SEED TRANSMISSION OF *FUSARIUM OXYSPORUM* F.SP. *BASILICI* IN SWEET BASIL

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SUMMARY

Three different agar media were compared on 13 commercial seed samples of sweet basil to detect *Fusarium oxysporum*. Komada medium shows a restricted growth of many fungi but only *F. oxysporum* and species within the *Liseola* section grow slightly faster and sporulate. Most *F. oxysporum* colonies were recognizable under the stereomicroscope after training. Komada medium was, therefore, chosen for testing. Out of 21 commercial samples, suspected of harbouring the pathogen, 14 resulted infected and 8 internally infected. Five of these latter gave rise, after sowing, to infected plants demonstrating the seed to plant transmission of the inoculum in commercial seeds. Sowing healthy seeds in soil where infected seed lots had previously been sown gave rise to infected plants supporting the role of infected seeds in soil contamination. Isolates of *F. oxysporum* from basil seeds were tested for pathogenicity. Most isolates tested (88.2%) belong to the f.sp. *basilici* but some were saprotrophs, in a few cases they represented the only *Fusaria* present on seeds. A seed health test based on the agar plate method can lead to the refusal of healthy seed lots actually contaminated by saprotrophic *F. oxysporum*. Artificial inoculations or VCG determination can furnish a correct identification of the pathogen but they are quite time consuming; a molecular identification of the *forma specialis* is therefore required.

RIASSUNTO

TRASMISSIONE PER SEME DI *FUSARIUM OXYSPORUM* F.SP. *BASILICI* IN BASILICO. Per il reperimento di *Fusarium oxysporum* su seme di basilico sono stati confrontati tre mezzi agarizzati su 13 campioni di seme commerciale. Il mezzo di Komada, scelto per le successive analisi, consente una crescita limitata di molti funghi ma solo *F. oxysporum* ed alcuni *Fusarium* appartenenti alla sez. *Liseola* crescono più velocemente e sporulano. *F. oxysporum*, con un po' di pratica, è riconoscibile allo stereomicroscopio. Su 21 campioni di seme commerciale, 14 erano infetti ed 8 avevano il patogeno internamente; 5 di questi ultimi hanno dato origine, dopo semina in terreno non sterile, a piantine infette, dimostrando così la trasmissione per seme del patogeno attraverso l'uso di seme commerciale. Seminando seme sano nello stesso terreno dove erano stati seminati campioni infetti sono state ottenute piante infette dimostrando così il ruolo del seme nella contaminazione del terreno. Gran parte (88,2%) degli isolati di *F. oxysporum* provenienti da seme di basilico appartenevano alla f.sp. *basilici* ma alcuni erano saprotrofi e questi rappresentavano, per alcuni campioni di seme, gli unici *F. oxysporum* presenti. L'analisi fitosanitaria su mezzo di Komada, portando al riconoscimento di *F. oxysporum* ma non della forma speciale, può, quindi, causare la non certificazione di lotti di seme contaminati da forme saprotrofe. Le inoculazioni artificiali o la determinazione del VCG degli isolati possono superare questo problema ma richiedono tempi lunghi; un metodo molecolare di identificazione del patogeno sarebbe utile.

Key words: Ocimum basilicum, seed health testing, seed certification.

INTRODUCTION

Sweet basil (*Ocimum basilicum* L.) is an economically important herb crop in Italy, France, Israel and USA (Garibaldi *et al.*, 1997). It is widely employed in Italy both fresh and processed and in 1993 it was grown on 86 ha with a production of 40,175 q of fresh product (Anonymous, 1995). Seed production reached 3.84×10^4 Kg in 1991 but today it is estimated to be about 3×10^4 Kg.

Fusarium oxysporum Schlechtend.:Fr. f.sp *basilici* (Dzidzariya) Armst. & Armst. (FOB) is the causal agent of Fusarium wilt and crown rot of basil. The disease was first recorded in southern European Russia in 1956 (Vergovskii, 1956) and in a region of Georgia facing the

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Black Sea in 1957 (Kvartskhava, 1957). Dzidzariya (1968) assigned the pathogen to the f.sp. *basilicum,* later renamed f.sp. *basilici* (Minuto *et al.,* 1994). The disease was then reported in Italy (Grasso, 1975; Tamietti and Matta, 1989), France (Mercier and Pionnat, 1982), USA (Wick and Haviland, 1992) and Israel (Gamliel *et al.,* 1996).

