Pathogenicity and fusaric acid production by *Fusarium proliferatum* isolated from garlic in Spain

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Abstract

Fusarium proliferatum has been reported on garlic in the north west USA, Spain and Serbia, causing as water-soaked tan lesions on cloves. Moreover, *F. proliferatum* is known to produce a range of toxins, including fumonisin B1, moniliformin, beauvericin, fusaproliferin and fusaric acid, which are implicated in pathogenesis. In this study six randomly selected *F. proliferatum* isolates from garlic were tested for pathogenicity and screened for fusaric acid production. Healthy seedlings of onion (*Allium cepa*), leek (*A. porrum*) and chives (*A. schoenoprasum*) and garlic clones (*A. sativum*) were inoculated . Onion seedlings and garlic clones were soaked in the conidial suspensions of each *F. proliferatum* isolate for 24 h and then planted in flats containing soil previously inoculated with the same isolate of *F. proliferatum*. Plants were maintained in a temperature and light-controlled greenhouse (12 h/12 h light/dark; 25/21ºC). The root and bulb/clove rot disease symptoms were graded into five classes following the method of Stankovick *et al.* (2007). A disease severity index (DSI) was calculated as the mean of three plants of each species and four test replicates. Symptoms on onion and garlic plants were observed three weeks after inoculation. The overall effects of isolate, host and variety were analyzed. Effects were significant for all the studied isolates. The correlations between isolate pathogenicity and production of FA are also discussed.

Introduction

Annual world garlic production is about 15,799,909 tons. Production in Spain is about 142,400 tons, where garlic is grown on 16,100 ha annually. Spanish garlic production is ranked first in the European Union and third in the world with exports of about 52,455 tons. Recent studies have identified, for the first time in Spain, the causal agent of cloves rot during storage (Palmero et al, 2010). Symptomatic cloves show internal tan rot progressed toward the clove apex, with occasional white mycelium in rot cavities. Isolates were identified as *Fusarium proliferatum* (T. Matsushima) Nirenberg. *F. proliferatum* was

previously reported on garlic in the northwestern USA (Dugan et al, 2007) and Serbia (Stankovic et al, 2007). There are few reports on the possibility that these same fungi could affect garlic plants during their field development or to other *Allium* crops present in the cultivation areas (onions, leeks, scallions and chives). For this reason, not only two commercial varieties of garlic but also commercial varieties of other Allium crops from the same area were inoculated.

On the other hand, Seefelder et al. (2002) have reported mycotoxins in garlic bulbs in Germany. Garlic could be consumed in fresh so the production of mycotoxins must be addressed.

In this study we have determined the amount of fusaric acid produced by the isolates. The possible correlation between the rates of fusaric acid production of the isolates with their pathogenicity in each of the hosts tested is also addressed. Such relation may open the way to control the disease by regulating fusaric acid gene expression.

Material and methods

Pathogenicity test: Six randomly selected *F. proliferatum* isolates from garlic were tested for pathogenicity and screened for fusaric acid production. Conidial suspensions used for the inoculum were adjusted to approximately 10^7 conidia ml^{-1}. Healthy seedlings of onion (*Allium cepa*), leek (*A. porrum*) and chives (*A. schoenoprasum*) were inoculated after cultivation in sterile soil for three weeks; garlic clones (*A. sativum*) were treated after two weeks of cultivation. Two different commercial varieties were used for each crop species. Onion seedlings and garlic clones were soaked in the conidial suspensions of each *F. proliferatum* isolate for 24 h and then planted in flats containing soil previously inoculated with the same isolate of *F. proliferatum*. Each flat was inoculated with 200 ml spore suspension (three replicate flats per isolate and onion/garlic/leek/chives cultivar seedlings). Plants were maintained in a temperature and light-controlled greenhouse (12 h/12 h light/dark; 25/21ºC). All tests were replicated four times. The root and bulb/clove rot disease symptoms were graded into five classes following the method of Stankovick *et al.* (2007). A disease severity index (DSI) was calculated as the mean of three plants of each species and four test replicates. Symptoms on onion and garlic plants were observed three weeks after inoculation.

Fusaric acid extraction and quantification: Fusaric acid (FA) extraction from *Fusarium proliferatum* strains was performed using a method modified from (Notz et al., 2002; and De Woort et al., 2004). FA in the extracts was analyzed using liquid chromatography – mass spectrometry (LC/MS) equipped with a C18 HD analytical column (250 mm x 4 mm) supplied by Agilent (USA). The LC apparatus comprised a Series 1100 LC pump Agilent

(USA), 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) and acidified water 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.). The retention time of Fusaric acid was 13 min at a flow rate 1 ml min^{-1}. FA was quantified based on a series of standard concentrations prepared with synthetic FA (Sigma –Aldrich) in methanol in the range 10–100 µg ml^{-1}. 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.

