

## SHORT COMMUNICATION

ANALYSIS OF VEGETATIVE COMPATIBILITY GROUPS OF ITALIAN AND DUTCH ISOLATES OF *FUSARIUM OXYSPORUM* f. sp. *LACTUCAE*I. Pintore<sup>1</sup>, G. Gilardi<sup>1</sup>, M.L. Gullino<sup>1,2</sup> and A. Garibaldi<sup>1</sup><sup>1</sup>AGROINNOVA - Centre of Competence for the Innovation in the Agro-Environmental Sector, University of Torino. Largo Paolo Braccini 2. 10095 Grugliasco (TO), Italy<sup>2</sup>DI.S.A.F.A. - Department of Agricultural, Forest and Food Sciences and Technologies, University of Torino. Largo Paolo Braccini 2. 10095 Grugliasco (TO), Italy

## SUMMARY

*Fusarium oxysporum* f. sp. *lactucae* (FOL), the causal agent of lettuce Fusarium wilt, has spread to several countries where lettuce is grown. To date, four races of FOL have been identified, but only race 1 has been detected in Europe; race 2 and race 3 have been identified in Japan and in Taiwan. A new physiological race has been isolated in the Netherlands. The vegetative compatibility group approach has been used to determine genetic diversity within a group of forty-eight FOL strains of different origin, with special attention to the Italian and Dutch isolates of the pathogen, in order to obtain a better understanding of the recent epidemics that have been observed in Europe. On the basis of the complementation pattern, all the Italian isolates of FOL tested were assigned to VCG 0300. FOL isolates belonging to races 2 and 3 belonged to VCG 0301 and 0302, respectively. The isolates obtained from lettuce in the Netherlands belonged to a new VCG, numbered 0303. The results support the hypothesis that FOL race 4 might have been selected locally in Dutch fields.

**Keywords:** VCG, leafy vegetables, Fusarium wilt, diagnostic tool.

The Fusarium wilt of lettuce, caused by *Fusarium oxysporum* f. sp. *lactucae* (FOL) is currently the most serious soil-borne disease of this crop in many areas. First reported in Japan (Matuo and Motohashi, 1967), the disease was observed in the United States of America (Hubbard and Gerik, 1993) 35 years later on, and then in Iran, Taiwan, Brazil, and Europe, where it was first detected in Italy in 2002 (Garibaldi *et al.*, 2002; Matheron and Gullino, 2012). The disease is currently spreading in many states in the USA (Matheron and Gullino, 2012), in South America

(Ventura and Costa, 2008; Malbrán *et al.*, 2014) and in Europe, where Fusarium wilt has been reported in Portugal (Marques Ramalhete *et al.*, 2006), the Netherlands (Gilardi *et al.*, 2017a) and France (Gilardi *et al.*, 2017b). The spread of the pathogen is favoured by the commercialization of contaminated seeds (Garibaldi *et al.*, 2004), while its survival in soil is helped by its capability to colonize the roots of many crops (*e.g.* melon, tomato, watermelon, cotton, broccoli, cauliflower and spinach) without producing symptoms (Hubbard and Gerik, 1993; Scott *et al.*, 2014). Severe field losses are also due to the widespread use of commercial varieties that are particularly susceptible to the pathogen (Matheron and Gullino, 2012; Cabral and Reis, 2013; Gilardi *et al.*, 2014).

Four races of the pathogen have been reported so far. Three of the four reported races (races 1, 2, 3) have been known for a long time in Japan. Race 1 has also been reported in Europe and in the USA (Matheron and Gullino, 2012). Lin *et al.* (2014) reported the presence of race 3 in Taiwan, while race 4 has only recently been observed in the Netherlands (Gilardi *et al.*, 2017b).

Several techniques, including pathogenicity testing and molecular methods, are used to differentiate *Fusarium oxysporum*, *formae speciales* and races (Leslie *et al.*, 2006; Lievens *et al.*, 2012), and many authors have applied molecular methods to characterize FOL (Shimazu *et al.*, 2005; Pasquali *et al.*, 2007, 2008; Mbofung and Pryor, 2010; Gilardi *et al.*, 2017a). Since the mid-1980s, vegetative compatibility grouping (VCG), which was first proposed by Puhalla (1985), has been used to characterize the *formae speciales* of *Fusarium oxysporum*. Moreover, their numbering was reviewed by Katan in 1999.