Fusarium wilt and crown rot of basil represents one of the major diseases of this herb. The widespread occurrence of the disease and its rapid spreading, exemplified by the succession of outbreaks in USA, where it has been recorded, after the first report in Massachusetts (Wick and Haviland, *l.c.*), in California (Davis and Marshall, 1993), Louisiana (Holcomb and Reed, 1994), Maryland (Dutky and Wolkow, 1994) and Florida (Datnoff and Liang, 1997), focuses the attention of many researchers upon the transmission of this pathogen by seeds. The only sound evidence about its seed-borne nature was furnished by Gamliel *et al.* (1996), who sowing seeds harvested from diseased plants obtained diseased plants. The presence of the pathogen on basil seeds has been reported repeatedly (Martini and Gullino, 1991; Elmer *et al.*, 1994; Keinath, 1994; Trueman and Wick, 1995). Physiological (Katan *et al.*, 1996) and molecular (Pan and Wick, 1995) data suggest that FOB is spreading as a single clone supporting the hypothesis that seeds play an important role in the rapid dissemination of the pathogen worldwide (Elmer *et al.*, 1994). Seed health testing of basil is, therefore, a key tool for preventing crop losses until registered efficient chemicals, biocontrol agents (Isarlishvili *et al.*, 1968; Minuto *et al.*, 1994) or resistant varieties become available (Reuveni *et al.,* 1997). The use of certified seeds has been claimed to be at the origin of the lowering incidence of Fusarium wilt and crown rot in Liguria (northern Italy) (Garibaldi *et al.*, 1997).

MATERIALS AND METHODS

Seed lots. Seed lots of sweet basil, produced mostly in northern Italy, were collected in 1995 and 1996 (Table 1). Seeds from India were harvested from plants grown from Italian seeds. 'Genovese' is the best represented cultivar as it is the most appreciated in Italy for fresh consumption. Seed lots were supplied by seed firms because suspected of harbouring the pathogen, and therefore are not representative of Italian production. Seed samples were maintained in a cold chamber at 8°C to minimize the possible decline of inoculum in seeds.

Table 1. Cultivar, geographical origin and year of harvesting of seed lots used in the experiments.

Seed lot no.	Cultivar	Origin	Year
195	genovese	northern Italy	1995
295	genovese	northern Italy	1995
395	genovese	northern Italy	1995
495	genovese	northern Italy	1995
595	genovese	northern Italy	1995
695	genovese	northern Italy	1995
795	genovese	northern Italy	1995
196	genovese	northern Italy	1996
296	genovese	northern Italy	1996
496	unknown	central Italy	1996
596	unknown	northern Italy	1996
696	unknown	northern Italy	1996
796	unknown	northern Italy	1996
1096	genovese	unknown	1996
1296	genovese	unknown	1996
1996	foglia di lattuga	India	1996
2196	greco	India	1996
897	genovese	northern Italy	1996
1097	genovese	northern Italy	1996
1197	genovese	northern Italy	1996
1297	genovese	northern Italy	1996

Seed health tests. Three different media were compared on 13 (from lot no. 195 to lot no. 796) of the seed lots reported in Table 1. Four hundred untreated seeds were sown, 10 per plate, on 9 cm diam. Petri dishes containing 15 ml of the following media: Komada (Komada, 1975), Nash and Snyder (Tuite, 1969) and Littman (Littman oxgall agar; Tuite, 1969). Plates were incubated at 25°C under cycles of 12 h darkness and 12 h n.u.v. light. After 7 and 10 days seeds were checked at the stereomicroscope for the presence of sporulating *F. oxysporum* colonies; when required, microscopic slides were arranged for a correct identification. Some troublesome isolates were sent to Prof. Fabrizio Marziano, Institute of Plant Pathology, University of Naples, for identification. On Komada medium *Fusarium* of section *Liseola* (mostly *F. proliferatum*) (Nelson *et al.,* 1983) sporulates and these colonies were recorded; on Nash and Snyder medium all *Fusarium* colonies, other than *F. oxysporum,* were recorded (some seeds gave rise to different species of *Fusarium*) without attempt to identify them at the species level. Seed lot infection was expressed as percent of infected seeds.

All the remaining seed lots were tested on Komada medium only. In order to determine the percentage of

seeds with internal inoculum, all were then tested on this medium after surface disinfection with an aqueous solution of NaClO (1% active chlorine) for 10 min without rinsing or wiping (Anonymous, 1996).

