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Research Papers

Fusarium proliferatum isolated from garlic in Spain: identification, toxigenic potential and pathogenicity on related *Allium* species

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Summary. *Fusarium proliferatum* has been reported on garlic in the Northwest USA, Spain and Serbia, causing water-soaked tan-colored lesions on cloves. In this work, *Fusarium proliferatum* was isolated from 300 symptomatic garlic bulbs. Morphological identification of *Fusarium* was confirmed using species-specific PCR assays and *EF-1α* sequencing. Confirmation of pathogenicity was conducted with eighteen isolates. Six randomly selected *F. proliferatum* isolates from garlic were tested for specific pathogenicity and screened for fusaric acid production. Additionally, pathogenicity of each *F. proliferatum* isolate was tested on healthy seedlings of onion (*Allium cepa*), leek (*A. porrum*), scallions (*A. fistulosum*), chives (*A. schoenoprasum*) and garlic (*A. sativum*). A disease severity index (DSI) was calculated as the mean severity on three plants of each species with four test replicates. Symptoms on onion and garlic plants were observed three weeks after inoculation. All isolates tested produced symptoms on all varieties inoculated. Inoculation of *F. proliferatum* isolates from diseased garlic onto other *Allium* species provided new information on host range and pathogenicity. The results demonstrated differences in susceptibility with respect to host species and cultivar. The *F. proliferatum* isolates tested all produced fusaric acid (FA); correlations between FA production and isolate pathogenicity are discussed. Additionally, all isolates showed the presence of the *FUM1* gene suggesting the ability of Spanish isolates to produce fumonisins.

Key words: Fusaric acid; fumonisin; clove rot, garlic, *Allium* spp.

Introduction

Annual world garlic production is 15.8 million tons. Production in Spain is the highest in the European Union and ninth highest in the world, with approximately 142,500 tons per annum grown on 16,100 ha. Exports account for 52,455 tons. In 2009, an investigation in 30 Spanish municipalities where garlic is cultivated identified *Fusarium proliferatum* (T. Matsushima) Nirenberg as the causal agent of clove rot during storage (Palmero *et al*., 2010). Over half (54%) of the fields producing this garlic were in the northwest of the Segovia region and the remainder in the province of Valladolid. The total area of garlic production in these provinces is 200 ha. In June 2010, similar symptoms were observed on stored bulbs of the cv. Morado de Cuenca grown in Castilla la Mancha, the biggest production area in Spain, which includes the Albacete province (4,900 ha) and the Cuenca province (2,632 ha), where average yields are over 8,000 kg ha^{-1}. Symptomatic cloves showed internal tan-coloured rot progressing toward the clove apex, with occasional white mycelium in rotted cavities. Isolates from the symptomatic cloves were identified as *F. proliferatum* (Palmero *et al*., 2010).

Prior to the report from Spain (Palmero *et al*., 2010), *F. proliferatum* was reported on garlic in the

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Northwest USA (Dugan *et al*., 2007), Egypt (Galad *et al*., 2002), in Poland and Serbia in Europe (Stankovic *et al*., 2007; Stepien *et al*., 2011), and recently in India (Ravi Sankar, 2012). It is also possible that this pathogen may affect garlic plants during growth in the field (Stankovic *et al*., 2007).

Seefelder *et al*. (2002) reported mycotoxins in garlic bulbs in Germany. *F. verticillioides* and *F. proliferatum* are reputed to be the main sources of fumonisins in food and feed products (Jurado *et al*., 2010). Moreover, *F. proliferatum* produces a number of toxins apart from fumonisins, such as moniliformin (Marasas *et al*., 1984), beauvericin (Plattner and Nelson, 1994; Logrieco *et al*., 1998), fusaric acid (FA) (Bacon *et al*., 1996) and fusaroproliferin (Ritieni *et al*., 1995). As fresh garlic is consumed worldwide, the production of mycotoxins in cloves infected with *F. proliferatum* requires serious consideration (Stepien *et al*., 2011).

Variability in fumonisin production by different isolates of *F. proliferatum* has been demonstrated, with the results indicating that *FUM1* is the key gene for fumonisin biosynthesis in this species (Jurado *et al*., 2010). Conventional PCR approaches for specific detection of *F. proliferatum* have been applied (Mulè *et al*., 2004; Jurado *et al*., 2006a), and recent work demonstrated that *F. proliferatum* isolated from garlic in Poland produced fumonisins. A real-time PCR assay developed for diagnosis and quantitation of *FUM1* gene expression (Jurado *et al*., 2010) can be used to determine the potential ability of Spanish isolates of this species from garlic to produce fumonisins.

