	Bergamo	Orosem (Azzano S. Paolo)
1387/2805PP	Charisma	Bergamo	Cora Seeds (Martorano)
R 102033	Giove	Bergamo	T&T (Sant'Anna Di Chioggia)
43/FRC-7	Extra	Bergamo	Franchi Sementi (Grassobbio)
B101448	Venere	Bergamo	T&T (Sant'Anna Di Chioggia)
31CM-1	Summer	Bergamo	Orosem (Azzano S. Paolo)

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202 **Table 2** List and layout of the trials carried out
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Trial N.	Seed samples evaluated	Number of seeds evaluated, disinfected (D) and not disinfected (ND)	Date
1	1A; 2S	400 D /400 ND	13/06/2012
2	1A; 2S	400 D /400 ND	24/07/2012
3	1A; 2S	400 D /400 ND	11/10/2012
4	Winter, Charisma, Giove, Extra, Venere, Summer	400 D /400 ND	25-29/07/2012
5	Winter, Charisma, Giove, Extra, Venere, Summer	400 D /400 ND	21-24/09/2012

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208 **Table 3** Evaluation of the presence of *Plectosphaerella cucumerina* from seed samples of wild
209 rocket obtained from a farm at Salerno, the area of first detection of the disease

Trial N.	Seed samples evaluated	Number of *P. cucumerina* colonies (isolate code) detected out of 400 seeds tested/sample	
		Not disinfected (ND)	Disinfected (D)
1	2S	3 (17RS2-ND; 18RS2-ND; 19RS2-ND)	0
	1A	0	0
2	2S	2 (22RS2-ND; 15RS2-ND)	0
	1A	1(6RS1-ND)	0
3	2S	2 (3RS2-ND; 4RS2-ND)	0
	1A	0	0
Total number of *P. cucumerina* colonies per seed sample	2S	7 out of 1200 seeds (0.58%)	0 out of 1200 seeds (0%)
	1A	1 out of 1200 seeds (0.08%)	0 out of 1200 seeds (0%)

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212 **Table 4** Evaluation of the presence of *Plectosphaerella cucumerina* from seed samples of wild
213 rocket from the Lombardy area

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Trial N.	Seed samples evaluated	Number of *P. cucumerina* colonies (isolate code) detected out of 400 seeds tested/sample	
		Not disinfected (ND)	Disinfected (D)
4	Summer	0	0
	Charisma	0	0
	Extra	0	0
	Venere	0	0
	Giove	1(6G-ND)	0
	Winter	1 (27W-ND)	0
5	Summer	0	0
	Charisma	0	0
	Extra,	0	0
	Venere	0	0
	Giove	0	0
	Winter	1(3W-ND)	0
Total number of *P. cucumerina* colonies per seed sample	Summer	0 out of 800 seeds (0.0%)	0 out of 800 seeds (0.0%)
	Charisma	0 out of 800 seeds (0.0%)	0 out of 800 seeds (0.0%)
	Extra	0 out of 800 seeds (0.0%)	0 out of 800 seeds (0.0%)
	Venere	0 out of 800 seeds (0.0%)	0 out of 800 seeds (0.0%)
	Giove	1 out of 800 seeds (0.13%)	0 out of 800 seeds (0.0%)
	Winter	2 out of 800 seeds (0.25%)	0 out of 800 seeds (0.0%)

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

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

[x] Control strains, RS-CC1and 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 Tukey's test ($P<0.05$).