Spread of pathogens through seeds

Occurrence of *Alternaria japonica* on seeds of wild and cultivated rocket

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Abstract

In vitro evaluation were carried out on seed samples of wild and cultivated rocket cultivars most frequently grown in Italy, obtained from farms affected by the leaf spot caused by *Alternaria japonica* in Piedmont and Lombardy during the fall of 2010. Twelve seed samples were collected and assayed for the presence of *A. japonica*. The pathogen was isolated only from not disinfected seeds. Among the two seed samples of cultivated rocket (*Eruca vesicaria*) only one was infected by *A. japonica* at a level of one infected seed out of 800. Four out of ten samples of wild (*Diplotaxis tenuifolia*) rocket seeds were contaminated by *A. japonica* with the highest level of infection detected in a single sample of 3 out of 800. All tested isolates of *A. japonica* obtained from seeds were pathogenic on both wild and cultivated rocket.

Key words: seed-borne pathogen; *Diplotaxis tenuifolia*; *Eruca vesicaria*; Alternaria black spot

Introduction

Wild [*Diplotaxis tenuifolia* (L.) D.C.] and cultivated [*Eruca vesicaria* (L.) Cav.] rocket are popular crops in Italy as well as in many Mediterranean areas, grown for fresh consumption as well as for dish decoration. The two crops have been interested during the past few years by many new soil-borne and foliar diseases, as a result of crop intensification (Gullino et al. 2014). During the fall-winter 2010-2011, extensive necrosis were observed on leaves of *D. tenuifolia* and of *E. vesicaria*, grown in commercial plastic-houses in Piedmont and Lombardy (northern Italy). The disease, new for Italy as well as for Europe, is caused by *Alternaria japonica* Yoshii (1941) and interested 30-40% of plants in the affected farms at temperatures ranging between 22 and 25°C and high relative humidity (R.H. >75%)(Garibaldi et al. 2011). The rapid spread of the disease in rocket cultivations suggested that the pathogen could be seed transmitted.

The present study was carried out in order to evaluate the contamination of rocket seeds by *Alternaria japonica* in order to consider the possible role of seed transmission in the epidemiology of the disease.

Material and methods

Seed infection evaluation

Seed samples of wild and cultivated rocket, belonging to the cultivars most frequently grown in Italy (Table 1), were obtained from farms affected by the disease in Piedmont and Lombardy during the fall of 2010. Twelve samples were collected and assayed for the presence of *Alternaria japonica* on Potato dextrose agar (PDA, Difco, Detroit, Michigan, USA) amended with 25 mgl^{-1} of streptomycin sulphate as described by Maude and Humpherson-Jones (1980). Subsamples represented by 400 seeds were tested on Petri plates (10 seeds/plate) in two trials. Isolations were

made from seeds either non disinfected or surface disinfected for 1 min in 1 % sodium hypochlorite, washed in sterile water for 5 min and dried under a sterile hood. The Petri dishes were incubated at 22°C in 12 h light and 12 h darkness at 75% R.H. for 7-10 days. The fungal colonies developing from seeds, morphologically identified as *Alternaria* sp. were transferred from Potato dextrose Agar to Potato Carrot Agar (PCA) and incubated at dark for 7-12 days before the observations at the stereo (Leica, M165C) and optical (NIKON, Eclipse55ι) microscopes. *Alternaria japonica* dark-brown colonies on PCA amended with 25 mgl^{-1} of streptomycin sulphate grew slowly. At the stereo microscope, *A. japonica* colonies presented the fungal mycelium usually grown into the media and conidia produced in singly or in short chains (2-3 elements). Conidia were dark brown, obclavate, obpyriform, ovoid or ellipsoid, with beaks 2-7 (average 3-4) transverse and 1-3 longitudinal septa, and measured 17.7-56.2 (average 30.9) x 6.6-17.8 (average 10.8) µm. Chlamydospores developed usually ten-twelve days after the incubation conditions previously reported. The isolate of *A. japonica* from *Brassica chinensis*, coded CBS118390, was used as reference control (Simmons, 2007).

