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Vegetative compatibility grouping of *Fusarium oxysporum* f. sp. *gladioli* from saffron

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Abstract

Fusarium corm rot of saffron (*Crocus sativus* L.), incited by *Fusarium oxysporum* f. sp. *gladioli*, causes severe yield losses in Italy. Major symptoms during flowering (October–November) include yellowing and wilting of shoots, basal stem rot and corm rot. Sixty-four isolates of *F. oxysporum* f. sp. *gladioli*, obtained from infected saffron crops located in Italy (Abruzzi, Tuscany and Umbria) and in Spain, were characterized by pathogenicity and vegetative compatibility. Chlorate-resistant, nitrate-nonutilizing (*nit*) mutants were used to determine vegetative compatibility among the isolates of the pathogen with the aim of examining the genetic relatedness among populations from different locations. All the isolates belonged to vegetative compatibility group 0340. Since saffron shares susceptibility to *F. oxysporum* f. sp. *gladioli* with other ornamental plants of the Iridaceae (*Crocus, Gladiolus, Iris* and *Ixia*), it is likely that a clone of the pathogen (VCG 0340) was introduced with other hosts and is responsible for the disease outbreak observed on saffron in Italy. Alternatively, or additionally, the clone of *F. oxysporum* f. sp. *gladioli* causing disease on saffron in other countries may have spread to the saffron fields in Italy through the import and dispersal of infested propagation material.

Introduction

The spice obtained from the stigmas of saffron (*Crocus sativus* L.) flowers is one of the world's most expensive agricultural products. Stigmas are harvested, dried and sold as the saffron spice for use as a culinary seasoning and food colouring. Since saffron flowers are sterile, the plant does not set viable seeds and is therefore propagated vegetatively. Each season, new corms are formed above the old ones, in a way similar to that of gladiolus corms. The cultivation of saffron requires a specific climate: warm and dry summers and cold winters (Simon et al., 1984). Saffron is cultivated in various geographical regions including France, Greece, Italy, Portugal, Spain, Turkey, Egypt, Iran, India and China, where annual yields of up to 15 kg ha^{-1} of stigma threads are obtained.

In Italy, saffron is cultivated as an annual high-cash crop. It is of great social and economic importance in the Navelli Valley in Abruzzi (Central Italy), where it was introduced about seven centuries ago (Sperati, 1998). In general, corms are planted during their dormant period in the summer (late August) and flowers are produced after 8–10 weeks, in the autumn (October–November). Flowers are hand-picked daily in the early morning. Immediately after harvest, the stigmas are separated from the flower and dried to about 20% of their weight. More than 85,000 flowers are needed to obtain just 1 kg of dried saffron stigma threads. After harvest, saffron plants are left in the soil undisturbed and allowed to grow throughout the winter. In the spring, 4–10 daughter corms are formed above the old ones, which shrivel and eventually rot away. In the summer (July), the daughter corms are harvested

and subjected to chemical disinfestation treatment by the fungicide prochloraz. Treated corms are dried and stored in the dark at room temperature until the next planting season (late August).

Fusarium corm rot, incited by *Fusarium oxysporum* Schlechtend: Fr. f. sp. *gladioli* Massey (*F.o.gladioli*) is the most destructive disease of saffron, causing severe yield losses in Italy (Cappelli, 1994). The disease has been referred to by various names, including dry rot, brown rot, basal rot and yellows. The major symptoms of the disease occur during the flowering period when the infected plants show drooping, damping-off, yellowing and wilting of shoots, as well as basal stem rot and corm rot. The pathogen survives in infected corms and in the soil as mycelium, chlamydospores, macroconidia and microconidia (Brayford, 1996). Plants may become infected in the field, when germinating spores or mycelia enter the roots directly or through wounds. The pathogen may be introduced into new saffron-growing regions via contaminated corms (Cappelli and Di Minco, 1999).

The disease was detected for the first time in Japan (Yamamoto et al., 1954) and was later reported in India (Shah and Srivastava, 1984), Spain (Garcia-Jimenez and Alfaro Garcia, 1987) and Italy (Cappelli, 1994). In surveys carried out from 1995 to 1997 in Abruzzi, Italy (Cappelli and Di Minco, 1999), the disease was present in 43 out of 79 (54%) visited fields. Up to 50% plant mortality was observed in a number of fields during severe outbreaks of the disease. In Umbria and Tuscany, the disease was first detected in 1997 and 1999, respectively. Currently, the disease is widespread, causing substantial yield losses in several Italian saffron-growing regions.

