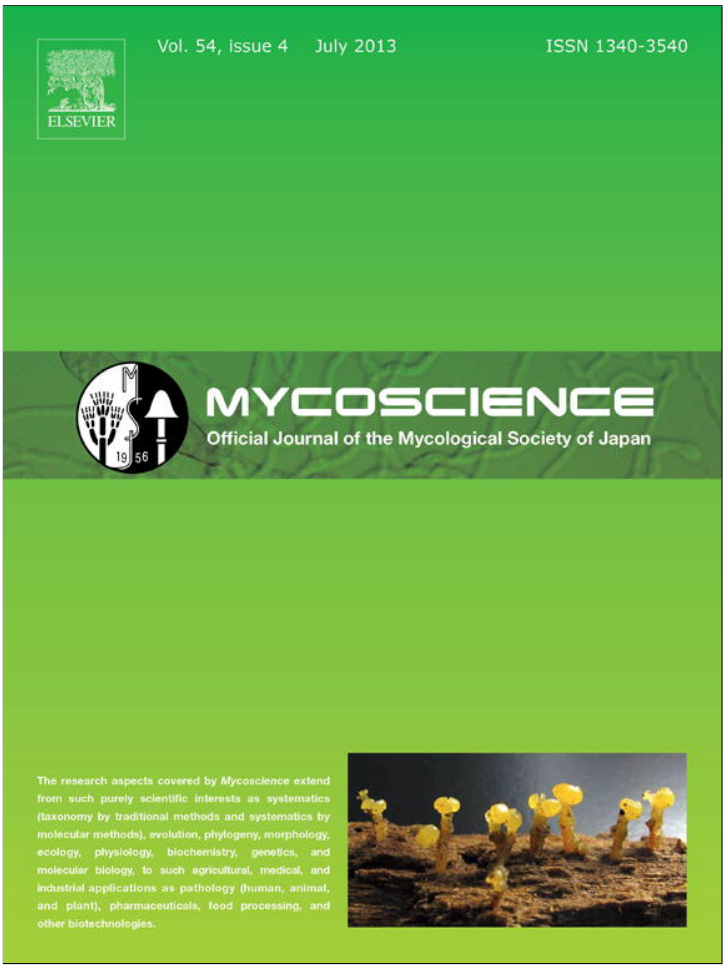

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Pathogenicity variation in *Fusarium verticillioides* populations isolated from maize in northern Italy

*Giovanni Venturini**, *Gemma Assante*, *Silvia Laura Toffolatti*, *Annamaria Vercesi*

Dipartimento di Scienze Agrarie e Ambientali – Produzione, Territorio, Agroenergia, Divisione di Produzione Vegetale – Patologia Vegetale, Università degli Studi di Milano, via G. Celoria 2, 20133 Milano, Italy

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ABSTRACT

One hundred and eighty one strains were selected among *Fusarium verticillioides* populations isolated from maize samples collected in three fields located in northern Italy. All the isolates were tested for their pathogenicity on maize seeds by assessing the seed germination percentages and the percentage infection indexes concerning seed colonization, radicle decay and coleoptile rot. *Fusarium verticillioides* strains did not affect seed germination even in presence of high seed colonization, but showed a variable pathogenic behavior according to the maize growth stages. Seedborne *F. verticillioides* population as well as strains isolated at maturity was effective in seed colonization and in inducing coleoptile rot, not causing however serious radicle decay. Only populations isolated at seedling and pre-silking stages showed high radicle decay ability. These results provide baseline information on *F. verticillioides* pathogenicity. They constitute an important input for further investigation of *F. verticillioides* biology in order to define its evolutionary potential.