Isolates used and their preservation

The strains of *A. japonica* from seeds were identified by morphological observations as well as by a phylogenetic analysis based on beta-tubulin gene sequences. (Ortu et.al., personal communication). The isolates obtained from seeds were coded as reported under tables 2 and 3. Genbank accession numbers of each isolate are shown in table 4. Three reference isolates of *A. japonica* were used: AltRuc1/10 (Genbank accession number KJ909926), obtained from leaves of *E. vesicaria*, AltRuc 1/11 (Genbank accession number KJ909927) obtained from infected leaves of *D. tenuifolia* and *A. japonica* coded CBS118390 (Genbank accession number KJ883438). The different isolates were maintained on PDA at 8 °C.

Inoculum production and pathogenicity test

The different isolates of *Alternaria* spp. were grown on PCA (Potato Carrot Agar) in a growth chamber in darkness at 22-24°C for one week, then kept for another week under 12 hours light /day to stimulate conidia production.

For the pathogenicity test, 30-day-old seedlings of cultivated (cv. Coltivata, Franchi) and wild (cv. Grazia, Maraldi) rocket, were transplanted into a steamed potting soil mix (peat: composted broadleaf bark: clay, 60:20:20 vol /vol) in plastic pots (2 L volume) and maintained in growth chamber at 24°C, with 12 hours/day of fluorescent light. Spores and mycelium fragments were removed from the surface of 15-day-old cultures of the different isolates of the pathogen with a spatula. The conidial suspensions obtained were filtered, conidia and mycelial fragments were counted by hemocytometer and adjusted with deionised water to $1x10^6$ CFU (colony forming units) ml^{-1}. Five plants of cultivated and wild rocket were inoculated with each isolate by spraying with a suspension of each of the strains obtained from seeds, and covered with plastic bags for 7 days after inoculation. Three reference strains of *A. japonica* were used. The pathogenicity test was carried out twice (Table 4).

Not inoculated plants were prepared similarly but sprayed with deionised water. The trials were carried out in a growth chamber at 24 ±1 °C, under 12 hours /day fluorescent light. Plants were checked two weeks after inoculation for disease development.

The data were analysed by univariate ANOVA with Tukey's HSD test using SPSS software 18.0.

Results and discussion

Among the two seed samples of cultivated rocket (*E. vesicaria*) only one was infected, at a low level (one infected seed out of 800) by *A. japonica* (Table 2).

Four out of ten samples of wild (*D. tenuifolia*) rocket seeds were contaminated by *A. japonica.* The pathogen was isolated only from not disinfected seeds (Table 3). Two out ten seed samples of wild rocket were contaminated by *A. japonica* at the highest level of 3 infected seeds out 800 (Table 3).

From disinfected seeds of cultivated and wilted rocket it was possible to isolate different strains of *Alternaria* spp., but no strain belonging to *A. japonica.*

The isolates of *A. japonica* obtained from the different seed lots were coded (Tables 2 and 3), maintained in culture and tested to evaluate their pathogenicity on wild and cultivated rocket.

All the plants of wild and cultivated rocket used, belonging to the cvs. Grazia and Rucola coltivata showed typical symptoms of black-brown leaf necrosis surrounded by a yellow halo after inoculation with the isolates of *A. japonica* obtained from seeds. The virulence of the isolates obtained from infected seeds was similar to that of the three reference strains. All isolates affected both wild and cultivated rocket (Table 4).

Wild and cultivated rocket were affected during the past few years as a consequence of crop intensification, by a number of new diseases, most of which are seed transmitted (Gullino et al. 2014). *A. japonica* is a seed-borne pathogen of plants in the Brassicaceae (David 2002;). This paper provides evidence that also *A. japonica* occurs on rocket seeds, which suggests that rocket seeds may be important in disseminating also this pathogen.

The pathogen was isolated only by not disinfected seeds, thus suggesting an external contamination of seeds. At the usual sowing rate for this crop of 2 g seeds per m^2 corresponding to 1000 seeds /m^2 for cultivated and 10,000 seeds/m^2 for wild rocket, also a low percent of contaminated seeds are enough to favour the spread of the disease, as already observed with other pathogens. For instance, in the case of *Plectosphaerella cucumerina* a percent of infected seeds as low as 0.15% may be important in disseminating the pathogen in wild rocket crops (Gilardi et al. 2013 b). Seed infections also contributed to the introduction in Italy of the *Fusarium* wilt agents for the first time observed on both the rocket species (Garibaldi et al. 2004).