Results

Pathogenicity test: The overall effects of isolate, host and variety were analyzed. Effects were significant for all the studied isolates. Pathogenicity tests conducted on garlic, leek, onion, chives and scallions showed the pathogenic capacity of *F.proliferatum* strains on all these crops. All isolates tested produced disease in inoculated varieties. Results conform to those obtained by Stankovik (2007) using isolates from Serbia, although the virulence of the Spanish isolates seems to be higher.

Isolate code	Onion								Garlic							
	cv. Albararcin				cv. Panter				cv. Garcua				cv. Plamegar			
Control	1,9	±	0,2	a	1,7	±	0,2	a	1,2	±	0,1	a	1,5	±	0,2	a
A3a1	3,8	±	0,4	bcd	4,0	±	0,5	bc	3,6	±	0,5	bc	3,7	±	0,5	c
A6m1	4,2	±	0,3	c	4,1	±	0,7	bc	3,9	±	0,3	c	4,1	±	0,1	d
A4a1H	4,3	±	0,2	d	4,8	±	0,2	c	2,7	±	0,1	b	3,6	±	0,1	bcd
A10a1	3,9	±	0,3	bcd	3,8	±	0,3	b	2,7	±	0,6	b	3,1	±	0,3	bc
A7a2	3,6	±	0,4	bc	3,7	±	0,3	b	2,8	±	0,3	b	2,9	±	0,4	b
A10a3	3,4	±	0,2	b	3,7	±	0,7	b	3,2	±	0,7	bc	3,1	±	0,3	bc

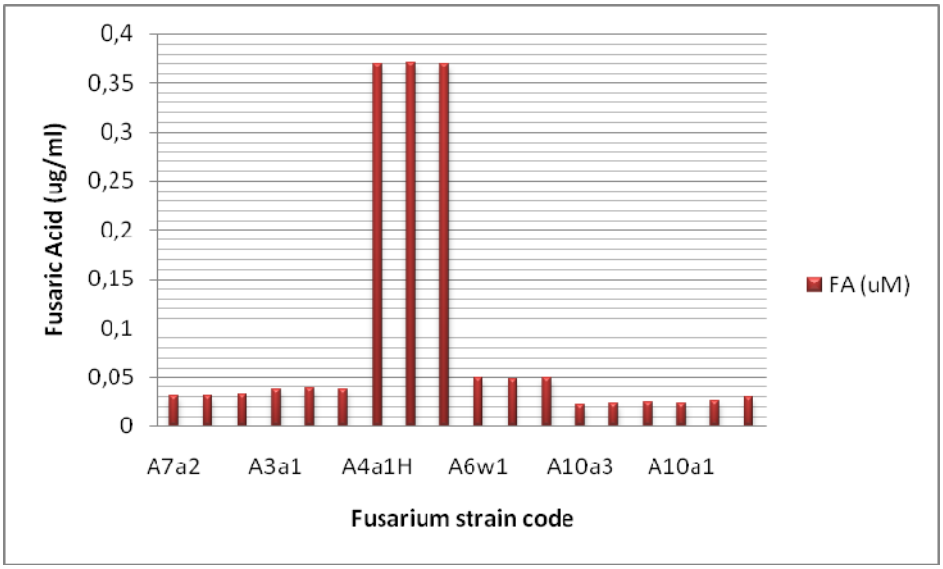
	Leek								Chives				Welsh onion			
	cv. Carental				cv. Genita											
Control	1,4	±	0,0	a	1,5	±	0,1	b	1,0	±	0,0	a	1,3	±	0,1	a
A3a1	2,9	±	0,2	b	2,4	±	0,2	c	3,0	±	0,4	b	2,6	±	0,3	bc
A6m1	3,0	±	0,1	bc	3,1	±	0,1	c	3,2	±	0,2	b	3,1	±	0,1	cd
A4a1H	3,5	±	0,2	cd	3,1	±	0,3	d	2,7	±	0,6	b	3,4	±	0,3	d
A10a1	4,3	±	0,2	f	3,9	±	0,2	cd	3,2	±	0,2	b	3,0	±	0,2	bcd
A7a2	4,1	±	0,2	ef	3,4	±	0,2	bc	3,7	±	0,5	bc	2,6	±	0,3	b
A10a3	3,6	±	0,4	de	2,9	±	0,5		4,5	±	0,4	c	3,0	±	0,2	bcd

This is the first report of the pathogenicity of *F. proliferatum* on leeks, chives and scallions. The results let us suggest that the propagules of the pathogen could find an alternative host on other alliaceous crops cultured in the area. Both germination and seedling emergence have been affected in onion and leek after inoculation with *F. proliferatum*. Indicating the serious problems that this fungus can lead to seedlings growers.