In the work reported here, isolates were identified as *F. proliferatum*, using both morphological means and from supplementary data provided by molecular analysis that enabled stringent identification. The main focus of this research was to determine the pathogenicity of the *Fusarium proliferatum* isolates on plants of different species of *Allium*. Pathogenicity tests were performed on non-germinated seeds, pre-germinated seeds and mature plants. In the work, FA production by isolates of *F. proliferatum* obtained from garlic was also evaluated and correlations drawn between isolate pathogenicity and formation of FA *in vitro*.

Material and methods

Morphological identification

In June 2009 symptoms of rot were observed on stored garlic (*Allium sativum*) cv. Morado de Cuenca from the Albacete province. The whole garlic production cycle of a cooperative of growers in Spain was analyzed in 2010. A minimum of one sample was collected for each stage in the production process (meristem culture plates to F5 field). Fifty bulbs from each sample were evaluated.

Symptomatic bulbs were surface sterilized for 2–3 min in 0.5% NaOCl in distilled water, rinsed in 4 changes of sterile distilled water and air dried under aseptic conditions. Pieces excised from lesion margins were transferred to potato dextrose agar (PDA) or Komada´s medium (Komada, 1975). Cultures were incubated at 25°C in the dark. Five to seven days later, single spore cultures were obtained from *Fusarium* colonies emerging from the infected tissues and examined morphologically.

The taxonomic criteria of Nelson *et al*. (1983), Gerlach & Nirenberg (1982) and Leslie & Summerell (2006) were followed to assign isolates to the *Fusarium* species level.

Pathogenicity was confirmed with 18 isolates of *Fusarium* recovered from different growth cycle samples using the methods of Dugan *et al*., (2007) with minor modifications (Palmero *et al*., 2010). Each isolate was inoculated into 5 cloves of the cv. Morado de Cuenca. Prior to treatment, cloves were surface sterilized in 0.5% NaOCl for 45 s, rinsed in 4 changes of sterile water, and wounded to a depth of 4.5 mm with a 1 mm diameter probe. The wound was inoculated with PDA colonized by the appropriate *Fusarium* isolate. Five cloves for each tested isolate were pseudoinoculated with sterile PDA as controls. Well-separated cloves were incubated in sealed, plastic boxes in a growth chamber at 25°C for 5 weeks, and observed for symptom development. The test was repeated once.

Molecular identification and PCR assays

Six *Fusarium* isolates were selected for molecular identification. Isolates were subcultured to PDA using the single spore technique (Leslie and Summerell, 2006). For DNA extraction, three mycelial disks were excised from the margin of a 3- to 5-d-old PDA culture and crushed against the wall of a 1.5-mL Eppendorf tube using a sterile pipette tip. DNA extraction was carried out as described by Querol *et al*. (1992).

Genomic DNA from the *Fusarium* isolates was subject to a specific PCR assay for *F. proliferatum* based on the IGS region (Intergenic Spacer of rDNA): Fp3-F (5′-CGGCCACCAGAGGATGTG-3′) and Fp4-

R (5′-CAACACGAATCGCTTCCTGAC-3′) according to protocol described by Jurado *et al*. (2006b).

Potential fumonisin-producing isolates were identified using the primers FUM5P2-F (5′-CCCCCATCATCCCGAGTAT-3′) and FUM5P2-R (5′-TGGGTCCGATAGTGATTTGTCA-3′) which amplify a partial sequence of the *FUM1* gene of *F. proliferatum* (López-Errasquín *et al*., 2007).

Amplification reactions were carried out in volumes of 25 mL containing 200 ng template DNA in 3 mL, 1.25 mL of each primer (20 mM), 0.2 mL of Taq DNA polymerase (5 U mL^{-1}) (Biotools, Madrid, Spain), 2.5 mL of 10× PCR buffer, 1 mL of $MgCl_2$ (50 mM), and 0.25 mL of dNTPs (100 mM) (Ecogen, Barcelona, Spain). PCR was performed in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). Amplification products were detected by electrophoresis on 1.5% (or 2.5% for *FUM1* amplification) agarose ethidium bromide gels in 40 mM Tris–acetate, 1.0 mM EDTA 1buffer.

Additionally, genomic DNA was used for PCR amplification of a partial region of the *EF-1α* gene using the primer pair EF-1/EF-2 and the conditions described by O'Donnell *et al*. (1998). The amplification products obtained were isolated using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). Sequencing of both strands was performed using the ABI 3700 DNA Sequencer (Applied Biosystems, USA) according to the manufacturer's instructions in the Genomic Unit of the Universidad Complutense of Madrid (Madrid, Spain). Sequences were edited and aligned using the Clusta lW method with the software included in the DNAstar package (Lasergene, Wisconsin, USA). A BLAST search for similarities was performed with the sequences obtained.