VCG has been very useful to obtain a better understanding of the genetic diversity of the pathogen population, even in the case of FOL. Ogiso *et al.* (2002) showed a close correlation between race and vegetative compatibility in FOL, and reported that the Californian and Japanese race 1 isolates of the pathogen belong to the same VCG group (VCG 0300), while all the isolates of race 2 belong to a separate VCG (Fujinaga *et al.*, 2005). Pasquali *et al.* (2005), by comparing different isolates of race 1 obtained

**Table 1.** *Nit* mutants obtained and vegetative compatibility groups (VCG) of *Fusarium oxysporum* f. sp. *lactucae* isolates from different geographical origins.

Isolate codes	Geographic origin	Year	Race	Number of <i>nit</i> mutants on MMC/PDC <sup>c</sup>	Nit M obtained on MMC/PDC	VCG
Mya 103040 (race 1) <sup>a</sup>	Italy	2002	1	3/2	3/0	0300
MAFF44086 (race 3) <sup>b</sup>	Japan	–	3	1/1	0/0	0302
MAFF44085 (race 3) <sup>b</sup>	Japan	–	3	0/0	0/0	0302
9501 (race 2) <sup>a,b</sup>	Japan	–	2	0/7	0/7	0301
2/10	Italy	2010	1	0/1	0/1	0300
4/10	Italy	2010	1	0/8	0/2	0300
7/10	Italy	2010	1	0/2	0/0	030-HSI
11/10	Italy	2010	1	2/5	2/2	0300
1/12	Italy	2012	1	0/0	0/0	– <sup>f</sup>
2/12	Italy	2012	1	8/3	0/1	0300
3/12	Italy	2012	1	2/2	2/0	0300
4/12	Italy	2012	1	0/3	0/0	0300
5/12	Italy	2012	1	0/1	0/0	0300
6/12	Italy	2012	1	4/5	1/0	0300
1/14	Italy	2014	1	2/0	0/0	0300
3/14	Italy	2014	1	0/7	0/0	0300
5/14	Italy	2014	1	2/0	0/0	0300
6/14	Italy	2014	1	0/8	0/0	0300
7/14	Italy	2014	1	2/5	2/1	030-HSI <sup>e</sup>
8/14	Italy	2014	1	9/1	6/1	0300
9/14	Italy	2014	1	2/2	2/1	030-HSI
1/03	Italy	2003	1	3/4	0/2	030-HSI
2/03 R2 <sup>d</sup>	Italy	2005	1	0/2	0/0	030-HSI
2/03	Italy	2005	1	8/1	8/1	030-HSI
4/03	Italy	2005	1	0/0	0/0	–
7/03	Italy	2005	1	0/1	0/0	030-HSI
8/03	Italy	2003	1	0/7	0/0	0300
10/03	Italy	2003	1	1/0	0/0	030-HSI
15/03	Italy	2003	1	0/6	0/4	0300
16/03	Italy	2005	1	1/7	1/2	0300
17/03	Italy	2005	1	1/0	0/0	0300
1/11	Italy	2011	1	0/2	0/0	030-HSI
3/11A R1	Italy	2011	1	2/0	0/0	030-HSI
3/11B R2	Italy	2011	1	0/1	0/0	0300
5/13	Italy	2013	1	0/0	0/0	–
3/14 R1	Italy	2014	1	0/0	0/0	–
3/14 R2	Italy	2014	1	3/0	2/0	0300
3/14 R3	Italy	2014	1	0/1	0/0	030-HSI
3/14 R4	Italy	2014	1	0/0	0/0	–
3/14 R5	Italy	2014	1	4/0	1/0	0300
3/14 R6	Italy	2014	1	2/0	0/0	0300
PD 015/04750896	The Netherlands	2015	4	0/6	0/0	0303
PD 015/04750888	The Netherlands	2015	4	0/1	0/0	0303
PD 015/04750888-R1	The Netherlands	2015	4	0/0	0/0	–
PD 015/04750888-R2	The Netherlands	2015	4	2/0	0/0	0303
1/14 R 3	Italy	–	1	6/0	0/0	0300
1/14 R 1	Italy	–	1	9/4	1/2	0300
1/14 R 2	Italy	–	1	0/0	0/0	–

<sup>a</sup>Race classification based on a report by Pasquali *et al.* (2007).

<sup>b</sup>Race classification based on reports by Fujinaga *et al.* (2001, 2003).

<sup>c</sup>MMC: minimal medium containing chlorate; PDC: potato dextrose agar with chlorate.

<sup>d</sup>R: Re-isolated from symptomatic lettuce plants (cv. 'Cavolo di Napoli') used in the pathogenicity assay according to the protocol reported by Garibaldi *et al.* (2004).