Seed to plant transmission. To evaluate the seed to plant transmission of the inoculum 400 seeds of those lots with a percentage of infection higher than, or equal to, 4% were sown in 35x25x9 cm brickstone trays (100 seeds per tray) containing not sterilized planting mix (garden soil:peat:sand 1:1:1) tested throughout all the experiments for the absence of FOB. Trays were maintained in the greenhouse at 26±4°C for two months. At regular intervals plants with symptoms were recorded and carefully uprooted, surface disinfected and plated on Komada medium to confirm the diagnosis. At the end of the experiment, plants originated from seed lots no. 795, 1096 and 1296 were carefully uprooted, the soil within each tray was mixed and each tray was sown with 100 seeds from a healthy basil seed lot of the cv. 'Genovese'. Four new brickstone trays filled with new pot mix from the same batch of the previous experiment were sown with 100 healthy seeds each and kept as control. Trays were maintained in the greenhouse at 26±4°C for two months. This last experiment was devised to verify the contamination of the soil due to the use of infected seeds.

Pathogenicity tests. All *F. oxysporum* colonies from seeds incubated on Komada medium were isolated, single spored and maintained in collection. All isolates from seed lots with a low percentage of infection and a set of the isolates from lots with high percentages of infection were tested for pathogenicity on basil plants. Three isolates from symptomatic plants (two from greenhouses in Riviera Ligure and one from a plant bought in a local market) were included for comparison. Isolates of *F. oxysporum* were grown on PDA under laboratory conditions for ten days. A spore suspension (about 10^6 conidia ml^{-1}) was used to inoculate 50 ml of Czapek broth in 150 ml flasks shaken on a rotary shaker (150 rpm) on a laboratory bench for 48 h. Cultures were filtered through sterile cheesecloth and the filtrate was centrifuged at 3000 rpm for 5 min and resuspended in sterile water two times consecutively, adjusting the final concentration of the suspension at 1-1.5 x 10^6 conidia ml^{-1}. Basil seedlings (5-6 cm tall) were uprooted from soil, their roots were washed free of soil and dipped in inoculum suspension for 30 s. Seedlings were then transplanted into 400 ml pots (5 seedlings per pot, 3 pots per isolate) maintained in a climatic chamber at 25-28°C. Uninoculated basil seedlings, maintained as control plants, did not show any symptoms. Plants were checked for the presence of symptoms at 15 day intervals up to 75 days after transplanting, symptomatic portions of diseased plants were plated on Komada medium and the fungus reisolated. Isolates that did not gave rise to symptomatic plants after the first inoculation were retested up to three times.

Statistical analysis. Confidence limits for proportions were calculated according to Zar, 1984. In Table 2 and Table 3 the 95% lower (L_1) and upper (L_2) confidence limits for proportions are reported.

RESULTS

Seed health tests. Litmann medium allows growth and sporulation of many different fungi, therefore identification of *F. oxysporum* was most troublesome and infection percentages (not presented) were not reliable. Results obtained with the other two media are reported in Table 2. Nash and Snyder medium allows growth and sporulation of *Fusarium* spp. Identification of *F. oxysporum* under the stereomicroscope was not always possible and frequently it required compound microscope examination. This makes the medium hardly workable as a routine seed health testing method. Komada medium allows a restricted growth of many fungi but only *F. oxysporum* and *Fusarium* species within *Liseola* section (mostly *F. proliferatum*) have a slightly faster growth and sporulation rate. After training most *F. oxysporum* colonies were distinguishable under the stereomicroscope from other *Fusarium* colonies. Few sporulated *Fusarium* colonies required a compound microscopic examination for a correct assignment. Several restricted sterile colonies morphologically similar to *Fusarium* were isolated and allowed to sporulate but never resulted to be *F. oxysporum.* Out of 13 seed lots tested, 7 were infected. Six of them were correctly scored on Komada medium while only 3 on Nash and Snyder medium. Interestingly the 4 infected lots scored healthy on Nash and Snyder medium showed high percentages of *Fusarium* contaminated seeds on Komada.

A total of 21 commercial seed samples were tested on Komada medium and 14 were found to be infected (Table 3). Treating infected seed lots with sodium hypoclorite lowered infection percentages dramatically. Only 8 (about 57%) infected lots tested resulted internally infected and showed an infection percentage with *F. oxysporum* higher than, or equal to, 4%. The fraction of seeds with internal inoculum varied from zero to about one third of infected untreated seeds with no significant correlation with infection percentages of untreated seeds.

Table 2. *Fusarium* infection of basil seeds incubated on two agar media.

