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Pathogenicity and genetic diversity of *Fusarium oxysporum* isolates from corms of *Crocus sativus*

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ABSTRACT

Crocus sativus is a triploid sterile plant and autumnal flowering geophyte with corms. As a subterranean organ, the corm is susceptible to diseases caused by fungi, bacteria, nematodes and viruses. *Fusarium* corm rot incited by *F. oxysporum* is the most destructive disease in saffron, having caused severe yield losses in saffron producer's countries. Infected plants die off early, resulting in reduction of corm yield, quality and flower and stigma production. Little is known about the *formae speciales* attacking saffron and less about the relationship among the fungus and the possible hosts. In this study we investigate the *formae speciales* which colonize saffron and their relationship with some members of iridaceae such as *C. vernus* (ornamental crocus), gladiolus and narcissus using pathogenicity tests; we determine whether different pathogenic isolates of *F. oxysporum* can be distinguished by ISSR, and we analyze the genetic relationships and variability among some isolates of these pathogens. We found two *formae speciales* iridiacearum and croci which attack different iridaceous crops and *Crocus* sp., respectively and we suggested the creation of a new *formae speciales* saffrani which shows only pathogenicity on saffron corms. From ISSR analysis, the unweighted paired group method with arithmetic averages cluster analysis (UPGMA) was used to discriminate the *F. oxysporum* isolates, we were able to differentiate among f. sp. from saffron corms and other *formae speciales* of *F. oxysporum* but not among *formae speciales* iridiacearum, croci and saffrani. Furthermore, we searched for *fum1*, *tri5*, *tri7* and *tri13* genes using PCR assays, however, all the isolates from saffron corms were negative.

1. Introduction

Crocus sativus is cultivated for its red style branches, which once dried, constitute saffron spice. Highly appreciated for its aromatic and flavoring properties, it is mainly used as a colorant for foodstuffs and is often used in the confectionary and liquor industries. Numerous studies have shown its implication in the reduction of cholesterol and triglyceride levels in the blood (Abdullaev and Frankel, 1999); its capacity to combat neural disorders (Abe and Saito, 2000), as well as being an attenuator for the adverse effects of cisplatin used in chemotherapy (Premkumar et al., 2003). Saffron has also used for medicinal purposes, is traditionally used as stimulant, aphrodisiac, antidepressant and anti-tumoral effects in various cellular models (Schmidt et al., 2007).

In general, the corms are planted during their dormant period in summer (late August) and the flowers emerge after 8–10 weeks in fall (October–November). Immediately after harvest, the stigmas are separated from the flower and dried. Over 85,000 flowers are needed to obtain just 1 kg of dried saffron stigma. After harvest, the crocuses are left to grow in the ground and let them grow throughout the winter. In spring, 4–10 new corms appear above the old ones, which wilt and eventually rot. In the third summer (July), new corms are harvested and subjected to chemical disinfestation treatment. Treated corms are dried and stored in the dark at room temperature until the next planting season (late August). As a subterranean organ, the corm is susceptible to diseases caused by fungi, bacteria, nematodes and viruses (López and Gómez-Gómez, 2009). Infected plants die off early, resulting in reduction of corm yield, quality and flower and stigma production.

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Several fungal species of the genera *Fusarium*, *Rhizoctonia*, *Penicillium*, *Aspergillus*, *Sclerotium*, *Phoma*, *Stromatinia*, *Cochliobolus*, and *Rhizopus* have been reported to be associated with saffron diseases (Ahrazem et al., 2010; Rubio-Moraga et al., 2013). Rot caused by *Fusarium oxysporum* is the most destructive disease in saffron, causing severe performance losses in most saffron fields (Cappelli, 1994). The main symptoms of the disease occur during the flowering period in which infected plants show a dropping, yellowing and wilting of shoots, basal and corm rot. The pathogen survives in infected corms and soil as mycelium, chlamydospores, macroconidia and microconidia (Brayford, 1996). Plants can become infected in the field at the time of germination of spores or mycelia, entering directly into the roots or through wounds in corms. The pathogen can be inserted into new growing regions through contaminated saffron corms (Cappelli and Di Minco, 1999). First detected in Japan (Yamamoto et al., 1954), the disease is currently widespread, causing substantial yield losses in several regions throughout the producer countries. Up to 50% of plant mortality was observed in the fields severely infected. Knowledge of the variation and distribution of pathogenic races of a plant pathogen is a crucial requirement for the efficient management of diseases (McDonald, 1997). In some pathosystems, race identification using pathogenicity testing requires a considerable effort and cost, providing much more limited information than neutral genetic markers. Selectively neutral genetic markers are particularly useful for identifying pathogenic races with strictly asexual reproduction, since no sexual recombination source occurs in the pathogen's genome (Milgroom and Fry, 1997). Natural populations of *Fusarium oxysporum* Schlechtend.:Fr contain pathogens that cause vascular diseases in a wide range of cultivated and uncultivated plants. Whereas species identification is based on morphological characteristics of the fungus, pathogenic *formae speciales* are classified based on their ability to attack a range of susceptible plants (Snyder and Hansen, 1940). The *formae speciales* and races are divided in accordance to their specific pathogenicity on group cultivars within a host.

Like many other plant pathogens, *F. oxysporum* secretes small proteins, commonly cystein-rich, to promote virulence by manipulating its plant host and suppressing host defense proteins (De Wit et al., 2009; Rep, 2005; Schmidt et al., 2013). The genes encoding these proteins are found to be proximal to transposable elements which have been considered to play a key role in the emergence of new virulence traits in many other plant pathogens (Schmidt et al., 2013). Transposable elements are usually flanked by inverted repeat sequences. Recombination between highly similar transposable elements leads to structural rearrangement and contributes to evolution of novel race and *formae speciales* (Feschotte and Pritham, 2007; Kang et al., 2001). This makes inter-simple sequence repeat (ISSR) markers very suitable for detecting intraspecific variability, particularly fungi such as *F. oxysporum* lacking sexual reproduction (Taylor et al., 1999). The ISSR technique has been applied extensively to the detection and genetic characterization of fungal pathogens, including the differentiation of several breeds of *formae speciales* in *F. oxysporum*, i.e., *formae speciales* cubense (E.F. Smith) W.C. Snyder & H.N. Hansen (Bentley et al., 1994), dianthi (Prill. & Delacr.) W.C. Snyder & H.N. Hansen (Manulis et al., 1994; Migheli et al., 1998), pisi (Linf.) W.C. Snyder & H.N. Hansen (Grajal Martin et al., 1993) and vasinfectum (Atk.) W.C. Snyder & H.N. Hansen (Assigbetse et al., 1994).