In vegetative compatibility grouping (VCG) studies using nitrate-nonutilizing (*nit*) mutants, six VCGs have been reported in *F.o.gladioli* (Katan and Di Primo, 1999). Four VCGs (0340, 0341, 0342 and 0343) were revealed among 39 isolates of *F.o.gladioli* from eight countries (Mes et al., 1994) and two additional, single-member VCGs (0344 and 0345) were reported later (de Haan et al., 2000). In those studies, the VCGs differed in their frequency, geographical distribution and host of origin. VCG 0340 is cosmopolitan and is represented in isolates from Australia, North and South America, Asia and Europe. VCG 0341, VCG 0344 and VCG 0345 were found only in the Netherlands, whereas four isolates from Italy belonged to VCG 0343 (de Haan et al., 2000; Mes et al., 1994) and two isolates from the Netherlands and Germany belonged to VCG 0342 (Mes et al., 1994). VCG 0340 included isolates originating from various Iridaceaeous hosts (*Gladiolus*, *Iris*, *Ixia* and ornamental *Crocus*), whereas isolates belonging to VCG 0341 came from a more restricted host range (*Gladiolus* and *Iris*) (de Haan et al., 2000; Mes et al., 1994). Isolates assigned to VCG 0342 were obtained only from ornamental *Crocus* (de Haan et al., 2000; Mes et al., 1994), whereas isolates belonging to VCG 0344 and VCG 0345 originated from *Freesia* sp. (Baayen et al., 2000; de Haan et al., 2000).

Roebroeck (2000) examined the virulence range of a collection of *F.o.gladioli* isolates representing all known VCGs. He suggested the existence of ten distinct virulence patterns ('races'), five of which were found in VCG 0340. Four isolates from ornamental crocus were pathogenic only to crocus ('race 3'), whereas three additional isolates ('race 4') were pathogenic to other Iridacea as well.

In Italy, only one VCG (0343) has so far been identified among four isolates of *F.o.gladioli* obtained from gladiolus (de Haan et al., 2000). No information is available on the VCG of isolates from *Crocus* in Italy. Even though *F.o.gladioli* induces heavy yield losses in many saffron-growing regions in that country, its population structure has never been examined, and the genetic relatedness between the populations from Italy and those from other countries is not known. Determining the VCG pattern of isolates of *F.o.gladioli* from saffron in Italy would offer several clues as to the population structure of the pathogen, its possible origin and its relatedness to isolates from other hosts. The purpose of this study was to characterize a collection of *F.o.gladioli* isolates obtained from symptomatic plants in the major saffron-producing areas in Italy by vegetative compatibility and pathogenicity testing. A preliminary report of this study has been published (Di Primo and Cappelli, 2000).

Materials and methods

Fungal isolates

The collection included 59 isolates of *F. oxysporum*, obtained during different growing seasons from 28 commercial saffron fields located in 12 sampling sites in Italy (Abruzzi, Tuscany, Umbria) (Cappelli and Di Minco, 1998; 1999). The sampling sites covered a range of environmental conditions with respect to temperature, precipitation, irrigation and soil type. Most of the isolates originated from seven sites in Abruzzi,

which is the major saffron growing area in Italy. *F. oxysporum* was isolated from diseased saffron plants by placing corm tissues (surface-disinfested with 1% sodium hypochlorite for 2 min, followed by three rinses in sterile distilled water) on potato-dextrose agar (PDA) (Cappelli, 1994). After 5–7 days of incubation at 20 ± 1 °C, the developing colonies were examined visually and microscopically for morphological characteristics typical of *F. oxysporum*. One single-spore isolate per plant was prepared for vegetative compatibility and pathogenicity testing. This study also included the following isolates of *F.o.gladioli*: five isolates from saffron in Spain (FOGL-339, FOGL-340, FOGL-453, FOGL-I2 and FOGL-I4) kindly provided by J. Garcia-Jimenez (Departamento de Produccion Vegetale, Universidad Politecnica de Valencia, Spain), and four reference isolates (LBO G2, LBO G5, LBO Cr1 and LBO G76 representing VCGs 0340, 0341, 0342 and 0343, respectively) (de Haan et al., 2000; Mes et al., 1994; Roebroeck and Mes, 1992) kindly provided by J.J. Mes (PRI-DLO, Wageningen, the Netherlands). The isolates are listed in Table 1.