Germination and seed-borne pathogenicity test

The same six selected *F. proliferatum* isolates from garlic were tested for pathogenicity against seeds and seedling. Isolates were inoculated onto two varieties of onion, two varieties of leek and one variety each of scallion and chives (Table 1). Seeds were surface sterilised in NaOCl (40–50 mg L^{-1} active Cl_2) for 3 min, rinsed in five changes of sterile distilled water and placed on previously surface sterilised plastic

Table 1. Plant material used in pathogenicity tests.

Pathogenicity test	Species	Variety	Source	Reference No.
Seedlings	Garlic (*Allium sativum* L.)	Garcua	National Institute for Agricultural Research (INIA)	Ref. 12.500.003
Seedlings	Garlic (*Allium sativum* L.)	Plamegar	INIA	Ref. 20.030.193
Germination and seedlings	Onion (*Allium cepa* L.)	Albarracin	INIA	Ref. 20352
Germination and seedlings	Onion (*Allium cepa* L.)	Panter	INIA	Ref. 20.279
Seedlings	Leek (*Allium ampeloprasum* var. *porrum)*	Carental 3	BATLLE.S.A (from market)	Lot 002-2I31
Seedlings	Leek (*Allium ampeloprasum* var. *porrum)*	Genita	Carrefour.S.A. (from market)	Lot 9B-254549
Germination	Leek (*Allium ampeloprasum* var. *porrum)*	Gennevillier	INIA	Ref. 6864
Germination	Leek (*Allium ampeloprasum* var. *porrum)*	Royal	INIA	Ref. 7054
Seedlings	Chives (*Allium schoenoprasum*)	Cebollino	BATLLE.S.A. (from market)	Lot 0010-4455/07
Seedlings	Scallions (*Allium fistulosum*)	Cebolleta	Carrefour.S.A. (from market)	Lot 9B/255383

trays previously filled to two-thirds capacity with autoclaved (105 kPa, 30 min at 120°C) vermiculite (Agroalse S.L., Moncada, Valencia, Spain). Conidia of *F. proliferatum* were harvested from actively growing 10–14 day-old cultures on PDA in sterile distilled water and filtered through two layers of muslin cloth. The concentration of conidia was adjusted to 10^7 conidia mL^{-1} using replicate haemocytometer counts. Conidial suspensions (300 mL) were used to water the trays; three trays of each cultivar and isolate were prepared per treatment, with 50 pre-germinated (5d) and 50 non-germinated seeds per tray.

Following watering, seeds or seedlings were covered with a 1 cm deep layer of autoclaved vermiculite. Control seeds or seedlings were treated with sterile water. Inoculated and control plants were maintained at 20–25°C under a 14-hour, 18.8 $mE.m^{-2}.s^{-1}$ light: 10 hours dark photoperiod. After 13 days, seedlings were rated for damping off (Schumann & D'Arcy, 2006) following the recommendations of the International Seed Testing Association standards (ISTA, 2004). The experiment was repeated twice.

Pathogenicity test on plants

Conidial suspensions were adjusted to approximately 10^7 conidia mL^{-1} and used to inoculate healthy seedlings of onion (*A. cepa*), leek (*A. porrum*), scallions (*A. fistulosum*) and chives (*A. schoenoprasum*) after cultivation in sterile (autoclaved twice at 105 kPa for 30 min) soil for three weeks; garlic clones (*A. sativum*) were treated after two weeks growth. Different commercial varieties were used for each crop species (Table 1). Seedling roots of onion, chives, scallions and leek and garlic cloves and roots were soaked in the conidial suspensions of each *F. proliferatum* isolate for 24 h before planting in flats containing soil previously inoculated with the same isolate of *F. proliferatum*. Each flat was inoculated with 200 mL of a spore suspension containing 10^7 conidia mL^{-1}. Three replicate flats were prepared for each isolate and each onion, garlic, leek or chive cultivar. Plants were maintained in a temperature and light-controlled greenhouse (12 h/12 h light/dark; 25/21°C). All treatments were replicated four times. Disease symptoms were graded into five classes following the method of Stankovick *et al*. (2007): 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 variety for each species and four test replicates. Symptoms on onion, chives, leeks, scallion and garlic plants were recorded three weeks after inoculation. At the end of the experiment, dry weights of plants were recorded following oven-drying at 80°C for 48h.

Fusaric acid extraction and quantification

The six isolates tested in pathogenicity tests were also screened for FA production. Extraction of FA from PDA single-spore cultures of *F. proliferatum* isolates (Codes: A3a1, A6m1, A4a1H, A10a1, A7a2, A10a3) was performed using modifications of the methods of Notz *et al*. (2002) and De Weert *et al*. (2004). Seven day-old cultures of *F. proliferatum* on PDA were chopped into small pieces using a scalpel and suspended in 10 mL phosphate buffer, pH 2.5. Three volumes of ethyl acetate were added and the mixture sonicated for 30 min. The organic phase was adjusted to pH 3.5 using 1M HCl and rotary evaporated to dryness at 40°C. The residue was redissolved in 1.0 mL of methanol and stored at -20°C until analysis.