Despite the severity of the disease little is known about the *formae speciales* attacking the saffron and much less about how many races could exist. The present study was designed for a threefold purpose: to investigate the *formae speciales* which colonize saffron and their relationship with some members of iridaceae such as *C. vernus* (ornamental crocus), gladiolus and narcissus using pathogenicity tests; to determine whether different pathogenic isolates of *F. oxysporum* can be distinguished by ISSR, and to analyze the genetic relationships and variability among representative isolates of these pathogens. We also look for some fumonisin and trichothecenes genes using PCR assays and HPLC analyses.

2. Materials and methods

2.1. Collection and isolation of Fusarium species

Fungi were isolated from 50 infected corms grown under field conditions in Minaya and Tarazona de la Mancha, Spain. Several fragments of symptomatic corms were superficially disinfected and inoculated in different culture media: general medium (PDA), *Fusarium* selective medium and King B medium for bacteria. After 5 days of incubation at 25 °C, colonies were examined visually and microscopically for morphological characteristics of isolates. Subsequently, single-spore cultures of different isolates were used to test their pathogenicity.

In addition two isolates of *F. oxysporum* f. sp. radici-lycopersici, two isolates of *F. oxysporum* f. sp. lycopersici, and one isolate of *F. oxysporum* f. sp. gladioli from University of Aberdeen (kindly provided by Dr. S. Woodward) and one isolate of *F. oxysporum* f. sp. radici-cucumerium and one isolate of *F. oxysporum* f. sp. melonis were used. The isolates were stored at 4 °C in PDA.

2.2. Pathogenicity test and data analysis

Healthy corms of saffron, ornamental crocus (*Crocus vernus* cv. Pickwick and cv. Jeanne d' Arc), daffodil (*Narcissus* sp.) and gladiolus (*Gladioulus* × grandiflorus cv. Peter Pears and *Gladiolus* × hortulanus cv. White Friendship) were used for pathogenicity testing. Corms were previously disinfected with 5% sodium hypochlorite for 15 min, followed by three washes with sterile water. We performed two repetitions of three plants for each combination isolated × cultivar or species. The corms were planted in sterile substrate and held for 3 weeks with controlled temperature and light (12/12 h light/dark, 25/21 °C). Seven isolates of *F. oxysporum* were selected from the damaged corms while one isolate of *F. oxysporum* f. sp. gladioli provided by Dr. Woodward (University of Aberdeen, Scotland) was used as a positive control of inoculation.

The inoculum consisted of a suspension of conidia obtained after a week in potato–glucose medium, stirred at 150 rpm. To remove the mycelium, the suspension was filtered with a double layer of cheesecloth. The conidia suspension was adjusted to 10^7 conidia/mL and used for inoculation of plant material. The plant roots were immersed for 24 h in the suspension of conidia (200 mL suspension) and then transplanted back and kept under the same conditions of temperature and light for 3 weeks. Root symptoms were then assessed. According to symptoms observed a scale of six categories were defined according to root rot: 0 = no symptoms, 1 = <20% of roots with rot, 2 = 21–40% of roots with rot, 3 = 41–60% of roots with rot, 4 = 61–80% of roots with rot, 5 = 81–99% of roots with rot, 6 = 100% of roots with rot. The disease severity rating (DSR) of each plant was calculated. Three plants were inoculated with each isolate in each experiment, which was conducted twice.

An analysis of variance (ANOVA) was performed to determine the effects of DSR of the isolates and cultivars. Comparison tests among the isolates and their hosts were carried out using Duncan's multiple ranges ($P < 0.05$) by Centurion StatsGraphics XV.II program (Statistical Graphics Corp. 195, Herndon, VA). The experimental data were analyzed according to the linear model $Yijk = aI + \mu + \beta j + (\alpha\beta)ij + \tau k + Yijk\varepsilon ijk$, where ijk is the observation, μ is the mean of DSR, aI is the corresponding isolate effect, βj is the effect of growing (in the analysis of ornamental gladiolus and crocus), $(\alpha\beta)ij$ is the effect of interaction among isolates × cultivars, τk is the experimental effect and εijk is the experimental error.

Table 1
Primers name and sequences of ISSR markers and mycotoxin genes.

Name	Primers
ISSR1	TCCTCCTCCTCCTCCTCCC
ISSR4	TGGTGGTGGTGGTGGTGGC
ISSR6	GAGAGAGAGAGAGAGAG
ISSR9	ATCATCATCATCATCATCATCATCATCATCG
ISSR10	ACACACACACACACACC
ISSR11	CTCTCTCTCTCTCTCTT
ISSR12	TTGTTGTTGTTGTTGTTGC
ISSR14	AGTGAGTGAGTGAGTGAGTGA
ISSR16	HBHGAGGAGGAGGAGGAG
ISSR17	DBDBCACCACCACCACCAC
ISSR18	DBDBCCACCACCACCACCA
ISSR20	DHBCGACGACGACGACGA
ISSR21	BDBACAACAACAACAACA
ISSR23	HBDBGACCGACCGACCGACC
ISSR26	GTGTGTGTGTGTGTGTYG
ISSR30	ACACACACACACACACYT
ISSR32	ACACACACACACACACYG
ISSR34	TGTGTGTGTGTGTGTGRC
ISSR39	ATGATGATGATGATGATG
ISSR40	CCGCCGCCGCCGCCGCCG
ISSR41	CTCCTCCTCCTCCTCCTC
ISSR49	CCCTCCCTCCCTCCCT
ISSR50	CACACACACACACACARC
ISSR51	CACACACACACACACART
ISSR54	CTCTCTCTCTCTCTCTRG
ISSR55	CTCTCTCTCTCTCTCTRC
ISSR56	CTCTCTCTCTCTCTCTRA
ISSR59	GAGAGAGAGAGAGAGAYT
ISSR65	GAGAGAGAGAGAGAGAA
FUM1F1	CACATCTGTGGGCGATCC
FUMR2	ATATGGCCCCAGCTGCATA
TRI13_NIVF	CCAAATCCGAAAACCGCAG
TRI13_NIVR	TTGAAAGCTCCAATGTCGTG
TRI13_DONF	CATCATGAGACTTGTKCRAGT
TRI13_DONR	GCTAGATCGATTGTTGCATTGAG
TRI7F	TGCGTGGCAATATCTTCTTCTA
TRI7R	TGTGGAAGCCGCAGA

2.3. Cellophane assay

For the cellophane penetration assay, DGasn plates were covered with sterile cellophane and inoculated into the center of the plate with 5 mm diameter mycelial disks of each strain. After the plates were incubated at 30 °C in the dark for 9 days, the cellophane disks were then removed, and the mycelia were separated from them (Díaz-Sánchez et al., 2012). The plates and discs were then photographed with a digital camera.

