Eur J Plant Pathol (2007) 118:165–172
DOI 10.1007/s10658-007-9126-8

FULL RESEARCH PAPER

Pathogenicity and mycotoxin production by *Fusarium proliferatum* isolated from onion and garlic in Serbia

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Received: 9 November 2006 / Accepted: 12 March 2007 / Published online: 11 April 2007

Abstract *Fusarium proliferatum* can occur on a wide range of economically important vegetable plants but its role in disease is not always well established. In 2000 and 2001, from forty-one field samples of wilting onion and garlic plants in Serbia, *F. proliferatum* as the predominant fungal species was isolated from root and bulbs. Seventy isolates were firstly characterized for their sexual fertility and were shown to be mostly members of *Gibberella intermedia* (sixty-seven of seventy isolates, the remaining three isolates were unfertile), the sexual stage of *F. proliferatum* (syn. mating population D of *G. fujikuroi* complex). A selected set of eleven *F. proliferatum* isolates from both hosts were also tested for their pathogenicity and toxigenicity. Although onion and garlic plants were susceptible to all isolates, onion plants showed a significantly higher disease severity index. Six of the eleven isolates of *F. proliferatum* produced fumonisin B_1 from 25 to 3000 μg g^{-1}, and beauvericin from 400 to 550 μg g^{-1}; ten isolates produced fusaric acid from 80 to 950 μg g^{-1} and moniliformin from 50 to 520 μg g^{-1}. Finally, all isolates produced fusaproliferin up to 400 μg g^{-1}. These results confirm *F. proliferatum* as an important pathogen of garlic and onion in Europe and that there is a potential mycotoxin accumulation risk in contaminated plants of both garlic and onion.

Keywords *Gibberella intermedia* · Mating type · Phytotoxicity · Fumonisin B_1 · Moniliformin

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Introduction

Fusarium proliferatum is a world-wide occurring fungal pathogen of several agriculturally important host plants. In particular, *F. proliferatum* has been reported as a pathogen of maize (Logrieco et al. 1995), rice (Desjardins et al. 1997), asparagus (Elmer 1990), date palm (Abdalla et al. 2000) and ornamental palms (Armengol et al. 2005). Moreover, this is a toxigenic species, producing a broad range of toxins, such as fumonisin B_1 (FB1; Leslie et al. 1996), moniliformin (MON; Marasas et al. 1984) beauvericin (BEA; Logrieco et al. 1995), fusaric acid (FA; Bacon et al. 1996), and fusaproliferin (FUP; Ritieni et al. 1995). Some of these toxins have a well-known phytotoxic activity; these include FA, which is implicated in the pathogenesis of tomato-wilt symptoms (Gaeumann 1957); MON, which is toxic towards tobacco plants (Cole et al. 1973), and FB_1, which has shown to be phytotoxic to maize and

tomato (Lamprecht et al. 1994). Recently, BEA was shown by Paciolla et al. (2004) to possess phytotoxic activity as it caused a severe reduction in tomato protoplast viability.

Fusarium proliferatum belongs to *Liseola* section of the *Fusarium* genus (Nelson et al. 1983) and its teleomorph, *Gibberella intermedia*, belongs to the *G. fujikuroi* complex composed of a number of reproductively isolated biological species designated by separate *Gibberella* species names (syn. mating populations [MPs]). Numerous additional *Fusarium* anamorphs within the *Liseola* section have been defined on the basis of morphology and sequence differences (Nirenberg and O'Donnell 1998; O'Donnell et al. 1998), showing undoubtedly that additional biologically significant entities remain to be identified and described. Therefore, it is important to assess the biological species for isolates identified as *Liseola* section members, in order to characterize the phytopathological and toxigenic risks that could affect the host plants. Wilting of young and adult plants of onion (*Allium cepa*) is caused by a complex of species of the genus *Fusarium* among which *F. oxysporum* f. sp. *cepae* is considered the most important onion parasite worldwide, causing rot of the basal plate of the onion bulb (Lacy and Roberts 1982). Rot of the basal plate of garlic (*A. sativum*) is most often caused by *F. culmorum* (Rengwalska and Simon 1986) which can be responsible for losses of garlic cloves of up to 40% in the field, and even higher in storage (Schwartz and Mohan 1995). Previous cases of *F. proliferatum* isolated from onion and garlic in storage were reported in USA, showing bulb rot of onions caused by this fungus (Dugan et al. 2003; du-Toit and Inglis 2003). In Europe, *F. proliferatum* was isolated from onion seeds (Mannerucci et al. 1987) and it was reported for the first time as an agent of bulb rot of garlic in Hungary during winter storage (Simey 1990). However, these reports were only based on morphological identification of *F. proliferatum* and pathogenic assays were not always performed, in order to complete Koch's postulates.

