Short Communication

OCCURENCE OF *FUSARIUM EQUISETI* AS A CONTAMINANT OF *DIPLOTAXIS TENUIFOLIA* SEEDS

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SUMMARY

Fusarium equiseti, the causal agent of leaf spot of both wild [*Diplotaxis tenuifolia* (L.) DC] and cultivated [*Eruca vesicaria* (L.) Cav.] rocket, is a new pathogen on these crops, which has increasingly been found on several commercial farms in Italy. In order to investigate the possible transmission of this pathogen through infested seeds, both wild and cultivated rocket seeds, from eight commercially available seed lots, have been collected from four farms suffering from severe field losses. *F. equiseti* was identified through morphological observations and molecular analysis based on the elongation factor 1 alpha gene (EF-1α). Four out of six seed lot samples of wild rocket were found to be contaminated, while the pathogen was not isolated from the two tested seed lots of *E. vesicaria.* The highest level of detected infestation was 5 out of 800 non-disinfected *Diplotaxis* sp. seeds. *F. equiseti* was not isolated from disinfected seeds. Nine of eleven isolates of *F. equiseti* obtained from seeds were pathogenic on wild rocket. This work demonstrates that *F. equiseti* can be transmitted by *Diplotaxis tenuifolia* seeds. The external nature of seed contamination of *Diplotaxis* sp. by *F. equiseti* has been proved. More extensive essays on seed lots of cultivated rocket are needed in order to have a better understanding of the seed transmissibility of the pathogen on such a host.

Keywords: seed-borne pathogens, leaf spot, seed health, leafy vegetables.

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Wild [*Diplotaxis tenuifolia* (L.) DC] and cultivated [*Eruca vesicaria* (L.)Cav.] rocket are increasingly grown in many countries, because of their extensive use as salad and/or as a garnish. In Italy, these leafy vegetables are cultivated in intensive cropping systems in open fields and under greenhouses over an area of approximately 3,600 hectares, with up to five successive crops per year on the same soil. Moreover, rocket seed production in Italy covers an area of about 500 hectares (Anonymous, 2012, 2014).

In recent years, several diseases caused by soil-borne and foliar pathogens have been observed for the first time on wild and cultivated rocket in Italy (Gilardi *et al*., 2013; Garibaldi *et al*., 2015). Many of these diseases have been shown to be seed-borne, and their transmission through infected seeds has been demonstrated (Gullino *et al*., 2014; Gilardi *et al*., 2015).

The leaf spot of wild and cultivated rocket, caused by *Fusarium equiseti*, is an increasingly important disease that affects many production areas in both Southern and Northern Italy. Yield losses of about 10% have been observed at temperatures between 15 and 28°C and under high relative humidity (Garibaldi *et al*., 2011, 2015). Black-brown and greasy lesions with a well-defined border, surrounded by a violet-brown halo, have been observed on plants at the cotyledon stage and after 20 to 25 days from sowing. The same pathogen is associated with leaf spot, root and crown rot and fruit rot, which result in seed decay and seedling infection on different crops, such as cereals, ginseng, cumin, hibiscus, cyperus, corn, watermelon and aleppo pine (Adams *et al*., 1987; Rai, 1979; Reuveni, 1982; Correll *et al*., 1991; Kosiak *et al*., 2003; Goswami *et al*., 2008; Punja *et al*., 2008; Hassan *et al*., 2014; Gupta *et al*., 2013; Lazreg *et al*., 2014; Li *et al*., 2014, 2015). Such a broad host range increases the risk caused by the recent introduction of *F. equiseti* into Italy. Its sudden appearance in different cultivation areas could be explained due to its spread through contaminated seeds.

In order to investigate the possible contamination of commercial seed lots of wild and cultivated rocket, a seed test was conducted on two seed samples of *E. sativa* and six of *Diplotaxis* spp. collected from commercial farms in

Table 1. List of the samples of seed lots tested for the presence of *Fusarium equiseti.*

Host	Cultivar	Seed lot	Seed company	Harvesting Year	Origin of the sample
Eruca vesicaria	Rucola coltivata	1	La Semiorto, Sarno, (Salerno), Italy	2014	Piedmont
E. vesicaria	Rucola coltivata	2	Franchi Sementi, Grassobbio (Bergamo), Italy	2015	Piedmont
D. tenuifolia	Extrema	3	Ortis, Cesena (Forlì-Cesena), Italy	2015	Veneto
Diplotaxis tenuifolia	Summer	4	Orosem, Azzano, San Paolo (Bergamo), Italy	2014	Lombardy
D. tenuifolia	Luna	5	T&T Vegetable seeds, Chioggia (Venezia), Italy	2014	Veneto
D. tenuifolia	Giove	6	T&T Vegetable seeds, Chioggia (Venezia), Italy	2014	Veneto
D. muralis	Rucola selvatica	7	Suba seed, Longiano (Forlì-Cesena), Italy	2014	Campania
D. muralis	Rucola selvatica	8	Suba seed, Longiano (Forlì-Cesena), Italy	2015	Campania

Veneto, Piedmont, Lombardy and Campania that have suffered severe losses in the field.