	Leek cv. Albarracín		**Leek cv. Panter**	
Isolate Code	**% emergence**	**% germination**	**% emergence**	**% germination**
Control	100	100	100	100
A3a1	0	21´74	0	20
A6m1	0	39´13	0	8
A4a1H	0	39´13	0	4
A10a1	18´75	52´17	45	24
A7a2	18´75	82´61	30	76
A10a3	6´25	21´74	10	4
	Onion cv. Royal		**Onion cv. Gennevillier**	
	% emergence	**% germination**	**% emergence**	**% germination**
Control	100	100	100	100
A3a1	0	21´74	0	20
A6m1	0	39´13	0	8
A4a1H	0	39´13	0	4
A10a1	18´75	52´17	45	24
A7a2	18´75	82´61	30	76
A10a3	6´25	21´74	10	4

Differences in varietal response against disease observed in the study allow us to suggest that there will be some differential varietal susceptibility.

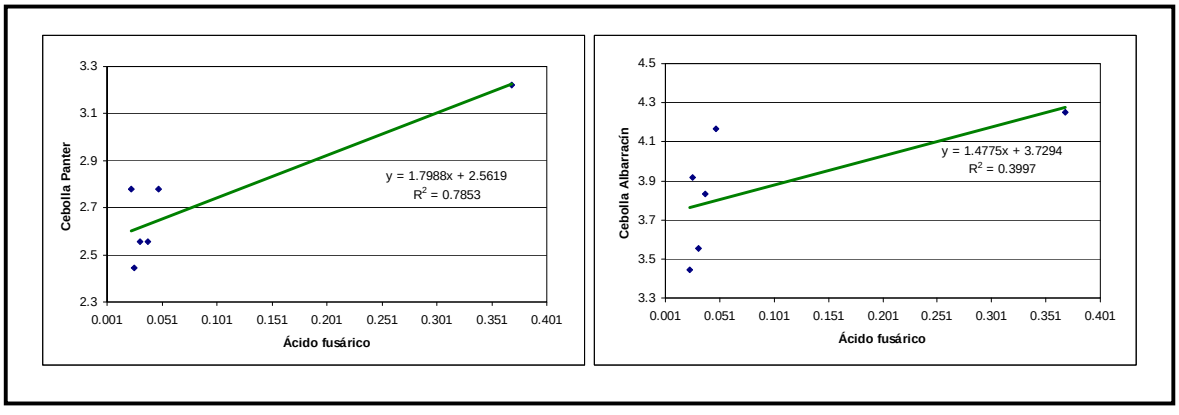
Fusaric acid quantification: The results showed that all six strains produced fusaric acid within a wide range of concentrations, 0.02–0.37 mM for FA. The amount of fungal secondary metabolite will probably differ according to the growth conditions and the plant host. Complementary studies on the extraction of fusaric acid directly from the garlic bulbs and from inoculated plants must be achieved. This is the first study carried out the Fusaric Acid-producing ability of *F. proliferatum* strains isolated from garlic in Spain. The information obtained is useful for assessing the risk of FA contamination in garlic.



The correlations between isolate pathogenicity and production of FA are also discussed. The comparison between fusaric acid production and pathogenicity of the isolates on garlic and leek varieties does not establish a direct relationship. Studies on mutations induced by UV light of *F.oxysporum* f.sp.*lycopersici* indicate that the ability to cause tomato wilt is not correlated with the production of fusaric acid (Kuo and Scheffer, 1964) On the other hand, there is a clear correlation between fusaric acid production and pathogenicity in onion and chives. Our results show a direct linear correlation. The greater the FA production, the greater pathogenicity of the isolates. In the variety Panter the determination coefficient indicates that the variation in production significantly (P = 0.033) explains 78% of variation in pathogenicity of the isolates.

Some authors suggested that the role of fusaric acid in the pathogenesis is closely linked to the eliciting effect of fumonisin produced by the fungus (Desjardins, 2006)

Tabla 41.- Relación entre la patogenicidad y el ácido fusárico en cebolla



Conclusion

5 different species of the genus *Allium* are likely to be attacked by *F.proliferatum*. All of the tested isolates were able to produce fusaric acid. The experimental results allow us to speculate with some specificity with respect to the plant genus.

References:

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W. Seefelder et al. J. Agr. Food Chem. 50: 2778. 2002

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