FA in extracts was analyzed using liquid chromatography-mass spectrometry (LC/MS) equipped with a 250 × 4 mm C18 HD analytical column (Agilent, Palo Alto, CA, USA). The LC apparatus comprised an Agilent Series 1100 LC pump, an LC 90 UV spectrophotometer (Jasco International Co. Ltd.) at room temperature. Samples (100 μL) were eluted in a linear gradient of 20 to 80% acetonitrile in water acidified with 0.1% trifluoroacetic acid (Sigma-Aldrich) over 30 min. Fusaric acid was detected by monitoring absorbance at 270 nm using a Jasco MD-910 multiwave length detector (Jasco International Co. Ltd.). At a flow rate 1 mL min^{-1}, the retention time of FA was 13 min. FA was quantified based on a series of standard concentrations between 10–100 μg mL^{-1} in methanol prepared with synthetic FA (Sigma-Aldrich). HPLC linear regression curves (absolute amount of standard against chromatographic peak area integrated from valley to valley) were calculated from three injections of different amounts of standard.

Statistical analysis

Data collected in experiments were subjected to two types of ANOVA. In the first experiment, treatments were analyzed according to the linear model

$\Upsilon_{ijk} = \mu + \alpha_I + \beta_j + (\alpha\beta)_{ij} + \tau_k + \varepsilon_{ijk}$ where Υ_{ijk} is the ijk-th observation, μ is the overall mean of the variable in question (DSI or weight), α_i is the effect of the i-th isolate, β_j is the effect of j-th cultivar (for garlic, onion and leek), $(\alpha\beta)_{ij}$ is the effect of the interaction isolate x cultivar, τ_k is the effect of the k-th block, and ε_{ijk} is the experimental error.

The second type of multiway ANOVA was used to assess the effect of the interaction isolate x host species as source of variability. Therefore, the control treatment was not taken into account. The linear model for this analysis was $\Upsilon_{ijk} = \mu + \alpha_I + \beta_j + (\alpha\beta)_{ij} + \tau_k + \varepsilon ijkl$ where Υ_{ijkl} is the ijkl-th observation, μ is the overall mean of the variable in question (DSI), αI is the effect of the i-th isolate, β_j is the effect of the j-th specie, $(\alpha\beta)_{ij}$ is the effect of the interaction isolate x specie, τ_k is the effect of the k-th block and ε_{ijkl} is the experimental error. ANOVA was carried out using the data from both experiments. Comparisons between mean results for each treatment were undertaken using multiple range tests (least significant difference method) (Montgomery, 1991). All ANOVA calculations and linear regression of pathogenicity (DSI) against FA production (μM mL^{-1}) for all the plant species and cultivars inoculated were carried out using StatsGraphics Centurion XV.II (Statistical 195 Graphics Corp., Herndon, VA, USA).

Results

Morphological, molecular and physiological identification of isolates

All analyzed symptomatic bulbs produced cultures characteristic of *Fusarium* with catenate microconidia borne on polyphialides, plus curved macroconidia usually 3- to 5-septate. Catenate microconidia were club shaped with a flattened base, aseptate, and were produced on mono- and polyphialides. Isolates were identified as *F. proliferatum* (T. Matsushima) Nirenberg (1998).

The species-specific PCR assays produced an amplicon of the size expected for *F. proliferatum*, confirming the morphological identification of all isolates. Moreover, *EF-1α* sequences had high similarity with *F. proliferatum.* Sequences were deposited in the NCBI GenBank database (accession numbers JF414783 for A3a1, A10a3, A4a14, A7a2 and A10a1 sequences and JF414784 for A6m1 sequence).

Pathogenicity confirmation tests were positive for all the 18 isolates used and clove rot was induced in all inoculated garlic cloves. Moreover, *F. proliferatum* was successfully re-isolated onto PDA from the symptomatic cloves. Each inoculated clove displayed symptoms typical of the bulb rot originally observed in cultivated garlic. An internal tan-coloured rot progressed from the inoculation site towards the clove apex, with white mycelium occasionally observed in the rot cavity. These symptoms matched those previously observed in Spanish garlic production areas. No fungi were isolated from control cloves; these cloves displayed no symptoms.

Pathogenicity tests

Damping off, evaluated in pathogenicity test using both un-germinated and pre-germinated seeds, increased significantly (P>0.05) after inoculation with the six isolates of *F. proliferatum* tested, although individual isolates showed different levels of virulence to the various *Allium* spp. (Figure 1).