Eight seed lots (Table 1) were assayed for the presence of *F. equiseti* on Potato Dextrose Agar (PDA, Merck, Germany), amended with 25 mg l^{-1} of streptomycin sulphate, as described by Mathur and Kongsdal (2003). Isolations were carried out on seeds that had only been washed in distilled water (not disinfected) or disinfected by soaking for 1 min in 1% sodium hypochloride, rinsed in sterile distilled water and dried on filter paper. Fifteen seeds were placed in each Petri plate, which was then sealed with parafilm and incubated at 25°C for a 12 h photoperiod for 7 days. The Petri plates were checked regularly for the development of fungal orange-brown colonies, with the typical morphological characteristics of *F. equiseti* (Leslie and Summerell, 2006). Any suspected colony of *F. equiseti* was purified, subcultured on PDA as a single-spore culture, coded and stored at 4°C. Genomic DNA of the isolates obtained from the seeds was extracted from a pure culture of selected isolates grown on PDA in order to confirm the morphological identification. After 7 days of incubation, macroconidia and microconidia of *F. equiseti* were produced in abundance on the PDA cultures and were accompanied by the presence of chlamydospores produced in chains. Four out of six wild rocket seed-lots were contaminated by the pathogen. A total of 11 isolates were obtained from the 6, 400 non-disinfected seeds (Tables 2 and 3), with infected seed percentage ranging from 0.125 (1 isolate out of 800 seeds) to 0.625% (5 isolates out of 800 seeds). Amplification of the elongation factor 1 alpha gene (EF-1α) was carried out for five isolates coded Rs1Feq, Rs5Feq, Rs6Feq, Rs7Feq and Rs9Feq with EF1/EF2 primers (O'Donnell *et al*., 1998) and deposited at GenBank with accession Nos. KX272609, KX272610, KX272613, KX272611, KX272612**.** BLAST analysis on EF 1-α sequences from all of the isolates showed a homology of 98 to 100% with the *Fusariun incarnatum - equiseti* species complex (FIESC). *F. equiseti* was not isolated from the tested lots of disinfected seeds.

The pathogenicity of the 11 isolates obtained from the seeds was tested on 30-day-old wild rocket plants, transplanted into 2-l pots, filled with a steamed peat substrate and maintained/kept in a greenhouse at 24-27°C. Four pots/treatment with 5 plants/pot were used. The artificial inoculation with the pathogen was carried out by spraying leaves with a spore suspension of 5×10^4 conidia/ml (1 ml per pot) prepared from 15-day-old cultures of each isolate on PDA. One strain of *Fusarium equiseti* obtained from infected leaves of wild rocket (coded Feq1/14, GenBank Accession No. KM583445) was used as a reference. Plants maintained in the same conditions, sprayed with distilled water, served as healthy controls. The inoculated plants showed the typical symptoms caused by the pathogen 7-10 days after the artificial inoculation, while the non-inoculated plants stayed healthy. The plants were checked for disease development, and the percentage of infected leaves and affected leaf area was evaluated 21 days after the artificial inoculation (Table 3). The data were statistically processed by means of variance analysis, that is, by ANOVA and the Tukey test ($p < 0.05$). *F. equiseti* was consistently re-isolated from the affected leaves. The virulence of the *F. equiseti* isolates from the seeds was similar to that of *F. equiseti* isolated from symptomatic leaves of *D. tenuifolia*, and ranged from 38.7% to 61.3% of affected leaves with 5.1 to 30.6% of affected leaf area. Two out of nine isolates from the seeds were not pathogenic (Table 3).

F. equiseti is a member of the FIESC, a genetically highly diverse group, with 30 phylogenetically distinct species (O' Donnell *et al*., 2009, 2012), divided into two clades designated as *F. incarnatum* and *F. equiseti* (Castella and Cabañes, 2014). Differences in pathogenicity among the tested isolates obtained from wild rocket seeds observed in the present study could be correlated with phylogenetic species known within the FIESC, as reported also in other pathosystem (Castella and Cabañes, 2014; Villani *et al*., 2016).