2.4. *fum1, tri5, tri7 and tri13* detection and extraction of trichothecenes

In order to determine the presence of *fum1*, *tri5*, and *tri13* all the isolates were tested PCR using primers listed in Table 1 (Stepien et al., 2013). Thermal cycling conditions were as follows: initial denaturation: 2 min 94 °C then 20 s 94 °C, 30 s 52 °C, 2 min 72 °C, 40 cycles and a final elongation step at 72 °C for 10 min. Amplified products were resolved by gel electrophoresis in a 1.5% agarose gel. The positive reactions were verified by DNA sequencing using an automated DNA sequencer (ABI PRISM 3730xl; Perkin–Elmer) from Macrogen. All reactions were performed three times.

Liquid cultures were obtained by inoculating a suspension of spores from all isolates obtained from saffron corms on DG medium (Candau et al., 1992), and keeping them under stationary conditions at 27 °C for 2 weeks. After removal of mycelia by centrifugation, the mycelia were lyophilized and then ground. 50 mL liquid cultures and lyophilized grounded mycelia were defatted with *n*-hexane, and then extracted 2 times with 50 mL chloroform. The extracts were evaporated to dryness and the residues were taken up with 1 mL HPLC-grade methanol. Before injections into HPLC the extracts were filtered through a 0.45 μm Millipore. HPLC separations were performed using a C18 Ascentis, 25 × 4.6, particle size 5 μm column (Supelco, Sigma–Aldrich) following the same conditions described by Visconti and Bottalico (1983).

2.5. DNA extraction and molecular identification

About 100 mg of mycelium was scraped from a Petri dish containing PDA, and DNA extracted from it using the DNeasy Plant Mini kit (QIAGEN) according to the manufacturer's instructions. For ISSR analysis, 15 and 30 ng of genomic DNA were amplified in a volume of 25 μL containing 10 mM Tris–HCl pH 9.0, 1.5 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP, dTTP, 0.4 μM primer, and 1 unit of Taq DNA polymerase by means of a thermal cycler (MJ-Mini, BioRad). The cycling program began with an initial 2 min at 94 °C followed by 40 cycles at 94 °C for 45 s, 48–62 °C for 45 s and 72 °C for 2 min plus a final 10 min at 72 °C and storage at 4 °C.

To amplify fumonisin and trichothecen genes, 10–20 ng of genomic DNA as template, 12.5 pmol of each forward and reverse primers, 2.5 mmol/L of each dNTPs and 1 unit of Taq DNA polymerase were used. The PCR conditions were as follows: 2 min at 94 °C followed by 40 cycles at 94 °C for 45 s, 58–62 °C for 30 s and 72 °C for 2 min plus a final 10 min at 72 °C and storage at 4 °C.

Primer sequences are shown in Table 1. A negative control was added in each run to test contamination in all analyses. Amplification products were separated by electrophoresis in 2% agarose gel containing 1 μg/mL ethidium bromide and TAE buffer.

2.6. Data analysis

We analyzed ISSR data based on both allele and phonotypic frequencies. Polymorphic bands were selected at the 95% level (two-tailed test) for use in further analyses. Data matrices were analyzed using POPGENE version 1.32 (Popgene-version 1.32, 1997) and GenAlEx 6.41 (Peakall and Smouse, 2006). The distance matrices were then used to construct a dendrogram by the unweighted pair group method with arithmetic mean (UPGMA) using the Tools for Population Genetic Analysis (TFPGA) program (Miller, 1997).

3. Results and discussion

As a subterranean organ, corms are prone to fungi attack due to permanent contact with soil, high temperature and soil humidity, plus the high water content of this organ (Ahrazem et al., 2010). The corm rot incited by *F. oxysporum* is the most destructive disease in saffron, having caused severe yield losses in Italy (Cappelli, 1994), Japan (Yamamoto et al., 1954), India (Shah and Srivastava, 1984) and Spain (García-Jiménez and Alfaro-García, 1987; Rubio-Moraga et al., 2013).

In Spain, *F. oxysporum* has been detected in many different saffron cultivation areas generating the highest losses in corm yield (Ahrazem et al., 2010) (Fig. 1). To identify *F. oxysporum* isolates from 50 damaged saffron corms sampled from Minaya and Tarazona de la Mancha (Spain), the criteria described by Leslie and Summerell (2006) have been followed. The mycelium of *Fusarium* isolates on PDA culture medium was abundant showing a purplish white color. After a week *F. oxysporum* produces a pale to dark violet pigment in the agar. Macroconidia are produced abundantly on sporodochia, showing three septa with half-length, slightly curved, thin walls while the apical cell is curved and has a slight hook and heel basal cell. The microconidia are oval or slightly elliptical and unicellular and are formed on short unbranched conidiophores called "false head" which are clusters of microconidia. The chlamydospores are

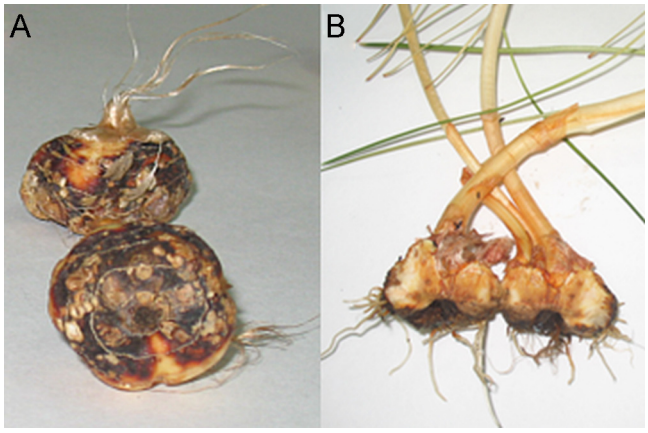

Fig. 1. Saffron corms collected from outfields at an advance stage of damage. (A) Damaged saffron corms sampled from Minaya (Spain) infected with *Fusarium oxysporum*. (B) Longitudinal section of affected corms.