Four isolates (A3a1, A6m1, A4a1H and A10a3) produced extensive damping off on pre-germinated onion seeds, with average damping-off incidence of up to 71% and 86% for cv. Albarracín and Panter, respectively. However, the effect on un-germinated seeds was lower, with emergence reductions of 28% and 11.54%, respectively. The effect on un-germinated leek seeds was higher than in onion. As found with onion, isolates A3a1, A6m1, A4a1H and A10a3 were the most virulent, with damping off averaging 67.3% and 80.29% for cv. Royal and Gennevillierr, respectively. With pre-germinated seeds, the differences in damping-off incidence were not as large, compared with controls, as those observed with un-germinated seeds. On scallion, isolates A6m1 and A4a1H caused significant declines in emergence for pregerminated seeds. A4a1H was the most pathogenic isolate on pre-germinated chives (77.7% damping off), but was only slightly pathogenic when inoculated before seed germination. Isolates A3a1 and A7a2 did not cause symptoms on un-germinated seeds of either scallions or chives.

Tests conducted on garlic, leek, onion, chives and scallions showed the pathogenic capacity of *F. proliferatum* strains on all these crops (Tables 2 and 3). All isolates produced symptoms with disease scores significantly different from controls (P<0.001) on all inoculated varieties.

Disease severity index (DSI) values from inoculated garlic varieties were significantly greater than