1. Introduction

Fusarium verticillioides (Sacc.) Nirenberg (synonyms *F. moniliforme* Sheldon, *Gibberella moniliformis* Wineland, *G. fujikuroi* mating population A) commonly infects a wide range of crops and plants throughout the world (Bacon and Nelson 1994). Although *F. verticillioides* shows no host specialization, it mainly occurs on maize and it is in general associated with several diseases including stalk, kernel, and ear rots known as Fusarium rots (White 1999). *Fusarium verticillioides* reduces seedling stands in maize causing seed decay, root rots, damping-off and seedling blight (Soonthornpoct et al. 2000). *Fusarium verticillioides* may occur on maize as a seedborne endophyte or infect the plant at various developmental stages without inducing visible disease symptoms (Munkvold et al. 1997; Venturini et al. 2011a). Moreover, *F. verticillioides* infection of maize may cause an accumulation of mycotoxins such as fumonisins. Contamination of field-grown maize with the mycotoxin fumonisin B_1 is of a greatest concern for food and feed safety because of its causal role in equine leucoencephalomalacia (Marasas et al. 1988), porcine pulmonary edema (Colvin and Harrison 1992), liver and renal carcinogenicity in laboratory rodents, and possibly even human carcinogenicity (IARC 2002) and neural tube birth defects (Marasas et al. 2004).

Many factors, including climate, cultural practices, and host susceptibility contribute to the disease progress and to the fumonisin accumulation in maize (Parsons and Munkvold 2010). Up to now it has not been clearly defined how the

* *Corresponding author*. Tel.: +39 (0) 250316792; fax: +39 (0) 250316781.
E-mail address: giovanni.venturini@unimi.it (G. Venturini).
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genetic variability in *F. verticillioides* population may play a role into disease incidence and mycotoxin production. While genetic variability of *F. graminearum* populations, the causal agent of maize Gibberella ear rot (or red ear rot), has been thoroughly investigated (Zeller et al. 2004; Akinsanmi et al. 2006; Fernando et al. 2006), no reliable information is available on *F. verticillioides* population genetics, with the exception of two studies carried out in Argentina (Reynoso et al. 2009) and in the Philippines (Cumagun et al. 2009).

Many phenotypic markers, such as pathogenicity, growth rate, mycelial aspect and conidia production may be used for describing the population biology in *F. verticillioides*; among them, pathogenicity may constitute one of the most suitable characters for investigating fungal population variability (Péros et al. 1997; Zhan et al. 2007; Haque et al. 2008). By considering pathogenicity as population marker, previous studies pointed out relevant differences between mating populations within *G. fujikuroi* clade (Leslie et al. 2005; Wulff et al. 2010). Such differences were aimed to characterize *Fusarium* species associated with plants providing data on *Fusarium* biodiversity and variability. The present work was undertaken to determine the variability in *F. verticillioides* populations associated with maize in northern Italy using pathogenicity on maize seed as population marker. Moreover, by considering the composite *F. verticilliodes* epidemiology, fungal populations were constituted taking into consideration different plant organs and maize growth stages at which strains were isolated.

Pathogenicity can be quantified by several methods, for example, by determining the disease severity (Zhan et al. 2002; Cumagun and Medianer 2003). In the current study pathogenicity was measured by assessing: seed germination inhibition; seed colonization, radicle decay, and coleoptile rot abilities of *F. verticillioides* strains following a modified version of the procedure described by Munkvold and O'Mara (2002). In a previous work, the same *F. verticillioides* populations were phenotypically characterized through determination of mating type and male/hermaphrodite polymorphism (Venturini et al. 2011b). These mating behavior data were further developed by the results of the present work providing a more detailed characterization of Italian *F. verticillioides* populations.