Table 2
ANOVA of DRS from the isolates of *F. oxysporum* isolates from damaged saffron corms.

Species	DRS
Saffron	5.54 c
Gladiolus × hortulanus cv. White Friendship	2.67 a
Gladioulus × grandiflorus cv. Peter Pears	2.67 a
Narcissus	2.19 a
Crocus vernus cv. Pickwick	4.07 b
Crocus vernus cv. Jeanne d' Arc	4.07 b
Significance	*
Isolate × species	*

Means followed by the same letter were not significantly different. NS: no significance.
* $P \leq 0.001$.

formed abundantly in nutrient-poor medium in 2 weeks, individually or in small groups.

Seven isolates of *F. oxysporum* named Ox2, Ox3, Ox4, Ox6, Ox7, Ox8 and Ox10, were selected based on slight phenotypical differences in order to perform the pathogenicity tests on saffron, gladiolus, narcissus and ornamental crocus together with *F. o.* gladioli. Three weeks after inoculation, the plants were examined visually scoring the yellowing and wilts. Later, corms were taken out to assess skin lesions and internal rot. Plants belonging to uninoculated controls did not show any symptoms after the culture period. After 3 weeks of inoculation, the isolates induced the onset of symptoms typically caused by *F. oxysporum*. Analyzed isolates caused a higher disease severity rating (DSR) in saffron and ornamental crocus than in gladiolus and narcissus (Table 2).

In saffron, all isolates tested produced significantly higher rates than uninoculated controls ($P < 0.001$). DSR obtained after inoculation with all saffron isolates tested were always significantly higher than the DSR from the isolate *F. oxysporum* f. sp. gladioli (Table 3). After 3 weeks of inoculation, saffron corms showed clear symptoms of wilting and rotten roots. In gladiolus plants, DSR observed were higher especially with the isolates Ox3 and Ox6. In general, no significant differences were observed ($P > 0.05$) in susceptibility between the two cultivars analyzed (cv. Peter Pears and cv. White Friendship). On the other hand, in narcissus, four isolates produced rot in roots, although much lower values were recorded when comparing them with those observed in saffron. The isolate of *F. oxysporum* f. sp. gladioli (used as a positive control) seriously damaged the inoculated plants. Regarding the inoculation of ornamental crocus, six of the seven isolates from saffron caused disease after inoculation in the two cultivars of *Crocus vernus* (Table 3). DSR varied between 4.5 and 5, the equivalent of almost the entire rotted root system. McClellan (1945) observed that isolates from gladioli were also pathogenic to crops of nine other genera of the Iridaceae, but that isolates from iris were not pathogenic to gladiolus. Apt (1958), whose research was based on cross-inoculation experiments, proposed the name *F. oxysporum* f. sp. gladioli for the pathogens of the genera *Gladiolus*, *Iris* and *Crocus*. Since then, it has been generally accepted that all isolates of *F. oxysporum* causing diseases in iridaceous crops belong to the *formae speciales* gladioli (Boerema and Hamers, 1989; Brayford, 1996; Linderman, 1981; Nelson et al., 1981). Later on, Roebroeck (2000) studied the pathogenicity of *F. oxysporum* on *Crocus* and other crops such as *Gladiolus*, *Iris* and *Freesia*, pointing out the presence of 10 different pathotypes. From the pathogenicity tests, the author proposes to erect a new *formae speciales*, f. sp. iridiacearum, accommodating all pathogenic *F. oxysporum* strains from iridaceous crops. One exception has been made for isolates causing so-called 'pseudorust' symptoms in some cultivars of *Crocus*. These isolates were considered distinct from isolates causing 'corm rot' and assigned to *F. oxysporum* f. sp. croci (Boerema and Hamers, 1989; Roebroeck, 2000). Our findings which show that the Ox2 isolate was not pathogenic to the tested gladiolus and narcissus, support the status of this isolate as a separate *formae speciales*, while the isolate Ox4 was not pathogenic to any of the iridaceae tested crops except *C. sativus*. This might justify the creation of a new *formae speciales* saffrani. Our data support the classification suggested by Roebroeck (2000) since designating *F. oxysporum* from other iridaceous crops as natural hosts as f. sp. gladioli could be confusing.

About the races infecting these crops, Roebroeck (2000) found out that all the isolates showing pathogenicity against *Crocus* spp. are included in race 3 while the isolates that are infecting members of Iridaceae are grouped in race 4. Following this classification, Spanish isolates used in this study could be classified as *F. oxysporum* f. sp. iridiacearum race 3 (isolates Ox7, Ox8, and Ox10) and race 4 (Ox3 and Ox6). The different pathogenic phenotypes can be classified as physiologic races within f. sp. iridiacearum even though, races within this *formae speciales* do not refer to cultivar-level specificity.

As far as we know, the only causal agent of basal rot in narcissus is *F. oxysporum* f. sp. narcissi. This pathogen causes rotting of bulbs in the soil and also in storage, being the major cause of phytosanitary failure in bulbs (Linfield, 1994). *F. oxysporum* f. sp. narcissi exists in the soil as chlamydospores which, in favorable conditions, can survive for up to 20 years. However, for practical reasons, growers may be able to achieve only 5–7 year rotations. Spores carried on the outside of both diseased and healthy bulbs together with chlamydospores in the soil are the main reservoir for infection. Seemingly, this is the first report showing that isolate *F. oxysporum* f. sp. iridiacearum is pathogenic in narcissus. When making a decision for crop rotation it is not recommendable using these cultures together in a crop rotation plan. From the inoculation of the two cultivars of gladiolus (Peter Pears and White Friendship), the data indicated that no significant differences in varietal susceptibility have been observed.