The objectives of this study were: (i) to determine the main *Fusarium* species occurring in onion and garlic infected plants in Serbia; (ii) to investigate the toxicological profile of the isolates; (iii) to study their possible fertility and assign them to a specific MP; (iv) to evaluate their pathogenicity to onion and garlic under greenhouse conditions.

Materials and methods

Fungal isolation and identification

Forty-one samples each representing a single field of onion or garlic were collected from various locations across Serbia during the growing seasons in 2000 and 2001. Each sample was composed of twenty plants and eight fragments of each plant were surface-sterilised and placed on water agar (WA) amended with streptomycin sulphate and incubated at ambient room conditions for 7 days. The *Fusarium* isolates obtained from onion and garlic were purified by hyphal tip isolation. The fungus was then single-spored and sub-cultured on potato dextrose agar (PDA) and carnation leaf agar (CLA) under permanent dark at 25 ± 1°C and an alternating temperature of 25 ± 1°C day and 20 ± 1°C night, with a 12 h photoperiod. The surface of the Petri dishes was 60 cm away from four cool-white tubes (Sylvania FR96T12/CV/VHO/235/1 215W, manufactured by the Mead Corporation, Gardner, MA, USA) and one black light tube (Phillips® TL 36W/80 RS F40 BLB). Based on morphological characters, *Fusarium* isolates were identified according to Nelson et al. (1983).

Fertility test

Tester strains for mating population tests were received from J. F. Leslie, Kansas State University. Isolates of *F. proliferatum* from onion and garlic were crossed on carrot agar as male parents with tester strains of MPs A to G as described by Klittich and Leslie (1988). A set of crosses were also performed as described by Leslie (1995) to verify the occurrence of female fertile strains among the *F. proliferatum* field isolates. All isolates were crossed twice by both testers from each MP. Crosses were examined weekly and were considered positive when mature dark blue perithecia were observed with oozing ascospores. Also the effective population number [$N_{e(mt)}$] of the *F. proliferatum* population was evaluated following Leslie and Klein (1996) by the equation: $N_e = (4\ N_m Nf)/(N_m + Nf)$, where N_m is the number of isolates with the mating type *MATD-1* and N_f is the number of isolates with the other mating type *MATD-2*.

Pathogenicity test

Eleven isolates were randomly selected from the population of *F. proliferatum* isolated from onion and garlic in the field. Conidial suspensions used for the inoculum were obtained from two week-old cultures. A mycelial plug of the sub-cultured monoculture of eleven isolates was transferred to potato-dextrose broth and incubated at 26°C on a rotary shaker at 120 rpm. After seven days, mycelia with spores and broth were homogenised for 2 min at low speed in a Waring Blender. The mixture was filtered through four layers of cheesecloth to remove hyphae. The spore concentration was adjusted with a haemocytometer to approximately 10^7 conidia ml^{-1}. Selected healthy seedlings of onion for the pathogenicity test were obtained from sterilised seeds after three weeks of cultivation in sterile soil. Garlic clones were obtained from sterilised cloves, after two weeks of cultivation. Onion seed and garlic cloves were surface-disinfected with 1% NaOCl for 1 min and then rinsed three times in sterile distilled water. Onion seedlings and garlic clones were soaked in the conidial suspension of each isolate of *F. proliferatum* for 24 h and then planted in flats (40 × 18 × 16 cm) with soil artificially inoculated with the single isolate of *F. proliferatum*. Each flat was prepared by moistening 1,400 g of sterilised grit with 100 ml of distilled water, autoclaving for 2 h on each of the two consecutive days. Each flat was inoculated with 200 ml of a spore suspension (three replications per isolate and onion/garlic seedlings). Plants were maintained in a temperature and light-controlled greenhouse (12 h/12 h light/dark 25/21°C). Tests were replicated four times.

Symptoms on onion and garlic plants were observed three weeks after inoculation. The root and bulb/clove rot disease symptoms were graded into five classes: 1 = no symptoms; 2 = <10% rotted roots; 3 = 10–50% rotted roots; 4 = >50% rotted roots and slight symptoms on bulbs/cloves; 5 = completely rotted roots and severe symptoms on bulbs/cloves. A disease severity index (DSI) was calculated as the mean of three plants of each species and four test replicates. Analysis of variance (ANOVA) was conducted on DSI data to determine the overall effects of isolate, host and isolate x host interaction. Comparisons of isolates for each host were made using Duncan's multiple range test ($P < 0.05$).