2. Materials and methods

2.1. Fusarium verticillioides *strains*

One hundred eighty one *F. verticillioides* strains were arbitrarily selected among the *F. verticillioides* strains held in the culture collection at the Department of Agricultural and Environmental Sciences Division of Plant Production – Plant Pathology, University of Milan. All the strains were isolated from maize samples collected during the 2007/2008 maize cropping season in three fields located in Lombardia (northern Italy), Sant'Angelo Lodigiano (SAL), Pontevico (PO), and Pieve d'Olmi (PDO) at the following growth stages (GS): maize residues cultivated in 2007 growing season, seeds (GS00) according to BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) scale (Lancashire et al. 1991), seedlings with 3 unfolded leaves (GS13), plants with the tip of tassel visible (GS53), fully ripe plants (GS89) (Venturini et al. 2011a). The isolates were maintained on potato dextrose agar (PDA, Difco, Becton & Dickinson Co., Sparks) at 4 °C and as conidial suspensions stored in 20% glycerol at −80 °C.

2.2. *Pathogenicity assays*

Untreated maize seeds used for experimental inoculations belonged to a non commercial hybrid line 'H6' kindly supplied by Dr. F. Introzzi (FMB-CRA, Sant'Angelo Lodigiano, LO, Italy). In order to reduce the seedborne *Fusarium* contamination in maize, seeds were sterilized following a modified version of the procedure described by Daniels (1983). Seeds were placed in sterile plastic cups, covered with distilled water and stirred for 3 min. Water was removed and 100% commercial bleach (6% sodium hypochlorite) was added to cover all the seeds, which were then stirred in bleach for 20 min on a reciprocal shaker. The bleach was removed, and the seeds were rinsed twice in sterile water. They were then covered with sterile distilled water and allowed to soak for 4 h at 25 °C. They were rinsed twice more, covered with sterile distilled water and placed in a 60 °C water bath for 10 min. After the heat treatment, water was removed and the seeds were immediately transferred to a Petri dish on a sterile filter paper. The kernels were dried under sterile laminar flow hood. The seeds were then placed on the surface of PDA plates previously colonized by *F. verticillioides* after an incubation at 25 °C in the dark for 5 d. The PDA plates were incubated in the dark for 9 d at 20 °C. The assays were carried out on three replicates consisting of 10 seeds for each *F. verticillioides* isolate. A set-up of one hundred sterilized H6 maize seeds plated on uninoculated PDA plates served as negative control. Pathogenicity was evaluated by assessing germination percentage (seeds were considered germinated if the radicle was >5 mm long). In addition, seed colonization (SC), radicle decay (RD) and coleoptile rot (CR) were assessed, for each seed, on a 0–4 scale where 0 = seed surface uncovered with mycelium or well developed root/coleoptile showing negligible decay symptoms; 1 = ≤25% seed surface covered with mycelium or root/coleoptile area showing decay symptoms; 2 = >25 to ≤ 50% seed surface covered with mycelium or root/coleoptile area showing decay symptoms; 3 = >50 to ≤ 75% seed surface covered with mycelium or root/coleoptile area showing decay symptoms and 4 = ≥75% seed surface covered with mycelium or root/coleoptile area showing decay symptoms. The disease severity index in percentage expressed by percentage infection index (I%I) was calculated according the Townsend–Heuberger formula (Townsend and Heuberger 1943).

2.3. *Statistical analyses*

All statistical analyses were performed using PAST software ver. 1.95 (Hammer et al. 2001). I%Is assessed were grouped by location, maize growth stages, plant organs from which *F. verticillioides* strains were isolated and by mating type and female fertility of the isolates. Box plots were drawn in order to graphically explore the distributions of the data. The agglomerative hierarchical clustering analysis using the Euclidean similarity coefficients under the rule of unweighted

pair-group method using arithmetic averages (UPGMA) was applied to the I%Is obtained during the pathogenicity assays. Principal component analysis (PCA) was also carried out by using a variance-covariance matrix. Clusters shown in the bi-plot of the first two principal components were made by using clustering method information in combination with PCA.