The external nature of seed contamination of *Diplotaxis* sp. by *F. equiseti* has been proved in the present study. This result is in agreement with similar results pertaining to other plant species, such as cowpea (Aigbe *et al*., 1999), ginseng (Punja *et al*., 2008) and *Carya illinoinensis* (Lazarotto *et al*., 2014). The pathogen was not isolated from seeds of two tested seed lot samples of *E. vesicaria.* Other assessments should be made in which the evaluation should be extended to new seed lots of cultivated rocket. The occurrence of *F. equiseti* on wild rocket seeds represents a potential source of inoculum, and favours the long distance spread of the pathogen. *Alternaria* sp. was also constantly isolated from the rocket seeds, thus confirming that the quick spread of this pathogen in Italy and in

Table 2. Number of *Fusarium equiseti* colonies detected on the tested wild and cultivated rocket seed lot samples.

Host and Cultivar	Seed lot	Total number of isolates on 800 seeds/sample	
		Non-disinfected (Isolate code)	Disinfected
E. sativa cv. Rucola coltivata	1	0	0
E. sativa cv. Rucola coltivata	2	0	0
Diplotaxis sp. cv. Extrema	3	0	0
Diplotaxis sp. cv. Summer	4	3(Rs1Feq; Rs2Feq;Rs3Feq)	0
Diplotaxis sp. cv. Luna	5	1(Rs5Feq)	0
Diplotaxis sp. cv. Giove	6	0	0
Diplotaxis sp. cv. Rucola Selvatica	7	5(Rs4Feq; Rs6Feq RS7Feq; RS8Feq;Rs10Feq)	0
Diplotaxis sp. cv. Rucola Selvatica	8	2 (Rs9Feq; Rs11Feq)	0
	Total	11 out of 6,400	0 out of 6,400

Table 3. Pathogenicity of different isolates of *Fusarium equiseti* obtained from wild rocket seeds, 21 days after the artificial inoculation, evaluated under growth chamber conditions at 25°C.

Isolate code	Obtained from	Infected leaves [%]			Affected leaf area [%]		
Feq 1-14	Infected wild rocket leaves	46.7	±1.3	b[a]	23.3	±1.2	bc
Rs1Feq	Non-disinfected seed	46.7	±9.6	b	22.9	±6.5	bc
Rs2Feq	Non-disinfected seed	41.3	±3.5	b	14.0	±3.1	a-c
Rs3Feq	Non-disinfected seed	0.0	±0.0	a	0.0	±0.0	a
Rs4Feq	Non-disinfected seed	38.7	±7.1	b	19.2	±4.7	a-c
Rs5Feq	Non-disinfected seed	42.7	±5.8	b	17.1	±2.8	a-c
Rs6Feq	Non-disinfected seed	60.0	±4.0	b	29.9	±6.8	c
Rs7Feq	Non-disinfected seed	41.3	±3.5	b	5.1	±1.4	ab
Rs8Feq	Non-disinfected seed	53.3	±5.8	b	18.1	±4.1	a-c
Rs9Feq	Non-disinfected seed	61.3	±4.8	b	30.6	±5.2	c
Rs10Feq	Non-disinfected seed	0.0	±0.0	a	0.0	±0.0	a
Rs11Feq	Non-disinfected seed	44.0	±10.6	b	13.7	±6.0	a-c
Non inoculated control	–	0.0	±0.0	a	0.0	±0.0	a

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

other countries could, at least partially, be explained by the use of contaminated seeds (Tidwell *et al*., 2014; Gilardi *et al*., 2015). Previous studies provided evidence that *Plectosphaerella cucumerina*, a pathogen of wild rocket that has frequently been detected on several farms that grow this crop for the ready to eat sector, is also a seed contaminant (Gilardi *et al*., 2013).

Owing to the extensive host range of F. *equiseti* and the economic value of rocket, this pathogen could also be a threat to this crop in other production areas, and the present results suggests that seeds may be important in disseminating different pathogens. Considering the external nature of the contamination of wild rocket seeds a conventional treatment of the seeds might eliminate the risk of infection in contrast with a more problematic endophytic contamination.

Identifying the primary source of inoculum is of critical importance for an effective disease management. Quick and reliable diagnostic tools and seed dressing methods need to be investigated and made available to seed companies and growers. Among the tested fungicides, benomyl, thiophanate-methyl, propiconazole and fludioxonil have been shown to be able to reduce the mycelial growth of *F. equiseti* from ginseng in *vitro* at 10 mg/l, while fludioxonil is already effective at 1 mg/l (Punja *et al*., 2008). Information regarding the chemical management of this pathogen is very scarce, especially in relation to the very limited availability of registered fungicides for rocket.

Moreover, disease management should rely above all on preventative methods, such as early diagnosis of the pathogen and seed dressing.

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