In vitro toxin production

The capability of different isolates to produce toxins was measured from fungal cultures on maize kernels. The isolates were grown in three replicates on 100 g of autoclaved yellow maize kernels adjusted to 45% moisture in 500 ml Erlenmeyer flasks and inoculated with 2 ml of the aqueous suspension containing approximately 10^7 conidia ml^{-1}. After four weeks of incubation in the dark at 25°C, cultures were harvested and dried in a fan oven at 60°C for 48 h, and ground and stored at 4°C until toxin analyses. Non-inoculated maize was used as the control.

Toxin analysis

For BEA and FUP extraction, 10 g of each culture was homogenized by shaking in 100 ml of methanol (99.5%) for 1 h. Samples were then filtered through Whatman No. 4 filter paper and methanol was removed under reduced pressure. Each extract was eluted in 1 ml of methanol and filtered through an Acrodisk filter (pore size, 0.22 μm), before high-performance thin layer chromatography (HPTLC) analysis, performed for BEA according to the procedure described by Logrieco et al. (1993) and for FUP following the procedure described by Logrieco et al. (1996). FA extraction and analysis were performed as previously described (Abdalla et al., 2000). FB_1 extraction and analysis were performed by HPTLC according to the procedure described by Munkvold et al. (1998) and MON extraction and analysis as described by Bottalico et al. (1982).

Results

Fungal identification

Fusarium proliferatum was the predominant species isolated from onion and garlic diseased plants collected in Serbia (seventy isolates collected). At a much lesser extent, isolates belonging to *F. oxysporum*, and *F. solani* (from onion and garlic) and *F. acuminatum* and *F. equiseti* (only from onion) were also isolated (data not shown).

Fertility test

Sixty-seven out of seventy isolates morphologically identified as *F. proliferatum* confirmed their identity by showing positive crosses with standard tester strains of *G. intermedia*. Of the population of *F. proliferatum* tested, no female fertile strains occurred. Of the isolates selected for the pathogenicity test, three isolates were MATD-1 and eight isolates were MATD-2 (ratio 2.7:1) (Table 1), according to the ratio found in the whole population tested (2.8:1). This ratio reduced the effective population number N_e to 87.27%.

Pathogenicity test

Three weeks after artificial inoculation, golden to yellowish brown (occasionally light to dark pink) lesions appeared on roots of onion and garlic. With time, they became dark brown and necrotic. As the disease progressed, roots become semi-transparent, shrunken, water-soaked and finally, they disintegrated. Water-soaked, tan to golden lesions and soft, shrunken tissue were observed on the bulbs/cloves of plants with severe symptoms. Generally, symptoms on onion were more severe than on garlic, since mean disease severity scores were 3.8 (isolates from onion) and 4.0 (isolates from garlic) for onion plants and 1.9 (isolates from onion) and 2.2 (isolates from garlic) for garlic plants (Table 2). Generally, onion was more susceptible to all investigated isolates than garlic. Re-isolation of the fungal isolates confirmed that symptoms on infected seedlings were caused by *F. proliferatum*.

Toxin production

The results of toxin production by isolates of *F. proliferatum* grown on autoclaved maize kernels are summarized in Table 1. All isolates but one produced FA from 80 to 950 μg g^{-1}; MON was produced by ten isolates from 50 to 420 μg g^{-1}. Three isolates did not produce FB_1, three isolates produced it at a relatively low level (25–250 μg g^{-1}) and five isolates produced it at high levels (1,000–3,000 μg g^{-1}). Eight of eleven isolates produced BEA from 400 to 550 μg g^{-1}, and all produced FUP, although three of them just in traces and eight isolates from 200 to 400 μg g^{-1}.