3. Results

The results obtained during the pathogenicity assays showed that seed germination still occurred even if *F. verticillioides* colonized the entire kernel. High germination rates, up to 86%, were detected in each pathogenicity assay showing a low variability degree (data ranged from 86.7% to 100%). High germination rates were also observed in negative controls (data ranged from 97% to 100%) with healthy seeds and asymptomatic seedling tissues. Seedborne *Fusarium* contamination in negative controls was reduced less than 10% *Fusarium*-infected seeds. Wide variations were observed in SC, RD and CR I%Is within *F. verticillioides* populations. Significant correlations were found between SC (I%Is) and both RD ($r = -0.311$, $P < 0.05$) and CR ($r = 0.694$; $P < 0.01$). These results suggest that an increment in SC is associated with a reduction in RD or to an increase of CR. A general high ability to colonize maize seeds characterized all the *F. verticillioides* strains, which showed an average SC I%I above 50%. RD showed low I %I values (average I%I = 23%) and seemed to be less severe than SC and CR (average I%I = 33%) ability (Table 1).

Table 1 – Mean values of percentage infection indexes (I% I), regarding each pathogenicity trait (SC I%I, seed colonization I%I; RD I%I, radicle decay I%I and CR I%I, coleoptile rot I%I) of 181 *Fusarium verticillioides* strains grouped by: (A) maize growth stages at which they were collected: residues; GS00, seeds; GS13, seedlings with 3 unfolded leaves; GS53, plants with the tip of tassel visible; GS89, fully ripe plants; (B) sampling sites; (C) mating type (Venturini et al. 2011b); (D) female fertility (Venturini et al. 2011b) and (E) plant organs from which strains were collected.

		Number of isolates	SC I%I	RD I%I	CR I%I
A	Maize residues	23	57.9	35.2	38.9
	GS 00	24	57.1	17.7	35.2
	GS 13	24	43.9	42.9	31.2
	GS 53	20	42.7	36.8	31.1
	GS 89	90	57.2	13.8	32.8
B	Pontevico	62	53.3	23.2	32.4
	Pieve d'Olmi	57	53.7	24.5	33.7
	Sant'Angelo Lodigiano	62	54.3	27.9	35.9
C	MAT1-1	120	53.6	23.2	33.5
	MAT1-2	61	53.8	23.6	33.7
D	Female fertile	36	53.5	23.6	33.4
	Female sterile	145	53.8	23.4	33.6
E	Stalk	47	53.2	22.8	33.3
	Ear	73	53.8	23.5	33.6
	Roots	33	53.1	22.3	32.5
	Leaves	28	47.6	32.0	32.4
	Total	181	53.8	23.4	33.6

Variations in pathogenicity were more emphasized considering the maize growth stage at which *F. verticillioides* strains were collected rather than their geographical origin, mating type, female fertility of the isolates and the plant organs from which the strains were isolated (Table 1). Box plots of the distributions of the I%Is *F. verticillioides* strains grouped by location, plant organs from which *F. verticillioides* strains were isolated, and by mating type and female fertility did not reveal differences among groups (data not shown). On the contrary, box plot analysis showed that "growth stage" contributed to the variation of pathogenicity among the *F. verticillioides* isolates particularly in the case of SC and RD (Fig. 1).

The SC I%I values varied from 6.7 to 95.8% showing dissimilar distributions among *F. verticillioides* populations sampled at different maize GS. GS13 and GS53 isolated *F. verticillioides* populations displayed similar quartile distributions (Fig. 1). In general *F. verticillioides* strains isolated from residues and at GS00 and GS89 reported higher median SC I%I values than in other phenological stages. At GS00 and GS89 three outliers could be identified, two of them showing reduced ability to colonize the seeds and one (GS00) characterized by a high SC (I%I = 83%).

The RD I%Is, ranging from 2.5 to 85.8%, displayed a peculiar pattern among *F. verticillioides* populations: GS00 and GS89 groups exhibited the lowest RD capability as showed by quartile distributions (Fig. 1). Residues group, GS13 and GS53 *F. verticilliodes* isolates displayed similar medians values and quartile distributions. As far as RD I%Is are concerned, six outliers isolated from residues group, GS00 and GS89 were characterized by higher capability of causing RD than the rest of the population. The majority of the outliers were strains isolated in the same field in PDO.