To test the virulence functions in the isolates from saffron corms, we measured the capacity to penetrate a cellophane membrane on agar medium supplemented with asparagine. Cellophane degradation implies the activity of extracellular hydrolytic enzymes, such as cellulases. Observations showed that Ox2, Ox3, Ox4 and Ox6 penetrated across the cellophane on DGasn agar and reached the agar surface after 5 days of incubation at 30 °C. Ox7, Ox8 and Ox10 were unable to cross the cellophane disks after 9 days of growth (Fig. 2). The difference in the capacity to penetrate cellophane was noticeable when mycelia were removed from the cellophane sheet; it was difficult to detach the positive strains mycelia from the cellophane surface while the negative ones were readily separated. There is a poor correlation of cellophane degradation with pathogenicity on

Table 3
DRS obtained from saffron, gladiolus, narcissus and *Crocus vernus* after inoculation with *F. oxysporum* from saffron corms and *F. o.* gladioli.

Species	Saffron	Gladiolus	Narcissus	*Crocus vernus*
Control	3.17 a	1.50 ab	0.75 ab	1.13 a
Ox2	5.84 b	1.17 a	0.75 ab	5.00 c
Ox3	6.00 b	4.92 d	3.50 cd	4.67 c
Ox4	5.67 b	1.33 a	0.25 a	1.58 a
Ox6	5.84 b	4.42 d	5.00 d	5.00 c
Ox7	5.84 b	2.83 c	2.25 bc	5.17 c
Ox8	6.00 b	2.50 bc	2.50 c	4.92c
Ox10	6.00 b	2.67 c	2.50 c	5.08 c
F. o. gladioli	3.00 a	1.25 a	5.00 d	2.88 b
Significance	***	***	***	***
Cultivar (Cv)		1: Peter Pears; 2: White Friendship		1: Jeanne d'Arc; 2: Pickwick
Cv1		2.88 a		4.50 a
Cv2		2.79 a		4.48 a
Significance	NT	NS	NT	NS
Isolate × Cv	NT	**	NT	**

Means followed by the same letter were not significantly different. NS: no significance.
** $P \leq 0.01$.
*** $P \leq 0.001$.

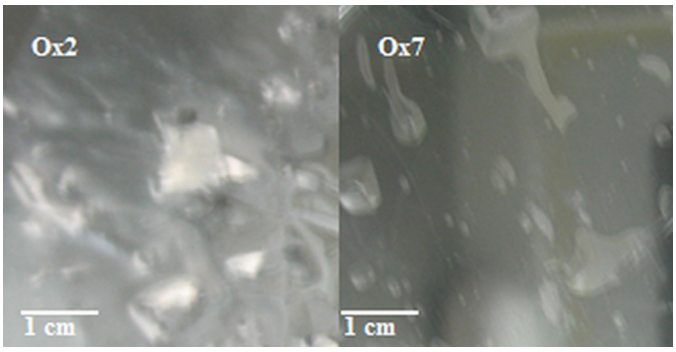

Fig. 2. Cellophane membranes after separation of Ox2 and Ox7 strains.

iridaceae crops by *F. oxysporum*. Díaz-Sánchez et al. (2012) studied the regulation of the polyketide synthase gene responsible for fusarin production in *Fusarium fujikuroi*, using mutants of the *fusA*. The *fusA* mutants were less efficient than the wild type at degrading cellophane on agar cultures; however, their capacities to grow on plant tissues were not affected. The authors concluded that fusarin, or a fusarin degradation product, could play a regulatory role on the activity of cellulases or other hydrolytic enzymes, either on gene expression or enzymatic activity. The poor correlation obtained might be explained by the existence of different physiological traits of our isolates which might affect the expression pattern of the hydrolytic enzymes.

According to the Food and Agriculture Organization of the United Nations (FAO), it is estimated that up to 25% of the world's food crop is contaminated with mycotoxins (FAO, 2013). It is well known that *Fusarium* species are able to produce certain toxins such as fumonisin, enniatin, beauvericin, fusarin, moniliformin, fusaric acid, fusaproliferin and trichothecenes (Desjardins, 2006). Among these mycotoxins, thrichothecenes and fumonisin are of greatest concern to food and feed safety. Trichothecenes are tricyclic sesquiterpenes and some *Fusarium* species can produce type A and/or the type B. Type A toxins, such as T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol are more acutely toxic than type B trichothecenes such as deoxynivalenol and nivalenol. However, nivalenol is present in more chronic toxicoses (Dinolfo et al., 2012; Prelusky et al., 1994). The main region containing genes involved in trichothecene biosynthesis is the TRI gene cluster, comprising 12 genes (*tri8*, *tri7*, *tri3*, *tri4*, *tri6*, *tri5*, *tri10*, *tri9*, *tri11*, *tri12*, *tri13* and *tri14*). Nivalenol production required *tri13* and *tri7* genes that produce the acetylation and oxygenation of the oxygen at C-4 to produce nivalenol and 4-acetyl nivalenol, respectively (Lee et al., 2009).

Fumonisins are associated with a number of mycotoxicoses, including (Nelson et al., 1993) human esophageal cancer, which has also been epidemiologically correlated with consumption of fumonisin contaminated maize in certain areas of South Africa and China (Marasas, 1996). *Fusarium oxysporum* is generally considered as a fumonisin-nonproducing species, although, two producing isolates of this fungus have been identified (Seo et al., 1996; Sewram et al., 2005). In order to investigate whether these isolates are or not mycotoxins producers, HPLC analysis were carried out by comparing the UV spectra of substances eluting at the retention time of fumosin, desoxynivalenol, nivalenol and 4-acetyl nivalenol with spectra of authentic standards, neither of these mycotoxins were detected in *Fusarium* isolates. The fact that no mycotoxines were extracted in any isolates of *Fusarium* may be explained by differences in extraction or culturing of the isolates or by the presence of a truncate gene in these strains (Fanelli et al., 2012; Kokkonen et al., 2010; Vogelgsang et al., 2008). To confirm these results PCRs using the primers listed in Table 1 were performed. None of the targets encoding *tri5*, *tri7* and *tri13* were detected in any of the isolates. The *FUM 1* encoding the polyketide synthase from the fumonisin biosynthetic gene cluster was not detected in any of the analyzed samples. This result is in agreement with those published up to date, since the strains examined from *F. oxysporum* are considered as non-producing fumonisins. Strain *F. oxysporum* O-1890 has the *FUM* genes and has the ability of producing primarily C fumonisins rather than B fumonisins (Proctor et al., 2004; Seo et al., 1996).