Table 1. Isolates of *Fusarium oxysporum* f. sp. *gladioli* listed according to vegetative compatibility group (VCG), origin and year of isolation

Isolate	VCG	Origin				
		Host	Country	Location	Field no.	Year
FOGL-SN	0340	*Crocus sativus*	Italy	Asciano (SI)[a]	1	1999
FOGL-SP	0340	*C. sativus*	Italy	Capodacqua Foligno (PG)	1	1999
FOGL-059	0340	*C. sativus*	Italy	Caporciano (AQ)	1	1995
FOGL-060	0340	*C. sativus*	Italy	Caporciano (AQ)	2	1995
FOGL-117	0340	*C. sativus*	Italy	Caporciano (AQ)	2	1995
FOGL-074	0340	*C. sativus*	Italy	Caporciano (AQ)	3	1995
FOGL-073	0340	*C. sativus*	Italy	Castelnuovo (AQ)	1	1995
FOGL-106	0340	*C. sativus*	Italy	Castelnuovo (AQ)	2	1995
FOGL-ANG	0340	*Gladiolus* sp.	Italy	Castiglione del Lago (PG)	1	1997
FOGL-001	0340	*C. sativus*	Italy	Civitaretenga (AQ)	1	1995
FOGL-015	0340	*C. sativus*	Italy	Civitaretenga (AQ)	1	1995
FOGL-017	0340	*C. sativus*	Italy	Civitaretenga (AQ)	1	1995
FOGL-008	0340	*C. sativus*	Italy	Civitaretenga (AQ)	2	1995
FOGL-200	0340	*C. sativus*	Italy	Civitaretenga (AQ)	2	1999
FOGL-121	0340	*C. sativus*	Italy	Civitaretenga (AQ)	3	1995
FOGL-124	0340	*C. sativus*	Italy	Civitaretenga (AQ)	3	1995
FOGL-125	0340	*C. sativus*	Italy	Civitaretenga (AQ)	3	1995
FOGL-032	0340	*C. sativus*	Italy	Goriano Valli (AQ)	1	1995
FOGL-102	0340	*C. sativus*	Italy	Goriano Valli (AQ)	2	1995
FOGL-103	0340	*C. sativus*	Italy	Molina Aterno (AQ)	1	1995
FOGL-034	0340	*C. sativus*	Italy	Molina Aterno (AQ)	2	1995
FOGL-104	0340	*C. sativus*	Italy	Molina Aterno (AQ)	2	1995
FOGL-114	0340	*C. sativus*	Italy	Navelli (AQ)	1	1995
FOGL-024	0340	*C. sativus*	Italy	Navelli (AQ)	1	1995
FOGL-068	0340	*C. sativus*	Italy	Navelli (AQ)	1	1995
FOGL-003[b]	0340	*C. sativus*	Italy	Navelli (AQ)	1	1995
FOGL-005	0340	*C. sativus*	Italy	Navelli (AQ)	2	1995
FOGL-002	0340	*C. sativus*	Italy	Navelli (AQ)	2	1995
FOGL-009	0340	*C. sativus*	Italy	Navelli (AQ)	2	1995
FOGL-019	0340	*C. sativus*	Italy	Navelli (AQ)	3	1995
FOGL-020	0340	*C. sativus*	Italy	Navelli (AQ)	3	1995
FOGL-046	0340	*C. sativus*	Italy	Navelli (AQ)	3	1995
FOGL-048	0340	*C. sativus*	Italy	Navelli (AQ)	4	1995
FOGL-067	0340	*C. sativus*	Italy	Navelli (AQ)	4	1995
FOGL-105	0340	*C. sativus*	Italy	Navelli (AQ)	4	1995
FOGL-115	0340	*C. sativus*	Italy	Navelli (AQ)	4	1995
FOGL-119	0340	*C. sativus*	Italy	Navelli (AQ)	4	1995
FOGL-122	0340	*C. sativus*	Italy	Navelli (AQ)	5	1995
FOGL-126	0340	*C. sativus*	Italy	Navelli (AQ)	5	1995

Table 1. (*Continued*)