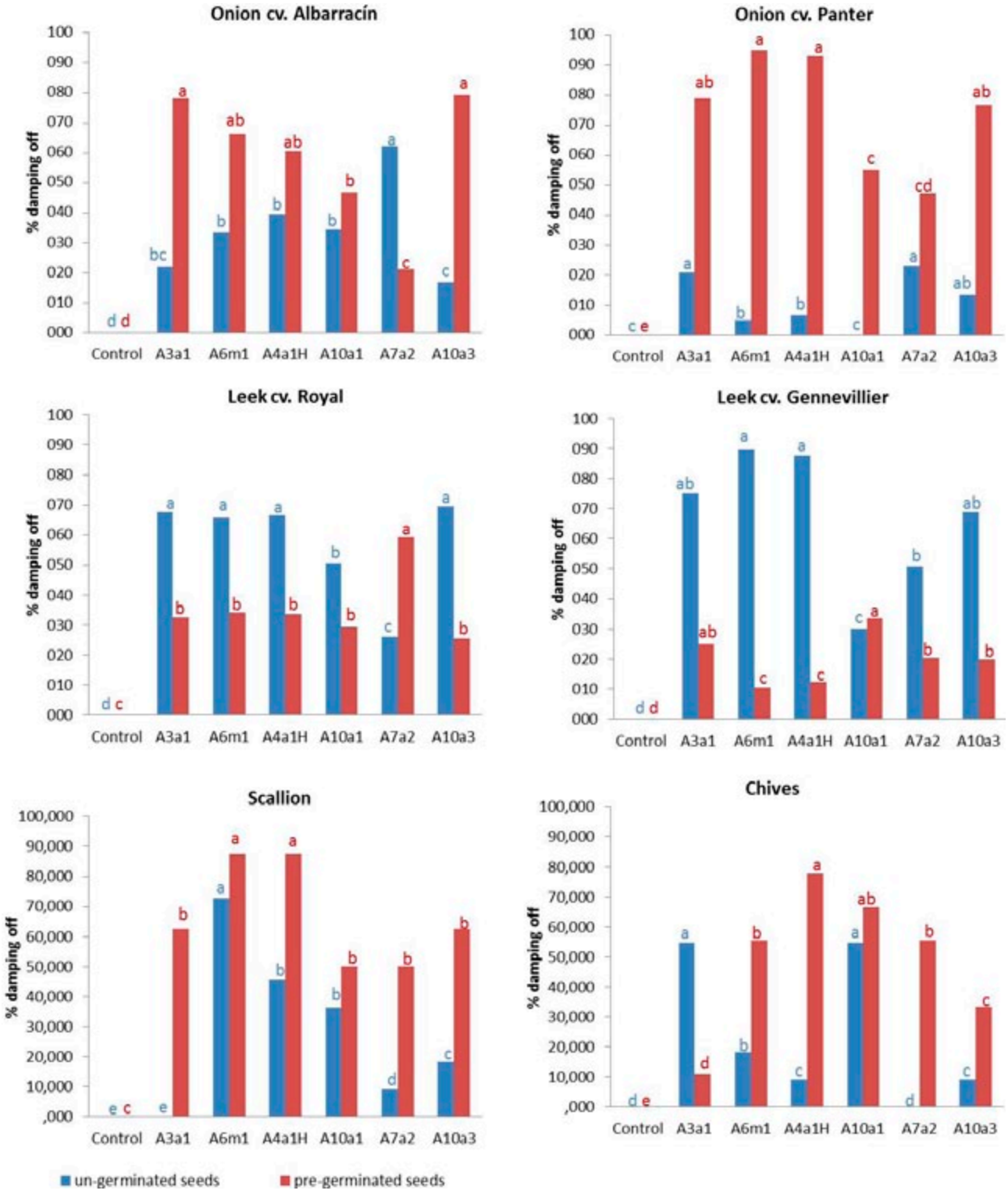

Figure 1. Effects of six isolates of *Fusarium proliferatum* recovered from garlic on damping off in onion, leek, chives and scallion seedlings. Bars topped by the same letter are not significantly different (Fisher´s protected least significant difference test; $P<0.05$).

Table 2. Disease severity index (DSI) on onion (*Allium cepa*), garlic (*A. sativum*), leek (*A. porrum*), chives (*A. schoenoprasum)* and scallion (*A. fistulosum*) seedlings following artificial inoculation with isolates of *F. proliferatum* originating from garlic.

Isolate code	Onion		Garlic		Leek		Chives		Scallion	
Control	1.80	c	1.30	c	1.40	e	1.00	a	1.30	a
A3a1	3.90	ab	3.60	a	2.70	d	3.00	b	2.60	bc
A6m1	4.10	ab	4.00	a	3.00	c	3.20	b	3.10	cd
A4a1H	4.50	a	3.10	b	3.30	c	2.70	b	3.40	d
A10a1	3.80	b	2.90	b	4.10	a	3.20	b	3.00	bcd
A7a2	3.60	b	2.90	b	3.70	b	3.70	bc	2.60	b
A10a3	3.60	b	3.10	b	3.20	c	4.50	c	3.00	bcd
Significance	***	***	***	***	***					
Cultivar[a]	cv. 1, Albarracín cv. 2, Panter		cv. 1, Garcua cv. 2, Plamegar		cv. 1, Carental cv. 2, Genita		cv. 1, Cebollino		cv. 1, Cebolleta	
cv. 1	3.60	a	2.90	a	3.20	a	3.04		2.71	
cv. 2	3.70	a	3.10	b	2.90	b				
Significance	NS	*	***	NT	NT					
Isolate × cv.	NS	NS	NS	NT	NT					

[a] Cultivars 1 and 2 are the same as those indicated in the previous line for each tested plant species.
DSI values with the same lower case letter did not differ significantly. ANOVA taking into account the 7 treatments according to the model $\Upsilon_{ijk} = \mu + \alpha_I + \beta_j + (\alpha\beta)_{ij} + \tau_k + \varepsilon_{ijk}$. NS, Not significant. *, **, *** $P \leq 0.05$, 0.01, and 0.001, respectively. NT, Not tested.

in controls (Table 2). Highest DSIs were recorded after inoculations with isolates A6m1, A3a1 and A10a3, with DSIs over three points higher than on un-inoculated control cloves.

Three weeks after inoculation of garlic, symptoms included water-soaked rotten roots, which eventually disintegrated. Rot progressed into the clove, resulting in a syrupy texture. No symptoms were observed on the aerial tissues. Overall, there was a significant difference in susceptibility ($P<0.05$) between the two cultivars, with cv. Garcua less susceptible to *F. proliferatum* than cv. Plamegar.

All *F. proliferatum* isolates caused disease on the two leek cv. tested (Table 2). Isolates A10a1 and A7a2 caused DSIs at least three points higher than control values. The two cultivars tested differed in susceptibility ($P<0.001$).

On onions, DSIs for all isolates tested were between 3.4 and 4.8, equivalent to almost 100% of roots with rot. Both onion cultivars tested were equally susceptible to all isolates of the pathogen. Symptoms on onion roots included a soft consistency, increasing transparency as the disease progressed, and finally disintegration of roots.

All isolates of *F. proliferatum* were pathogenic on chive and scallion. Isolates produced DSIs at least 1.3 above those of the controls.

There was no differential varietal response against one or more pathogen isolates for any cultivar of chive or scallion.

Fusarium proliferatum caused large and significant ($P<0.001$) dry weight reductions on onion and garlic cultivars (Table 3). On leek, five and three isolates caused significant decreases ($P<0.01$) in mass on the two tested cultivars, Carental and Genita. There was also a differential varietal response ($P<0.01$) against *Fusarium* isolates. In contrast, inoculations did not cause similar reductions in dry weights of chives or scallion seedlings; only two isolates of *F. proliferatum* caused severe decreases in the dry weights of seedlings of these species.