<sup>e</sup>HIS: heterokaryon self-incompatible.

<sup>f</sup>No mutant was obtained.

in Italy, the USA, Japan and Taiwan (isolate type 2) found that they belong to the same VCG group (VCG 0300), while Taiwanese type 1 isolates belong to VCG 0301. Isolates of race 3, which have only been found in some regions of Japan and in Taiwan (Fujinaga *et al.*, 2001, 2003,

2005; Yamauchi *et al.*, 2001; Lin *et al.*, 2014), and of race 2, which has been reported in Japan (Fujinaga *et al.*, 2005), belong to a different VCG group. The present study was undertaken in order to update the situation of VCG in FOL, working with strains of different origin, with special

attention being devoted to Italian and Dutch isolates of the pathogen, in order to evaluate the level of genetic variability of the recent epidemics observed in Europe.

Forty-eight strains of *Fusarium oxysporum* f. sp. *lactucae* (FOL), listed in Table 1, obtained from wilted lettuce plants were used in this study. Four strains (PD015/04750896 and PD015/04750888 and two re-isolates of PD015/04750888) originated from lettuce plants grown in the Netherlands; MAFF 744085, MAFF 744086 and 9501 were obtained from Japan, while all the other 41 were obtained from wilted lettuce grown in Italy. FOL re-isolates (R) from artificially inoculated plants, according to the protocol reported by Garibaldi *et al.* (2004), were also included into the assay. Monoconidial isolates were obtained from each FOL strain and used for the VCG tests. Each monoconidial isolate was stored in glycerol at  $-80^{\circ}\text{C}$ . A Basal Medium (BM) was prepared with 1 l of distilled water, 30 g sucrose, 1 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g KCl, 10 mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 20 g agar and a 0.2 ml trace element solution (95 ml of distilled water, 5 g citric acid, 5 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g  $\text{Fe}(\text{NH}_4)_2 (\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , 0.25 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 50 mg  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 50 mg  $\text{H}_3\text{BO}_4$ , 50 mg  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , as described by Correll *et al.*, 1987). A Minimal Medium (MM) was used to recognize *nit* mutants and for a complementation (heterokaryon) test. This medium was prepared by adding 2 g of  $\text{NaNO}_3$  to 1 l of BM (Correll *et al.*, 1987). The chlorate media used for *nit* production were: MMC (1 l of MM amended with 15 g  $\text{KClO}_3$  and 1.6 g L-asparagine) and PDC (1 l of  $\text{H}_2\text{O}$ , 24 g potato-dextrose broth, 20 g agar and 15 g  $\text{KClO}_3$ ) (Correll *et al.*, 1987). Media with different nitrogen sources were used to determine the *nit* mutants phenotype: nitrate (MM), nitrite (BM amended with 0.5 g/l  $\text{NaNO}_2$ ) and hypoxanthine (BM + 0.2 g/l hypoxanthine) (Correll *et al.*, 1987). If no mutants grew on the full dose nitrite, 75% nitrite (0.375 g/l  $\text{NaNO}_2$ ), 50% nitrite (0.25 g/l  $\text{NaNO}_2$ ) and 25% nitrite (0.125 g/l  $\text{NaNO}_2$ ) were used. Each monoconidial isolate of FOL was grown in Petri plates (90 mm) containing MM for 3-4 days at room temperature ( $22-28^{\circ}\text{C}$ ) (Puhalla, 1985). Four small ( $1 \text{ mm}^3$ ) pieces of mycelium were cut from the culture of each strain, transferred to plates containing the chlorate-amended media (MMC and PDC) where they were spaced (Puhalla, 1985). The plates were incubated at  $25^{\circ}\text{C}$  in a 12 h dark/light cycle for 7-15 days (Vannacci and Cristani, 1998), and were periodically inspected to verify the appearance of sectors with faster growth (chlorate resistant) compared to the remaining colony (Correll *et al.*, 1987).

The chlorate-resistant sectors were detected in each PDC and MMC plate, transferred to an MM plate (90 mm with 3 sectors) and incubated at room temperature in order to define the kind of mutant (Puhalla, 1985). After 3-4 days, the mutant was checked: a thin but expansive growth, without aerial mycelium on MM indicated that sectors were unable to reduce nitrate, and they were therefore considered *nit* mutants (Correll *et al.*, 1987; Puhalla, 1985). When this standard procedure did not allow for

**Table 2.** Frequency data of *nit* mutants from media containing chlorate.

Medium	Mutation frequency, number (%)			
	<i>nit1</i>	<i>nit3</i>	Nit M	Total
PDC	25 (42)	54 (81)	27 (47)	106 (43)
MMC	35 (58)	13 (19)	31 (53)	79 (57)

mutants to be obtained, the plates were exposed to ultraviolet (UV) irradiation under a UV-C lamp for 1 min (Ioannis *et al.*, 2015). The *nit* mutants thus obtained were grown on potato dextrose agar (PDA), and stored as conidial suspensions in potato dextrose broth (PDB) with 50% glycerol, at  $-80^{\circ}\text{C}$ .