Discussion

Fusarium proliferatum was the predominant species isolated from visibly young and adult infected plants of onion and garlic in our survey. Although in previous reports, wilting of onion plants showing rot of the basal plate of the bulb in the field has been

Table 1 Mating type and toxin production by isolates of *Fusarium proliferatum* from onion (*Allium cepa*) and garlic (*A. sativum*)

Code No.	No. of MRIZP collection	Host species	Mating Type	Mycotoxins (μg g^{-1})[a]				
				BEA[b]	FA	FB_1	FUP	MON
17	96	*Allium cepa*	MATD1	400	230	3000	200	250
14	93	*Allium cepa*	MATD2	500	80	nd	200	280
15	94	*Allium cepa*	MATD2	400	270	nd	400	nd
68	135	*Allium cepa*	MATD2	nd	150	25	traces	420
13	82	*Allium cepa*	MATD2	400	950	250	400	370
16	134	*Allium cepa*	MATD2	400	330	1000	traces	520
18	87	*Allium sativum.*	MATD1	400	Nd	1400	400	50
21	86	*Allium sativum*	MATD1	550	230	1500	200	220
22	76	*Allium sativum*	MATD2	500	470	nd	400	150
69	92	*Allium sativum*	MATD2	nd	140	25	traces	500
20	139	*Allium sativum*	MATD2	nd	350	2500	200	60

[a] Isolates grown on autoclaved maize kernels in the dark at 25°C for 4 weeks; FB_1 = fumonisin B_1; BEA = beauvericin; FUP = fusaproliferin; ND = Not detected

[b] Detection limits: FB_1 = 25 ppm; BEA = 10 ppm; FUP = 10 ppm; FA = 0.01 ppm

Table 2 Disease severity on onion (*Allium cepa*) and garlic seedlings (*A. sativum*) under artificial inoculation with isolates of *F. proliferatum* originating from onion and garlic

Code No.	Collection Number[A]	Host species	Disease severity index	
			Onion	Garlic
15	94	*Allium cepa*	3.10[a]	2.20[abc]
16	134	*Allium cepa*	3.83[a]	1.67[bc]
68	135	*Allium cepa*	3.91[a]	1.65[bc]
14	93	*Allium cepa*	3.96[a]	1.63[bc]
13	82	*Allium cepa*	4.00[a]	3.07[a]
17	96	*Allium cepa*	4.13[a]	2.60[ab]
		Mean	3.82	2.14
69	92	*Allium sativum*	3.81[a]	1.75[bc]
18	87	*Allium sativum*	4.57[a]	1.76[bc]
20	139	*Allium sativum*	3.73[a]	2.30[ab]
21	86	*Allium sativum*	4.00[a]	2.00[abc]
22	76	*Allium sativum*	3.90[a]	1.70[bc]
		Mean	4.0	1.90
Test			1.26[b]	1.07[c]

[A] All cultures are deposited in Culture Collection of the Maize Research Institute, Zemun Polje, Belgrade-Zemun

[a,b,c] Values followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test

Disease severity index = Σ (seedlings/class x class score)/total score)

related mostly to *F. oxysporum* f. sp. *cepae* (Lacy et al. 1982) and the rot of the basal plate of garlic is most often caused by *F. culmorum* (Rengwalska and Simon 1986), we rarely isolated *F. oxysporum* from garlic and onion, while we never isolated *F. culmorum* from either host plant. The data of the pathogenicity tests that we performed showed that *F. proliferatum* should be regarded as a potentially serious pathogen of onion and garlic in Serbia. All evaluated isolates of *F. proliferatum* were pathogenic to both onion and garlic seedlings. However they were more pathogenic to onion than to garlic. As far as we are aware, this is the first report of the occurrence of *F. proliferatum* on garlic plants in the field, while previous reports from USA and Hungary have shown garlic rot caused by *Fusarium* species in storage conditions, and the first report of *F. proliferatum* isolated from infected plants of onion in the field in Europe.

Previous reports on the occurrence and pathogenicity of *F. proliferatum* on onion worldwide (du-Toit and Inglis 2003) were based only on morphological identification. According to several authorities, *F. proliferatum* is a species that is easily misidentified as *F. verticillioides* due to closely related morphological traits (Nelson et al. 1983; Leslie 1995). These species, although morphologically and phylogenetically closely related, have a different toxin profile (Leslie et al. 1996; Moretti et al. 1996). Therefore, using another tool for the more accurate identification of field isolates of *F. proliferatum* is important not only for a correct taxonomic approach, but more importantly for correctly evaluating the toxigenic risks related to a specific *Fusarium* contamination of food commodities. We selected the fertility test as a tool for confirming the identity of the *F. proliferatum* isolates. Based on our studies, isolates of *F. proliferatum* isolated from onion and garlic in Serbia belong to the MP D of *G. fujikuroi* complex, being therefore *G. intermedia*. Data showed also that *MATD-2* was predominant over the *MATD-1* in this population (ratio 2.8:1) with a similar estimated ratio in a population of *F. proliferatum* isolated from maize in Argentina (Chulze et al. 1998), but different from that (1.2:1) observed in a pathogenic population from ornamental palms in Spain (Armengol et al. 2005). However, this ratio reduced the effective population numbers (Leslie et al. 1996) and could be the consequence of the lack of female fertility that was

shown among the set of isolates analyzed. The lack of female fertility could be a limiting factor for the sexual recombination to occur in the field. However, the occurrence of both mating types in both garlic and onion fields, show that the potential for sexual recombination of *F. proliferatum* in the field is high and could consequently improve the genetic pool available for the pathogenic population of *F. proliferatum* and for its toxigenicity.