The occurrence of different species of *Alternaria* on seeds and their role in the spread of diseases was reported also in the case of *Ocimum basilicum* (Gilardi et al. 2013 a), *Brassica oleracea* (Humpherson-Jones and Maude, 1982; Kohl et al. 2010), *Cichorium* spp. (Barreto et al.2008), and *Helianthus annuus* (Jackson et al. 1987).

The outbreak of *A. japonica* on rocket represents a new, potential threat to rocket production. The fact that the pathogen infects both wild and cultivated rocket and is seed associated increases its potential to cause severe losses on a crop intensively grown. Quick and reliable diagnostic tools and seed dressing, carried out with chemical, physical or biological means might help preventing field losses, as already observed in the case of other seed-borne *Alternaria* spp. (Vannacci and Harman, 1987).

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References

Barreto R W, Santin AM, Vieira BS. (2008) *Alternaria cichorii* in Brazil on *Cichorium* spp. seeds and cultivated and weedy hosts. J Phytopathol 156: 425–430.

David JC. (2002) *Alternaria japonica*. Distribution Maps of Plant Diseases, No. 862. Wallingford, UK: CAB International.

Garibaldi A, Gilardi G, Bertoldo C, Gullino ML. (2011) First report of leaf spot of wild (*Diplotaxis tenuifolia*) and cultivated (*Eruca vesicaria*) rocket caused by *Alternaria japonica* in Italy. Plant Dis 95:1316.

Garibaldi A, Gilardi G, Pasquali M, Keiji S, Gullino ML. (2004) Seed transmission of *Fusarium oxysporum* of *Eruca vesicaria* and *Diplotaxis muralis*. J Plant Dis Protect 111: 345-350.

Gilardi G, Garibaldi A, Gullino ML.(2013 b) Seed transmission of *Plectosphaerella cucumerina*, causal agent of leaf spot of *Diplotaxis tenuifolia* in Italy. Phytoparasitica 41: 411-416.

Gilardi G, Gullino ML, Garibaldi A. (2013 a) Occurrence of *Alternaria* spp. in the seeds of basil and its pathogenicity. J Plant Pathol 95: 41-47.

Gullino ML, Gilardi G, Garibaldi A. (2014) Seed-borne fungal pathogens of leafy vegetable crops. In: Gullino ML, Munkvold G. (eds). Global perspectives on the health of seeds and plant propagation material. Springer, Dordrecht, The Netherlands, in press.

Humpherson-Jones FM, Maude RB. (1982) Studies on the epidemiology of *Alternaria brassicicola* in *Brassica oleracea* seed production crops. Ann Appl Biol 100: 61-71.

Jackson KJ, Irwin JAG, Berthelsem JE. (1987) Sources of *Alternaria carthami* inoculum in safflower. Aust J Exp Agric 27: 149-154.

Kohl J, van Tongeren CAM, Groenenboom-de Haas BH, van Hoof RA, Driessen R, van der Heijden L. (2010) Epidemiology of dark leaf spot caused by *Alternaria brassicicola* and *A. brassicae* in organic seed production of cauliflower. Plant Pathol 59: 358-367.

Maude RB, Humpherson-Jones FM. (1980) Studies on the seed-borne phases of dark leaf spot (*Alternaria brassicicola*) and grey leaf spot (*Alternaria brassicae*) of brassicas. Ann Appl Biol 95: 311-327.

Simmons EG. (2007) *Alternaria*: An Identification Manual. APS PRESS, St Paul, MN, USA, 775 pages.

Vannacci G, Harman GE. (1987) Biocontrol of seed-borne *Alternaria raphani* and *A. brassicicola*. Can J Microbiol 33: 850-856.