Identification of the *nit* mutant phenotype was made by growing each mutant on nitrate (MM), nitrite and hypoxanthine medium (Correll *et al.*, 1987). Small pieces ( $2 \text{ mm}^3$ ) of *nit* mutant grown on PDA were transferred to different nitrogen media, and the plates were incubated for 4 days at  $25^{\circ}\text{C}$ . *Nit* mutants were identified as *nit1*, *nit3*, or Nit M, according to their growth on nitrate, nitrite or on the hypoxanthine medium (Correll *et al.*, 1987).

Different types of *nit* mutants from each tested isolate were paired in all the possible combinations. *Nit* mutants recovered from the same parents were paired with at least one *nit1*, one *nit3* and one Nit M mutant from the parent. *Nit* mutants of the same phenotype were also paired (Correll *et al.*, 1987).

Using *nit* mutants stored at  $-80^{\circ}\text{C}$ ,  $5 \mu\text{l}$  from two different *nit* mutants was placed 3 cm apart on MM and the plates were incubated at  $25^{\circ}\text{C}$  and monitored for 7-14 days.

The development of a dense aerial mycelial growth at the line of contact between two *nit* mutant colonies indicated the formation of a heterokaryon. The strains whose mutants formed a heterokaryon were assigned to the same VCG. An isolate was defined self-incompatible (HSI) when no compatible mutants were obtained.

The tested FOL isolates, listed in Table 1, produced spontaneous resistant sectors on the media containing potassium chlorate, with a frequency that varied according to the isolate and medium that were used. Mutant phenotypes were assigned to the *nit1*, *nit3* and Nit M classes, according to the colony morphology on the media containing different nitrogen sources (Correll *et al.*, 1987). The number of Nit M mutants obtained from both chlorate substrates was evaluated to compare the efficiency of different media in the production of the *nit* mutants that would be useful for the VCG analysis. Several Nit M mutants were produced on the MMC medium (Table 2), while PDC generated a higher frequency of chlorate-resistant mutants that were not useful for the VCG analysis. At last, 184 mutants were obtained from 40 isolates, and they were crossed in all the possible combinations.

On the basis of the complementation pattern, all the Italian isolates tested for FOL were assigned to VCG 0300. The FOL isolates belonging to races 2 (9501) and 3

(MAFF 744086) belonged to VCG 0301 and 0302, respectively. The MAFF 744085 isolate, which belongs to race 3, did not produce any mutants. FOL isolates 04750896 and 04750888, obtained from wilted lettuce in the Netherlands, belonged to a new VCG, which was numbered 0303 (Table 1).

The obtained results, which are in line with those of Pasquali *et al.* (2005), confirm the presence of a new race in the Netherlands. Pasquali *et al.* (2008) developed a sequence-specific amplified polymorphism (SSAP) technique to study the clonality of FOL VCG 0300, which permitted race 1 to be discriminated from race 2, and confirmed the worldwide clonality of VCG 0300 race 1.

In this study, the VCG technique was a useful tool to characterize the race 4 of the pathogen. This study confirms the high degree of VCG homogeneity among Italian isolates of FOL collected in fields for more than ten years from the first observation of lettuce Fusarium wilt, all belonging to race 1 and to VCG 0300. The results have once again provided evidence of the pathogen having reached Italy through one single introduction, and of its further spreading, mostly through the use of infected seeds. On the contrary, the new race 4, which belongs to a different VCG, recently detected in the Netherlands, could have originated locally. Isolates of all four races of FOL belong to different VCG, indicating that FOL races are a genetically homogeneous group of isolates. Even if there is evidence that the race-VCG relationship is not an appropriate tool for identifying physiologic races (Katan *et al.*, 1994), VCG remain useful to gain a better understanding of the origin of races within this *forma specialis*. The presence of a new FOL race in the Netherlands suggests the necessity of adopting preventative measures, such as the use of healthy propagation material and seed dressings (Katan *et al.*, 2012) in order to prevent its spread to new cultivation areas, in consideration of the important role of this country in the production and exporting of seeds.

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