Isolate	VCG	Origin				
		Host	Country	Location	Field no.	Year
FOGL-110	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	1	1995
FOGL-116	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	1	1995
FOGL-118	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	1	1995
FOGL-123	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	1	1995
FOGL-049	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	1	1995
FOGL-050	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	2	1995
FOGL-055	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	2	1995
FOGL-043	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	3	1995
FOGL-109	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	4	1995
FOGL-113	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	4	1995
FOGL-120	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	4	1995
FOGL-128	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	5	1995
FOGL-127	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	5	1995
FOGL-129	0340	*C. sativus*	Italy	Prata d'Ansidonia (AQ)	5	1995
FOGL-107	0340	*C. sativus*	Italy	Tussio (AQ)	1	1995
FOGL-039	0340	*C. sativus*	Italy	Tussio (AQ)	2	1995
FOGL-038	0340	*C. sativus*	Italy	Tussio (AQ)	2	1995
FOGL-101	0340	*C. sativus*	Italy	Villa S. Angelo (AQ)	1	1995
FOGL-025	0340	*C. sativus*	Italy	Villa S. Angelo (AQ)	1	1995
FOGL-028	0340	*C. sativus*	Italy	Villa S. Angelo (AQ)	1	1995
FOGL-339[c]	0340	*C. sativus*	Spain	Cuenca	Unknown	1983
FOGL-340[c]	0340	*C. sativus*	Spain	Cuenca	Unknown	1983
FOGL-453[c]	0340	*C. sativus*	Spain	Cuenca	Unknown	1983
FOGL-I2[c]	0340	*C. sativus*	Spain	Valencia	Unknown	1998
FOGL-I4[c]	0340	*C. sativus*	Spain	Valencia	Unknown	1998
LBO G2[d]	0340	*Gladiolus* sp.	The Netherlands	Unknown	Unknown	Unknown
LBO G5[d]	0341	*Gladiolus* sp.	The Netherlands	Unknown	Unknown	Unknown
LBO Cr1[d]	0342	*Crocus* sp.	The Netherlands	Unknown	Unknown	Unknown
LBO G76[d]	0343	*Gladiolus* sp.	Italy	Unknown	Unknown	Unknown

[a]The sites (provinces in parentheses) represent the main saffron-growing areas in Abruzzi (AQ, L'Aquila); Tuscany (SI, Siena); and Umbria (PG, Perugia) in Italy.
[b]Isolate deposited at the American Type Culture Collection (accession number ATCC 201001).
[c]Isolates obtained from J. Garcia-Jimenez.
[d]Reference isolates obtained from J.J. Mes.

Pathogenicity tests

Pathogenicity tests were conducted using disease-free corms of *C. sativus* (Cappelli and Di Minco, 1999). Corms obtained from disease-free saffron fields were surface-disinfested with 1% sodium hypochlorite for 2 min, followed by three rinses in sterile distilled water. For each *F. oxysporum* isolate, 10 corms were dipped for 20 min in an inoculum suspension (10^6 conidia ml^{-1}) prepared by macerating the contents of a 7-day-old PDA culture (9-cm plate) with 75 ml of water. Inoculated corms were planted (five per pot) in sterile potting soil (85% peat, 10% clay, 5% sand) in 12-cm pots and maintained in the greenhouse at 20–26 °C (16-h photoperiod). Eight weeks after planting, the shoots were examined visually for yellowing and wilt appearance, and the corms were uprooted and assessed for superficial brown lesions and rot symptoms. Isolates that caused disease symptoms in 80–100% of the inoculated corms, from which typical cultures of *F. oxysporum* were reisolated, were considered *F.o.gladioli.* Uninoculated control corms, dipped in sterile distilled water and maintained under the same conditions, remained healthy throughout the pathogenicity tests.

Isolation of nit *mutants and vegetative compatibility tests*

nit mutants were generated, phenotyped and used in complementation (heterokaryon) tests according to standard procedures (Correll et al., 1987;

Di Primo et al., 2001). *nit* mutants *(nit1, nit3* and NitM) generated from each isolate were paired among themselves to test for self-compatibility. Pairings between *nit* mutants from the same or different isolates were performed on Fusarium minimal medium (Correll et al., 1987) plates (5-cm diameter), incubated at 27 °C and scored for complementation 5–14 days later. When the mycelia of two *nit* mutants formed dense aerial growth where they had met and anastomosed, their parents were assigned to the same VCG. Four pairs of complementary *nit* mutants, representing the four established VCGs of *F.o.gladioli* (Baayen et al., 2000; Mes et al., 1994) were prepared from the reference isolates (Table 1) and used to examine the compatibility of the isolates from Italy with these VCGs.

Results

Pathogenicity tests

Eight weeks after dip-inoculation of saffron corms, typical disease symptoms were observed. They included progressive yellowing and downward turning of leaves; brown rot of the corm basal plate and core, extending upward into the leaf bases via vascular tissues; and shoot wilting. All 59 isolates of *F. oxysporum* from Italy in the collection induced typical disease symptoms in 80–100% of the inoculated corms, and were thus identified as *F.o.gladioli* (Table 1).