Table1 List of the sample of seeds tested for the presence of *Alternaria japonica*

Code	Host	Cultivar	Seed company
PMP	*Eruca vesicaria*	Rucola coltivata	PMP SEMENTI SAS, Forlì (Cesena) , Italy
LAS	*Eruca vesicaria*	Rucola coltivata	La Semiorto, Sarno, (Salerno) , Italy
ORT	*Diplotaxis tenuifolia*	Ortis	Ortis, Cesena (Forlì-Cesena), Italy
36/Q	*Diplotaxis tenuifolia*	Venere	T&T Vegetable seeds, Chioggia (Venezia) , Italy
37/Q	*Diplotaxis tenuifolia*	Giove	T&T Vegetable seeds, Chioggia (Venezia) , Italy
38/Q	*Diplotaxis tenuifolia*	Frastagliata	Mazzocchi, Casalpusterlengo (Lodi) , Italy
39/Q	*Diplotaxis tenuifolia*	Brenta	Orosem, Azzano, San Paolo (Bergamo) , Italy
40/Q	*Diplotaxis tenuifolia*	Reset	Maraldi Sementi, Cesena (Forlì-Cesena), Italy
41/Q	*Diplotaxis tenuifolia*	Extra	Franchi Sementi Sp.a., Grassobbio (Bergamo), Italy
42/Q	*Diplotaxis tenuifolia*	Standard (rif. 283/CR)	Olter Sementi, Asti, Italy
43/Q	*Diplotaxis tenuifolia*	Winter (rif. 71/CB)	Orosem, Azzano, San Paolo (Bergamo) , Italy
44/Q	*Diplotaxis tenuifolia*	Rucola Selvatica	Olter Sementi, Asti, Italy

Table 2 Number of *Alternaria japonica* colonies detected on cultivated rocket seed samples tested

Seed sample code	Total number of *Alternaria japonica* isolates on 800 seeds/sample	
	Not-disinfected	disinfected
PMP	1 (PMP19-NL)	0
LAS	0	0
Total	1 out 1,600 seeds	0 out 1,600 seeds

Table 3 Number of *Alternaria japonica* colonies detected on wild rocket seed samples tested

Seed sample Code	Total number of *Alternaria japonica* isolates on 800 seeds/sample	
	Not-disinfected	disinfected
ORT	0	0
36/Q	**1**(36Q-4NL)	0
37/Q	**3** (37Q-16NL; 37Q-13NL; 37Q-22NL)	0
38/Q	**3**(38Q-1NL; 38Q-9NL; 38Q-19NL)	0
39/Q	0	0
40/Q	0	0
41/Q	0	0
42/Q	0	0
43/Q	**2**(43Q-1NL;43Q2-NL)	0
44/Q	0	0
Total	9 out 8,000	0 out 8,000

Table 4 Pathogenicity of different isolates of *Alternaria japonica* obtained from infected rocket seeds, expressed as percentage of infected leaves 21 days after artificial inoculation, evaluated under greenhouse conditions at 24°C. The data are the average of two trials

Isolate code	Genbank accession number[b]	Obtained from	% infected leaves on			
			Cultivated rocket cv.Coltivata		Wild rocket cv. Grazia	
AltRuc 1/10	KJ909926	Infected leaves of cultivated rocket	57.5	ab[a]	45.0	ab
AltRuc 1/11	KJ909927	Infected leaves of wild rocket	45.0	ab	72.5	bc
CBS118390	KJ883438	CBS collection	52.5	ab	35.0	a
PMP19	KJ909928	Not disinfected seed	30.0	ab	57.5	a-c
36Q-4NL	KJ909929	Not disinfected seed	60.0	ab	50.0	a-c
37Q-13NL	KJ883443	Not disinfected seed	70.0	b	47.5	a-c
37Q-16NL	KJ909930	Not disinfected seed	65.0	ab	42.5	ab
37Q-22NL	KJ883441	Not disinfected seed	50.0	ab	57.5	a-c
38Q-1NL	KJ883442	Not disinfected seed	40.0	ab	32.5	a
38Q-9NL	KJ909931	Not disinfected seed	40.0	ab	82.5	c
38Q-19NL	KJ909932	Not disinfected seed	27.5	a	55.0	a-c
43Q-1NL	KJ883440	Not disinfected seed	57.5	ab	32.5	a
43Q-2NL	KJ909933	Not disinfected seed	57.5	ab	37.5	ab

[a]Means of the same column, followed by the same letter, do not significantly differ following Tukey's test ($P<0.05$)

[b]GenBank/ accession numbers of the beta tubulin sequences