To gain insights into the identification of isolates from saffron corms, a set of ISSR primers have been used to determine whether the data obtained by pathogenicity tests are supported by molecular analysis or not. Out of 96 primers, 29 primers were able to produce robust and clear bands. A total of 167 bands were obtained, of which 99.40% polymorphic loci were scored. The total number of amplified bands with every primer varied from 3 with primer ISSR10, to 10 with primer ISSR50. As a representative, the amplification of the studied isolates with primer ISSR32 is shown in Fig. 3.

In order to assess the genetic relatedness between *Fusarium* isolates included in this study, the distance matrix was calculated based on the obtained fingerprints and was found to range from 0.066 to 0.494. Using TFPGA software, a phylogenetic analysis was carried out and a dendrogram was constructed using only the same isolates selected in pathogenicity tests. The dendrogram of ISSR analyses grouped the 14 isolates into two clusters (Fig. 4). Cluster I contained eight isolates and cluster II contained six isolates of *Fusarium*. The first cluster grouped all the Fusaria isolated from

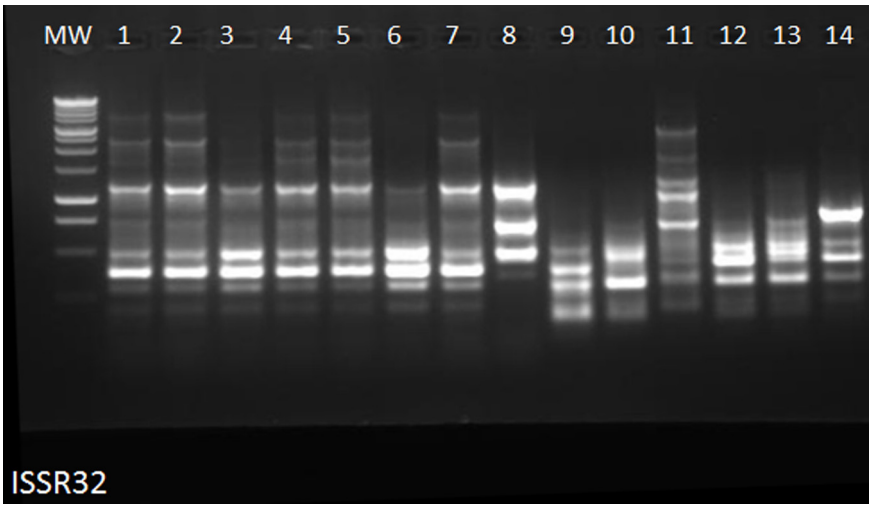

Fig. 3. Amplification profile using ISSR32 primer. M_w: Molecular weight (1 kb, Promega), 1: Ox2; 2: Ox3; 3: Ox4; 4: Ox6; 5: Ox7; 6: Ox8; 7: Ox10; 8: *F. o.* sp. Gladioli; 9: *F. o.* sp. radicis-cucumerinum; 10: *F. o.* sp. Melonis; 11: *F. o.* sp. Lycopersici; 12: *F. o.* sp. radicis-lycopersici; 13: *F. o.* sp. lycopersici and 14: *F. o.* sp. radicis-lycopersici.

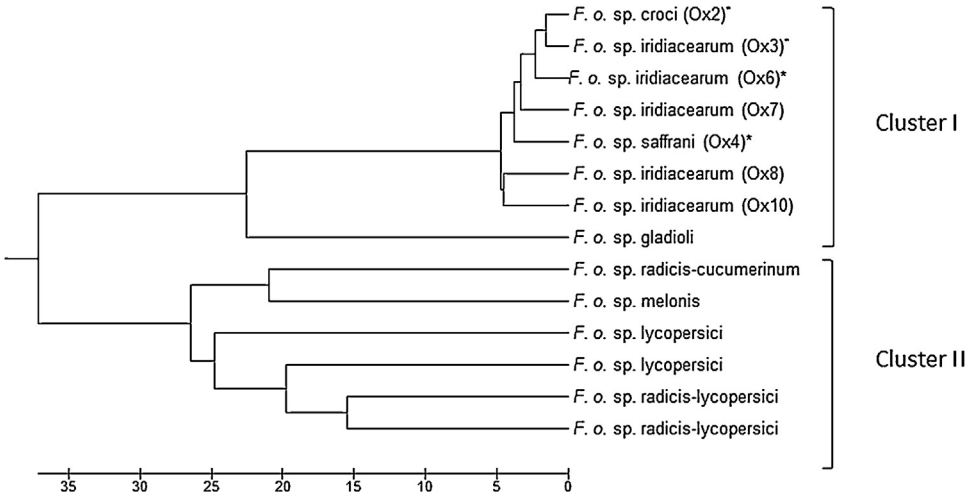

Fig. 4. Dendrogram of ISSR showing relationships among 14 *Fusarium* spp. isolates. Genetic distances were obtained by the UPGMA method. (*) degrading cellophane strains.

saffron (Ox2, Ox3, Ox4, Ox6, Ox7, Ox8 and Ox10) plus the isolate of *F. o.* gladioli and the second cluster the rest of the species. The dendrogram is validated since in cluster II the isolates of *F. o.* f. sp. *lycopersici* were grouped together in the same clade and also those from f. sp. *radicis-lycopersici*. In cluster I the isolates from saffron are clearly differentiated from *F. o.* gladioli. From ISSR results, the isolates were not grouped by races neither by specific hosts following the classification resulting from the pathogenicity tests. Comparing the data from ISSR and those obtained by pathogenicity tests, Ox2 and Ox3 were clustered together, nevertheless, according to pathogenicity tests results, Ox2 isolate affected only crocus and was considered as a *F. oxysporum* f. sp. croci, while Ox3 isolate was able to colonize other iridaceae plants and was classified as a *F. oxysporum* f. sp. iridiacearum.