Seed lot no.	Agar medium							
	Komada				Nash & Snyder			
	F. oxysporum			*F. liseola*	*F. oxysporum*			*Fusarium* spp.
	L_1%[a]	%[b]	L_2%	%	L_1%	%	L_2%	%
195	0.01	0.25	1.38	0.25	0.00	0.00	0.92	13.00
295	0.00	0.00	0.92	0.00	0.00	0.00	0.92	0.00
395	0.00	0.00	0.92	0.00	0.00	0.00	0.92	0.25
495	0.00	0.00	0.92	0.00	0.00	0.00	0.92	0.00
595	0.00	0.00	0.92	0.00	0.00	0.00	0.92	0.00
695	0.06	0.50	1.78	0.00	0.00	0.00	0.92	19.00
795	35.05	39.00	42.31	1.50	36.00	40.00	43.33	37.00
196	0.00	0.00	0.92	0.00	0.01	0.25	1.38	3.00
296	0.15	0.75	2.16	0.25	0.00	0.00	0.92	11.00
496	3.10	5.00	7.56	0.00	0.00	0.00	0.92	16.00
596	0.00	0.00	0.92	0.00	0.00	0.00	0.92	2.25
696	2.26	4.00	6.50	0.00	0.15	0.75	2.16	3.25
796	0.00	0.00	0.92	0.00	0.00	0.00	0.92	1.00
mean		3.46		0.15		3.15		8.13

[a] Lower (L_1) and upper (L_2) 95% confidence limits for proportions (see Zar, 1984).
[b] 400 seeds tested.

Table 3. Influence of surface disinfection on *F. oxysporum* seed infection and performance of selected seed lots following sowing into unsterilized soil.

Seed lot no.	Infected seeds[a]									Symptomatic plants out of [b]			
	Untreated			Treated[c]			Emergence			Sown seeds[d]	Emerged seedlings[e]		
	L_1%	%	L_2%	L_1%	%	L_2%	L_1%	%	L_2%	(%)	L_1%	%	L_2%
195	0.01	0.25	1.38	0.00	0.00	0.25		n.t.[f]		n.t.		n.t.	
695	0.06	0.50	1.78	0.00	0.00	0.25		n.t.		n.t.		n.t.	
795	35.05	39.00	42.31	0.56	1.50	3.22	68.62	72.75	76.57	0.00	0.00	0.00	1.25
296	0.16	0.75	2.16	0.00	0.00	0.25		n.t.		n.t.		n.t.	
496	3.10	5.00	7.56	0.06	0.50	1.78	72.99	78.25	81.86	0.25	0.01	0.32	1.76
696	2.26	4.00	6.50	0.06	0.50	1.78	88.59	91.75	94.36	0.50	0.07	0.54	1.94
1096	18.48	22.00	25.66	0.71	1.75	3.53	48.20	54.50	58.88	0.50	0.11	0.92	3.26
1296	5.86	8.25	11.19	1.40	2.75	4.82	63.58	68.75	72.76	0.00	0.00	0.00	1.34
1996	0.01	0.25	1.38	0.00	0.00	0.25		n.t.		n.t.		n.t.	
2196	3.10	5.00	7.56	0.30	1.00	2.52	38.65	44.75	48.79	0.75	0.35	1.68	4.80
897	3.89	6.00	8.68	0.87	2.00	3.86	75.67	80.25	83.70	0.75	0.20	0.93	2.68
1097	0.01	0.25	1.38	0.00	0.00	0.25		n.t.		n.t.		n.t.	
1197	0.01	0.25	1.38	0.00	0.00	0.25		n.t.		n.t.		n.t.	
1297	2.54	4.25	6.50	0.16	0.75	2.16	71.97	77.00	80.64	0.00	0.00	0.00	1.18
Mean		11.69			1.34					0.34		1.10	

[a] 400 seeds on Komada medium.
[b] 400 seeds in unsterilized soil.
[c] After surface disinfection with a aqueous solution of NaClO (1% active chlorine) for 10 min.
[d] Plants with symptoms divided by the number of seeds sown (400).
[e] Plants with symptoms divided by the number of emerged seedlings (400 x emergence percentage).
n.t. : not tested.

Samples 395, 496 and 696 were tested again on Komada medium after 18 months of storage at 8°C and results of both tests did not statistically (P = 95%) differ.