Mating population D of *G. fujikuroi* has been reported occurring on several host plants (Leslie 1995), including maize (Leslie et al. 1990), asparagus (Elmer 1995), palms (Abdalla et al. 2000; Armengol et al. 2005) and rice (Desjardins et al. 1997), where the most common MP is C. *Fusarium proliferatum* has been considered by several authorities as the anamorph of both C (syn. *F. fujikuroi*) and D MPs, that differ significantly in toxin production (Desjardins et al. 1997, Moretti et al. 1996). This is the first report of MP D on garlic and onion. It is interesting to observe that *F. proliferatum* isolates from onion and garlic belong to MP D together with many other populations of this species isolated from a very broad range of plant hosts, on which they show a high level of pathogenicity (e.g., asparagus, maize, palms, rice, sorghum, and tomato; Armengol et al. 2005; Desjardins et al. 1997; Elmer 1995; Leslie et al. 1995; Logrieco et al. 1995; Moretti et al. 1998).

Among the toxins studied here, some of them have well known phytotoxic activity, including FA, implicated in the pathogenesis of tomato wilt symptoms (Gaeumann 1957), MON, toxic toward tobacco plants (Cole et al. 1973), FB_1, shown to be phytotoxic to maize and tomato (Lamprecht et al. 1994) and causing chromosomal aberrations in onion cells (Lerda et al. 2005), and BEA that affects the viability of tomato protoplasts (Paciolla et al. 2004). All of these secondary metabolites have phytotoxic activity and all tested isolates proved to be producers; therefore, we suspect their involvement in the expression of symptoms on onion and garlic plants. However, before drawing any conclusions about the role played by these toxins in onion and garlic wilt, it would be necessary to study the in vivo toxin production of the isolates in pathogenicity tests and compare the results with tests using pure toxins, also evaluating possible additive and/or synergistic effects of the toxins on the plants. In addition to the phytotoxic effects, all five toxins analyzed have mycotoxic activity. In particular, FB_1 has been linked to human oesophageal cancer (Rheeder et al. 1992) and is involved in several mycotoxicoses (Ross et al. 1990). FA affects both brain and pineal neurotransmitters of rats (Porter et al. 1995), MON was reported to cause haematological disorders, myocardial hypertrophy, and mortality in farm animals (Ledoux et al. 1995), BEA was toxic to several human cell lines and induces apoptosis in mammalian cells (Logrieco et al. 2002), and FUP showed cytotoxic activity on human B-lymphocytes (Logrieco et al. 1996) and teratogenic effects on chicken embryos (Ritieni et al. 1997). It is of concern that all isolates of *F. proliferatum* we studied can produce at least three of these toxins and that three isolates were able to produce all of them. In preliminary experiments, Seefelder et al. (2002) reported FB_1 contamination in cloves of garlic grown in soil artificially inoculated by *F. proliferatum*, showing a capability of this fungus to accumulate its mycotoxins in edible part of garlic plants. Therefore, natural FB_1 contamination of garlic infected by *F. proliferatum* may be possible.

The pathogenic and toxigenic data reported here warrant further investigations on the occurrence of *F. proliferatum* metabolites in the edible parts of garlic and onion worldwide to evaluate the risk of consumption of contaminated plants. Finally, more attention should be given to the identification of *F. proliferatum*, using tools other than morphological identification (e.g. molecular approaches and fertility tests) and further investigation is needed on the distribution of *F. proliferatum* in other geographical areas where garlic and onion are cultivated. The pathogenicity tests suggest the isolates have varying ability to produce disease on both plant hosts. Furthermore, previous reports (du-Toit and Inglis 2003) showed that the type of onion can significantly affect the expression of pathogenicity (e.g. symptoms did not occur on yellow and red cultivars, but only on white onions). As a consequence, further research is required to accurately establish the role of this fungus as a pathogen of these two similar crops.

Acknowledgements The authors would like to thank J.F. Leslie, Kansas State, University, Manhattan, KS, for providing mating population tester strains and Vincenzo Ricci for valuable technical assistance.

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