4. Conclusion

Taken as a whole the obtained results demonstrate that three different *formae speciales* iridiacearum, croci and saffrani are present in saffron corms rot caused by *F. oxysporum*, the first f. sp. infects all the iridaceae crops tested, the second one attacks *Crocus* sp. species and the last one is pathogenic to saffron. Differences on cellophane degradation response were obtained suggesting different physiological traits on virulence of our isolates. Using ISSR marker, these isolates were clearly separated from the other *formae speciales* but not among them. None of the mycotoxins studied were detected neither by HPLC analysis nor by PCR assay. These results should be confirmed using *F. oxysporum* isolated from other saffron from different regions.

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References

Abdullaev, F., Frankel, G., 1999. Saffron in biological and medicinal research. In: Negbi, M. (Ed.), Saffron: *Crocus sativus* L. Harwood Academic Publishers, Amsterdam, pp. 103–113.

Abe, K., Saito, H., 2000. Effects of saffron extract and its constituent crocin on learning behaviour and long-term potentiation. Phytother. Res. 14, 149–152.

Ahrazem, O., Rubio-Moraga, A., Castillo-Lopez, R., Trapero-Mozos, A., Gómez-Gómez, L., 2010. *Crocus sativus* pathogens and defence responses. In: Husaini, A.M. (Ed.),