Seed to plant transmission. Seed lots with a percentage of infection with *F. oxysporum* higher than, or equal to, 4% were sown in unsterilized pot mix. Sowing density, about 1100 seeds m^{-2}, is less than one sixth of the density usually adopted in specialized farms in Italy (Garibaldi *et al.*, 1997). Percentages of infected plants recorded two months after sowing are reported in Table 3; symptomatic plants appeared scattered over the whole surface of trays indicating that data were not biased by plant to plant transfer of the inoculum. Five seed lots (62.5%) gave rise to infected plants. Seed lots showed variable emergence, therefore percentage of infected plants was calculated out of the total number of sown seeds (400) or the number of emerged seedlings. No significant correlation exists either between percentage of infected seeds, either untreated or treated, and percentage of infected plants, calculated in either way, but this could be also due to the low number of infected plants recorded, nor between seedling emergence and percentage of infection. It is interesting to note the extensive overlapping of confidence intervals (L_1 and L_2, P = 0.05) calculated for percentages of internally infected seeds and for infected plants.

Sowing healthy seeds in the soil which had previously hosted infected seed lots 795, 1096 and 1296 gave rise, after two months of growth, to infected plants (4.25, 3.00 and 6.75%, respectively); control trays gave healthy plants only.

Pathogenicity tests. Pathogenicity tests furnished quite variable data. Symptomatic plants appeared at different rates during growth (data not shown) and isolates that were non pathogenic in the first inoculation were tested a second and, possibly even, a third time. Isolates that at the third inoculation did not induce disease symptoms were considered non pathogenic. Results are summarised in Table 4. Most isolates tested (88.2%) belong to the f.sp. *basilici* but some were saprotrophs. When mixed with pathogenic isolates, saprotrophic strains of *F. oxysporum* represent a very small fraction of the whole inoculum; in a few cases they represent the only *Fusaria* present on seeds.

Table 4. Pathogenicity of *F. oxysporum* isolates from basil seeds.

Seed lot no.	Isolates[a]		
	Tested	Pathogenic	
	no.	no.	%[b]
195	1	0	0
695	2	0	0
795	12	11	91.7
296	3	0	0
496	7	7	100.0
696	16	16	100.0
1096	16	16	100.0
1296	14	13	92.9
1996	1	1	100.0
2196	9	9	100.0
897	6	6	100.0
1197	1	0	0
1297	5	3	60.0
total	93	82	88.2

[a] From seed health testing on Komada medium.
[b] Out of tested isolates.

DISCUSSION

Commercial seed lots of basil can be infected and/or contaminated by *F. oxysporum* f.sp. *basilici* and these seeds can give rise to infected plants. The availability of seeds certified as *F. oxysporum* f.sp. *basilici* free is, at the moment, a key factor for a disease control strategy.

Seed health testing can be performed on Komada medium where most sporulating colonies of *F. oxysporum* can be differentiated from those of *Fusarium* species within *Liseola* section, which seem to be the only *Fusaria* from basil seeds that sporulate on this medium. Anyway, seed health testing based on colony and fructification morphology does not allow the identification of the *forma specialis basilici*. For these reasons the method is prone to furnish false positive results. This will not constitute a major drawback when pathogenic and saprotrophic isolates are present on the same sample as it has to be rejected anyway, but artificial inoculations performed with all isolates from seed health testings showed that four samples harboured saprotrophic isolates only; this means that such a test can lead to the rejection of seed lots (not certifiable as *F. oxysporum* free) that are actually healthy. Artificial inoculations overcome this problem but they require too long a time, therefore a rapid diagnostic tool allowing the identification of the *forma specialis* would be welcome. The determination of the VCG group (Elmer *et al.*, 1994;

Katan *et al*., 1996) is faster than artificial inoculation but requires the production of *nit* mutants of the isolate to be tested; DNA probes are promising (Pan and Wick, 1995) and initial results on molecular variability of the pathogen (Cristani *et al.*, 1998) suggest a very limited variability confirming the molecular approach could be a reliable diagnostic strategy.

Seed to plant inoculum transmission experiments show that the number of infected seeds does not correlate with the number of infected plants arising from that seed lot. *F. oxysporum* f.sp. *basilici* can be recovered after surface disinfection of seeds but, usually, only a small fraction of the inoculum is located internally. A better estimate of infected plants, considering the statistical variability of data, is this fraction of seeds harbouring internal inoculum. These data suggest that external inoculum plays a minor role in the establishment of the disease on a growing crop.

Infected plants represent a source of inoculum for the growing crop, and are also important from the point of view that the pathogen can be air-borne (Gamliel *et al.*, 1996). The following crops may be infected as soil becomes contaminated by fungal mycelium and conidia.

The presence of diseased plants after sowing healthy seeds in soil where infected seed lots had previously been sown demonstrates the role of infected seeds in soil contamination, even when the crop growing from infected seeds does not show any sign of the disease. In such a case ungerminated seeds, seed integuments (which remain in the soil at emergence) or unemerged seedlings harbouring the pathogen act as sources of inoculum for the next crop.

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