Vegetative compatibility

A set of nine pathogenic isolates, originating from various locations, were chosen for the initial complementation tests. A total of 23 *nit* mutants (*nit1* and NitM) derived from these isolates (2 or 3 mutants for each isolate) were paired in all possible intra and inter-isolate combinations to determine complementation within and between isolates. Heterokaryon formation was recorded for up to 14 days, although the reactions could usually be clearly defined as either positive (complementation) or negative (no complementation) after 4–7 days. The pattern of complementary heterokaryon formation revealed the presence of only one VCG among the nine isolates of *F.o.gladioli*. Two complementary NitM testers (FOGL-008/33 and FOGL-105/1), which have demonstrated the ability to form strong heterokaryons with many other mutants, were chosen as local representative tester strains. Pairing of *nit* mutants of the remaining 50 pathogenic isolates of our collection, as well as the five isolates from Spain, with these testers resulted in strong complementation. Consequently, all the isolates of the collection were assigned to the same VCG. To compare this VCG with previously described VCGs, 13 randomly chosen *nit1* mutants from our collection were paired with the two tester strains representing each of the four reference isolates (Table 1). Based on strong complementation with the testers of isolate LBO G2, all *F.o.gladioli* isolates of the collection were assigned to VCG 0340 (Table 1). No self-incompatible isolates were found.

Discussion

One VCG (0340) was found among 64 isolates of *F.o.gladioli* originating from 28 Fusarium corm rot-affected saffron fields in 12 locations covering three geographical regions (Abruzzi, Tuscany and Umbria) in Italy and from two locations in Spain. This cosmopolitan VCG has already been reported from other geographical regions, including Europe, America, Asia and Australia (Mes et al., 1994), among isolates from four ornamental genera of the Iridaceae (*Gladiolus, Ixia, Crocus* and *Iris*). However, this is the first report on the occurrence of VCG 0340 in Italy and in Spain. Its source or way of introduction into Italy or other European countries is not clear. None of our isolates corresponded to VCG 0343, previously reported as occurring on *Gladiolus* in Italy, to VCG 0342 previously reported from ornamental *Crocus* in the Netherlands (Baayen et al., 2000; Mes et al., 1994), or to VCGs 0341, 0344 or 0345. Due to the scarcity of population studies on *F.o.gladioli*, only limited data are available on the host plants and geographical distribution of VCGs of this pathogen. Information is particularly lacking on the population structure of the pathogen in other major saffron-growing regions (India, Japan and Spain) where the disease occurs. Such information should enhance our comprehension of the origin(s) and worldwide spread of *F.o.gladioli*. In general, the appearance of a Fusarium disease in a new growing region can be explained either by imports from an external source (pathogen dispersal) or by new local emergence (independent origin) (Gordon and Martyn, 1997). The fact that saffron shares susceptibility to *F.o.gladioli* with other Iridaceaeous plants supports the hypothesis that a clone of the pathogen (VCG 0340) was introduced with other hosts and is responsible for the disease outbreak observed on saffron in Italy.

The occurrence of *F.o.gladioli* on *Iris* flowers in Italy was reported in as early as 1983 (Gullino et al., 1983). Alternatively or additionally, the clone of *F.o.gladioli* causing disease on saffron in other countries may have spread to saffron-growing sites in Italy through import and dispersal of infested propagation material (Cappelli and Di Minco, 1998; 1999).

The results of our pathogenicity test, using only saffron as the test plant, are insufficient to decide whether the isolates from saffron correspond with any of the virulence types ('races') found among VCG 0340 isolates from ornamental crocus in the Netherlands (Roebroeck, 2000).

Recognizing the epidemiological significance of seedborne inoculum of the pathogen, as reported here, is important for improved strategies to control Fusarium corm rot on saffron. The best means of disease prevention is offered by the use of pathogen-free corms. Chemical treatment with prochloraz provides an effective means of eradicating or reducing latent *F.o.gladioli* inside saffron corms (Cappelli and Di Minco, 1998). The indexing of treated corm lots may provide a useful tool for assessing the efficiency of the treatments, thus reducing the risk associated with *F.o.gladioli* dispersal to disease-free growing regions. Recently, de Haan et al. (2000) described a quick multiplex PCR assay capable of detecting the occurrence of *F.o.gladioli* race 1 in symptomless infected *Gladiolus* corm samples. Hence, efforts should be aimed at developing quick and reliable diagnostic tools for specific detection of cryptic *F.o.gladioli* infection in saffron corm lots from various sources. The time lapse between corm harvest in July and planting in late August is long enough to allow for detection and identification of *F.o.gladioli* infection in propagation material by VCG and molecular assays.

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