Evaluations of susceptibility of the *Allium* species to the pathogen demonstrated significantly different specific responses ($P<0.001$) against one

Table 3. Dry weight of onion (*Allium cepa*), garlic (*A. sativum*), leek (*A. porrum*), chives (*A. schoenoprasum)* and scallion (*A. fistulosum*) seedlings following artificial inoculation with six isolates of *F. proliferatum* originating from garlic.

Isolate code	Onion		Garlic		Leek		Chives		Scallion	
Control	31.1	a	616.9	a	55.6	a	35.4	b	60.7	b
A3a1	13	c	365.9	b	39.7	ab	17	ab	50.2	ab
A6m1	12.4	c	265.7	c	36.4	ab	16.5	ab	57.3	b
A4a1H	10.4	c	340.6	bc	28.8	bc	12.8	ab	40.2	a
A10a1	12.9	c	342.8	bc	11	c	16.5	ab	55.7	ab
A7a2	12.7	c	332.8	bc	15.7	c	13.7	ab	94.9	c
A10a3	18.5	b	362.3	b	24.1	bc	17.3	ab	45	ab
Significance	***	***	***	***	***					
Cultivar[a]	cv. 1, Albarracín cv. 2, Panter		cv. 1, Garcua cv. 2, Plamegar		cv. 1, Carental cv. 2, Genita		cv. 1, Cebollino		cv. 1, Cebolleta	
cv. 1	18.3	a	417.2	a	34.1	a	15.63		57.22	
cv. 2	13.5	b	333.4	b	26.3	b				
Significance	***	***	**	NT	NT					
Isolate × cv.	NS	**	NS	NT	NT					

[a] See Table 2.
ANOVA, See Table 2. NS, See Table 2.*, **, ***, See table 2. NT, See Table 2.

or more pathogen isolates (Table 4). Onion was the most susceptible host, with a dry weight reduction relative to uninoculated controls of up to 57.1%. Chives were the second most susceptible species, followed by garlic and leek, with average weight reductions of 55.9; 43.50 and 50.5% from controls, respectively. Scallion was the least susceptible species to the disease, with the lowest DSI value (Table 4) and also the lowest weight reduction after inoculation (4.1%).

Toxigenic potential

The six isolates of *F. proliferatum* produced FA over a wide range of concentrations, between 0.02–0.37 mM mL^{-1} culture fluids (Table 5). There was no correlation between FA production by *F. proliferatum* isolates and virulence on garlic, leek or scallions. In contrast, a positive linear correlation between FA production and virulence was observed in onion cv. Panter (R^2=0.3193, P=0.0145) and chives (R^2=0.3384, P=0.0113). For these two *Allium* species, greater virulence of the *F. proliferatum* isolate was associated with greater FA production in culture.

In PCR assays using primers based on a partial sequence of the *FUM1* gene, the expected *FUM1* fragment for *F. proliferatum* (FUM5P2/FUM5P2-R primers) was amplified from all isolates tested (A3a1, A6m1, A4a1H, A10a1, A7a2, A10a3).

Discussion

This paper is the first report of garlic rot caused by *Fusarium proliferatum* in the main garlic cultivation area of Europe. *F. proliferatum* was isolated from symptomatic garlic bulbs at all points in the production cycle, in all analyzed samples. The disease was previously reported, however, on Alliaceae in other production areas (Dugan *et al*., 2007; Stankovic *et al*, 2007; Dissanayake *et al*., 2009; Palmero *et al*., 2010).

Table 4. Multiway ANOVA for the evaluation of *Allium* species susceptibility (Disease Severity Index – DSI) following artificial inoculation with isolates of *F. proliferatum* originating from garlic.

Isolate code	DSI	
A3a1	3.30	a
A6m1	3.60	a
A4a1H	3.50	a
A10a1	3.50	a
A7a2	3.30	a
A10a3	3.40	a
Significance	NS	
Species tested		
Onion	3.60	a
Garlic	3.00	b
Leek	3.10	b
Chives	3.00	b
Scallion	2.70	c
Significance	***	
Isolate × Species	***	

ANOVA, See Table 2.
NS, See Table 2.
*, **, ***, See table 2.
NT, See Table 2.

Table 5. Fusaric acid production by isolates of *Fusarium proliferatum* from garlic (*A. sativum*).

Strain code	Mean Fusaric acid µM mL^{-1}	Standard error
A7a2	0.030994	0.000889
A3a1	0.037736	0.000663
A4a1H	0.368993	0.000663
A6w1	0.047979	0.00051
A10a3	0.023106	0.000769
A10a1	0.025956	0.002259

The PCR assays reported in this work allowed a rapid and accurate diagnosis of fumonisin-producing *Fusarium* species isolated from garlic. Supplementary data provided by these molecular analyses confirmed the identity of the isolates.