Functional Plant Science and Biotechnology. Special Issue: Saffron. Global Science Books, Ltd., London.
Apt, W.J., 1958. Studies on the *Fusarium* Diseases of Bulbous Ornamental Crops. Washington State University, Pullman, pp. 88.
Assigbetse, K.B., Fernández, D., Dubois, M.P., Geiger, J.P., 1994. Differentiation of *Fusarium oxysporum* f. sp. vasinfectum races on cotton by random amplified polymorphic DNA (RAPD) analysis. Phytopathology 84, 622–626.
Bentley, S., Pegg, K.G., Dale, J.L., 1994. Optimization of RAPD-fingerprinting to analyze genetic variation within populations of *Fusarium oxysporum* f. sp. cubense. J. Phytopathol. 142, 64–78.
Boerema, G.H., Hamers, M.E.C., 1989. Checklist for scientific names of common parasitic fungi, Amaryllidaceae and Iridaceae. Neth. J. Plant Pathol..
Brayford, D., 1996. *Fusarium oxysporum* f. sp. gladioli. Mycopathologia 133, 47–48.
Candau, R., Avalos, J., Cerdá-Olmedo, E., 1992. Regulation of gibberellin biosynthesis in *Gibberella fujikuroi*. Plant Physiol. 100, 1184–1188.
Cappelli, C., 1994. Occurrence of *Fusarium oxysporum* f. sp. gladioli on saffron in Italy. Phytopathol. Mediterr. 33, 93–94.
Cappelli, C., Di Minco, G., 1999. Results of a triennial study on saffron diseases in Abruzzi. Inf. Fitopatol. 49, 27–32.
De Wit, P.J., Mehrabi, R., Van den Burg, H.A., Stergiopoulos, I., 2009. Fungal effector proteins: past, present and future. Mol. Plant Pathol. 10, 735–747.
Desjardins, A.E., 2006. *Fusarium* Mycotoxins: Chemistry, Genetics and Biology. American Phytopathological Society (APS Press), St Paul, Minnesota.
Díaz-Sánchez, V., Avalos, J., Limón, M.C., 2012. Identification and regulation of fusA, the polyketide synthase gene responsible for fusarin production in *Fusarium fujikuroi*. Appl. Environ. Microbiol. 78, 7258–7266.
Dinolfo, M.I., Barros, G.G., Stenglein, S.A., 2012. Development of a PCR assay to detect the potential production of nivalenol in *Fusarium poae*. FEMS Microbiol. Lett. 332, 99–104.
Fanelli, F., Schmidt-Heydt, M., Haidukowski, M., Geisen, R., Logrieco, A., G, M., 2012. Influence of light on growth, fumonisin biosynthesis and FUM1 gene expression by *Fusarium proliferatum*. Int. J. Food Microbiol. 153, 148–153.
FAO, http://www.fao.org/food/food-safety-quality/a-z-index/mycotoxins/en
Feschotte, C., Pritham, E.J., 2007. DNA transposons and the evolution of eukaryotic genomes. Annu. Rev. Genet. 41, 331–368.
García-Jiménez, J., Alfaro-García, A., 1987. *Fusarium oxysporum* Schlecht. as Causal Agent of a Seedborne Disease of Saffron (*Crocus sativus* L.). 7th Congress of the Mediterranean Phytopathological Union, Granada, Spain, pp. 156.
Grajal Martin, M.J., Simon, C.J., Muehlbauer, F.J., 1993. Use of random amplified polymorphic DNA (RAPD) to characterize race 2 of *Fusarium oxysporum* f. sp. pisi. Phytopathology 83, 612–614.
Kang, S., Lebrun, M.H., Farrall, L., Valent, B., 2001. Gain of virulence caused by insertion of a Pot3 transposon in a *Magnaporthe grisea* avirulence gene. Mol. Plant.Microbe Interact. 14, 671–674.
Kokkonen, M., Ojala, L., Parikka, P., Jestoi, M., 2010. Mycotoxin production of selected *Fusarium* species at different culture conditions. Int. J. Food Microbiol. 143, 17–25.
Lee, J., Chang, I.-Y., Kim, H., Yun, S.-H., Leslie, J.F., Lee, Y.-W., 2009. Genetic diversity and fitness of *Fusarium graminearum* populations from rice in Korea. Appl. Environ. Microbiol. 75, 3289–3295.
Leslie, J.F., Summerell, B.A., 2006. The Fusarium Laboratory Manual. Blackwell Publishing, Ames, IO, USA.
Linderman, R.G., 1981. *Fusarium* diseases of flowering crops. In: Nelson, P.E., Toussoun, T.A., Cook, R.J. (Eds.), *Fusarium*: Diseases, Biology and Taxonomy. Pennsylvania State University Press, Pennsylvania, pp. 129–141.
Linfield, C.A., 1994. Fungal and nematode pathogens of *Narcissus*: current progress and future prospects for disease control. In: Monographs – British Crop Protection Council. British Crop Protection Council, Canterbury, UK, pp. 247–256.
López, R.C., Gómez-Gómez, L., 2009. Isolation of a new fungi and wound-induced chitinase class in corms of *Crocus sativus*. Plant Physiol. Biochem. 47, 426–434.
Manulis, S., Kogan, N., Reuven, M., Ben Yephet, Y., 1994. Use of RAPD technique for identification of *Fusarium oxysporum* f. sp. dianthi from carnation. Phytopathology 84, 98–101.
Marasas, W.F.O., 1996. Fumonisins: history, worldwide occurrence and impact. In: Jackson, L.S., De Vries, J.W., Bullerman, L.B. (Eds.), Fumonisins in Food. Plenum Press, New York, pp. 1–17.
McClellan, W.D., 1945. Pathogenicity of the vascular *Fusarium* of gladiolus to some additional iridaceous plants. Phytopathology 35, 921–930.
McDonald, B.A., 1997. The populations genetics of fungi: tools and techniques. Phytopathology 87, 448–453.
Migheli, Q., Briatore, E., Garibaldi, A., 1998. Use of random amplified polymorphic DNA (RAPD) to identify races 1, 2, 3 and 8 of *Fusarium oxysporum* f. sp. dianthi in Italy. Eur. J. Plant Pathol. 104, 49–57.
Milgroom, M.G., Fry, W.E., 1997. Contributions of population genetics to plant disease epidemiology and management. Adv. Bot. Res. 24, 2–30.
Miller, M.P., 1997. Tools for Population Genetic Analyses (TFPGA), Version 1.3. A Windows Program for the Analysis of Allozyme and Molecular Population Genetic Data.
Nelson, P.E., Desjardins, A.E., Plattner, R.D., 1993. Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry and significance. Annu. Rev. Phytopathol. 31, 233–252.
Nelson, P.E., Horst, R.K., Woltz, S.S., 1981. *Fusarium* diseases of ornamental plants. In: Nelson, P.E., Toussoun, T.A., Cook, R.J. (Eds.), *Fusarium*: Diseases, Biology and Taxonomy. Pennsylvania State University Press, Pennsylvania, pp. 121–128.
Peakall, R.O.D., Smouse, P.E., 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6, 288–295.
Popgene-version 1.32, 1997. The User-Friendly Shareware for Population Genetic Analysis. Molecular Biology and Biotechnology Center, University of Alberta, Edmonton, Canada, Available from: http://www.ualberta.ca/~fyeh
Prelusky, D., Rotter, B., Rotter, R., 1994. Toxicology of mycotoxins. In: Miller, D., Trenholm, H. (Eds.), Mycotoxins in Grain: Compounds Other than Aflatoxin. Eagan Press, Saint Paul, pp. 359–403.
Premkumar, K., Abraham, S.K., Santhiya, S.T., Ramesh, A., 2003. Protective effects of saffron (*Crocus sativus* Linn.) on genotoxins-induced oxidative stress in Swiss albino mice. Phytother. Res. 17, 614–617.
Proctor, R.H., Plattner, R.D., Brown, D.W., Seo, J.A., Lee, Y.W., 2004. Discontinuous distribution of fumonisin biosynthetic genes in the *Gibberella fujikuroi* species complex. Mycol. Res. 108, 815–822.
Rep, M., 2005. Small proteins of plant-pathogenic fungi secreted during host colonization. FEMS Microbiol. Lett. 253, 19–27.
Roebroeck, E.J.A., 2000. *Fusarium oxysporum* from Iridaceous Crops: Analysis of Genetic Diversity and Host Specialisation. Faculty of Science, University of Amsterdam, The Netherlands, pp. 125.
Rubio-Moraga, T., Gómez-Gómez, L., Trapero, A., Castro-Díaz, N., Ahrazem, O., 2013. Saffron corm as a natural source of fungicides: the role of saponins in the underground. Ind. Crops Prod. 49, 915–921.
Schmidt, M., Betti, G., Hensel, A., 2007. Saffron in phytotherapy: pharmacology and clinical uses. Wien. Med. Wochenschr. 157, 315–319.
Schmidt, S.M., Houterman, P.M., Schreiver, I., Ma, L., Amyotte, S., Chellappan, B., Boeren, S., Takken, F.L., Rep, M., 2013. MITEs in the promoters of effector genes allow prediction of novel virulence genes in *Fusarium oxysporum*. BMC Genomics 14, 119.
Seo, J.A., Kim, J.C., Lee, Y.W., 1996. Isolation and characterization of two new type C fumonisins produced by *Fusarium oxysporum*. J. Nat. Prod. 59, 1003–1005.
Sewram, V., Mshicileli, N., Shephard, G.S., Vismer, H.F., Rheeder, J.P., Lee, Y.W., Leslie, J.F., Marasas, W.F.O., 2005. Production of fumonisin B and C analogues by several *Fusarium* species. J. Agric. Food Chem. 53, 4861–4866.
Shah, A., Srivastava, K., 1984. Control of corm rot of saffron. Progress. Hortic. 16, 141–143.
Snyder, W.C., Hansen, H.N., 1940. The species concept in *Fusarium*. Am. J. Bot. 27, 64–67.
Stepien, L., Koczyk, G., Waskiewicz, A., 2013. Diversity of *Fusarium* species and mycotoxins contaminating pineapple. J. Appl. Genet. 54, 367–380.
Taylor, J.W., Jacobson, D.J., Fischer, M.C., 1999. The evolution of asexual fungi: reproduction, speciation and classification. Annu. Rev. Phytopathol. 37, 197–246.
Visconti, A., Bottalico, A., 1983. Detection of *Fusarium trichothecenes* (nivalenol, deoxynivalenol, fusarenone and 3-acetyldeoxynivalenol) by high-performance liquid chromatography. Chromatographia 17, 97–100.
Vogelgsang, S., Sulyok, M., Bänziger, I., Krska, R., Schuhmacher, R., Forrer, H.R., 2008. Effect of fungal strain and cereal substrate on in vitro mycotoxin production by *Fusarium poae* and *Fusarium avenaceum*. Food Addit. Contam. A: Chem. Anal. Control Expo. Risk Assess. 25, 745–757.
Yamamoto, W., Omatsu, T., Takami, K., 1954. Studies on the corm rots of *Crocus sativus* L. On saprophytic propagation of *Sclerotinia gladioli* and *Fusarium oxysporum* f. sp. gladioli on various plants and soils. Sci. Rep. Hyogo Univ. Agric. 1, 64–70.