The pathogenicity of *F. proliferatum* isolates obtained from stored garlic was tested here for the first time. The presence of the pathogen in the cloves used for planting in the next planting season, coupled with a lack of information on the ability of this fungus to infect other *Allium* species, suggested that *F. proliferatum* may have a pernicious effect during the early stages of crop growth in the field. Stankovic *et al*. (2007) reported inoculations on garlic and onion, using isolates obtained from diseased plants in the field, although only a single cultivar of each crop species was inoculated. Galván *et al*. (2008) inoculated twelve plants per cultivar from seven *Allium* species screening for resistance but again only one isolate of *F. proliferatum* was used in that study.

The results obtained in the present work compared the effects of inoculation with six different isolates on five different *Allium* species and provided novel information on epidemiological aspects of *F. proliferatum* isolated from stored garlic. Moreover, the use of at least two different cultivars of leek, garlic and onions enabled the detection of possible differential varietal responses to the pathogen. All varieties tested were susceptible to *F. proliferatum* but there was some variation in response for leek and garlic varieties. Variations in varietal response to inoculation observed in the study, however, indicate differential varietal susceptibility which could be exploited for reducing losses in areas where the pathogen is established. A greater range of varieties must be tested, however, to determine if selection for resistance to *F. proliferatum* is a possibility. The inoculation of *F. proliferatum* isolates from diseased garlic on other *Allium* species provided further new information on the pathogenicity of the pathogen and its host range. Inoculation tests on garlic, onion, leek, chives and scallions showed that all 5 *Allium* species were attacked by *F. proliferatum*. These experimental results also demonstrated some differences in susceptibility between species.

In general *A. schoenoprasum* and *A. fistulosum* cultivars had the lowest DSIs; in contrast, *A. cepa* had the highest DSI scores. These results are in agreement with Galván *et al*. (2008) who stated that the aggressiveness of each isolate of *F. oxysporum* (3 isolates were tested) was dependent on specific isolate-cultivar combinations.

The clear susceptibility to *F. proliferatum* of leeks, chives and scallions suggests that propagules of the pathogen could find alternative hosts to garlic cultured in the same soils. Germination and seedling emergence were seriously affected in onion and leek after inoculation of un-germinated and pregerminated seeds with *F. proliferatum*, indicating the serious problems that this fungus poses to seedling growers.

FA production appears to be widely distributed in the genus *Fusarium* (Bacon *et al*., 1996, Capasso *et al*., 1996; Desjardins and Proctor, 2001; Amalfitano *et al*., 2002). FA production has also been reported in fumonisin-producing species, such as *F. proliferatum* and *F. verticillioides*, and trichothecene-producing species, such as *F. crookwellense* or in other species such as *F. solani* or *F. oxysporum*.

All isolates of *F. proliferatum* tested in the present work produced FA, although correlations performed with DSI data did not demonstrate any model which explained more than 35% of the variability observed. It is likely that other factors such as host species, cultivar or age of the isolate in terms of incubation time, have important roles in disease. Amplification of the *FUM1* gene indicated the presence of this gene and the potential ability of the isolates to produce fumonisins. A previous exhaustive analysis of fumonisin production carried out with *F. proliferatum* suggested that most isolates were able to produce fumonisins (Jurado *et al*., 2010). Complementary studies on the extraction of FA and fumonisins directly from garlic bulbs and from inoculated plants are required to determine the actual threat posed to humans by these mycotoxins. Although in the case of FA, the toxicity determined in a wide range of animals was apparently low (Voss *et al*., 1999; Bryden *et al*., 2001); however, fumonisins (particularly fumonisin B1) are considered highly toxic to humans and other animals (D'Mello *et al*., 1999; Dragan *et al*., 2001;). The occurrence of *F. proliferatum* on garlic may result in fumonisin contamination similar to *F. verticillioides* in other crops (Miller *et al*. 1995; Moretti *et al*. 1997; Jurado *et al*. 2010). Dissanayake *et al*. (2009) reported the fumonisin B1-producing ability of *F. proliferatum* strains isolated from scallions. The risk for human consumption of fumonisins and FA in garlic and other *Allium* species, however, requires further elucidation. *F. proliferatum* is one of the main fumonisin producing species in the *Fusarium* genus and, in the near future, the mycotoxigenic hazard of *Fusarium* infections in garlic should be determined.

An epidemiological survey based on the approach presented in this work is currently in progress to determine the effects of temperature on growth of the isolates and on fungal colonization of plants at different stages of the cultivation cycle. Studies on the efficacy of different fungicides against *F. proliferatum* on garlic and onion are also currently underway *in vitro* and in field trials.

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