The CR I%Is, value range from 2.5 to 85.0%, showed similar quartile distributions in all the examined *F. verticillioides* populations. Just a single strain, namely GV1343 isolated at SAL during GS89 from external husk, was able to cause 85% CR.

The PCA showed that the SC ability of *F. verticillioides* isolates was positively correlated to CR ability. The second eigenvector (PC2) explaining a further 32% of variation was associated with RD ability of strains. On the contrary, germination inhibition ability of the isolates had an irrelevant weight in explaining data variation. Cluster analysis showed that isolates can be grouped in three main clusters (Fig. 2). Cluster I included two *F. verticillioides* strains, both isolated from ear during GS89, causing the most severe symptoms as accounted for PC1. One of the strains grouped in cluster I was the outlier observed in CR I%I distribution (Fig. 1). Cluster II grouped the majority of the strains able to cause symptoms with very variable severity. Cluster III consisted of 17 *F. verticillioides* strains characterized by high RD I%Is and mainly isolated at GS13 and GS53.

4. Discussion

This research aimed at determining the pathogenicity variation in *F. verticillioides* population associated with maize in northern Italy. Pathogenicity variation, extensively studied within *G. fujikuroi* clade in order to characterize biological

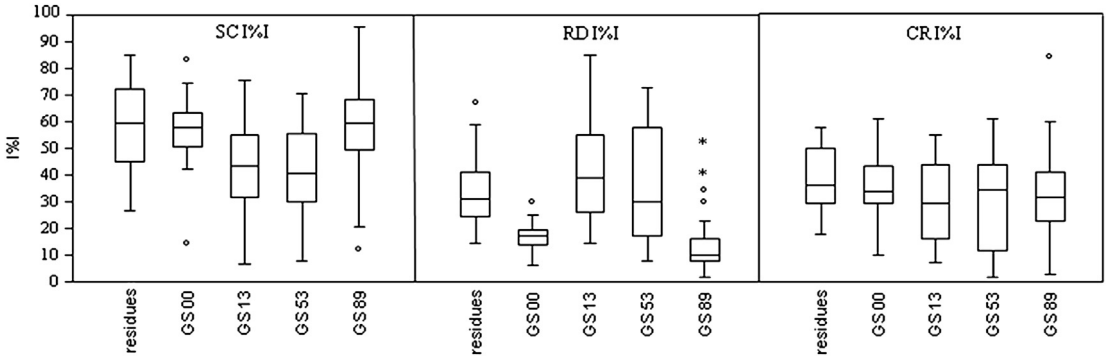

Fig. 1 – Box plots of the distributions of percentage infection index (I%I) of seed colonization (SC), radicle decay (RD) and coleoptile rot (CR) of 181 *Fusarium verticillioides* strains grouped by maize growth stages (GS) at which they were collected: residues; GS00, seeds; GS13, seedlings with 3 unfolded leaves; GS53, plants with the tip of tassel visible; GS89, fully ripe plants. The box plots depict the five-number summaries, namely the minimum and maximum values, the upper and lower quartiles and the median. The median is identified by a line inside the box. The length of the box represents the interquartile range (IQR). Values more than 3 IQRs from either end of the box are labeled as extremes and are denoted by an asterisk (*). Values more than 1.5 IQRs but less than three IQRs from either end of the box are labeled as outliers (○).

species (Leslie et al. 2005; Iglesias et al. 2010), has rarely been investigated in *F. verticillioides* populations or, in general, on relatively small strain sets (Asran and Buchenauer 2002; Cumagun et al. 2009; Covarelli et al. 2012). Thus, the wide collection of isolates taken into account in the present study seemed to be better suited for evaluating the possible differences in *F. verticillioides* pathogenicity at the population level.

Depending on the biotic and abiotic environment of the plant, *F. verticillioides* on maize can behave either as a pathogen or an endophyte. Such behavioral variability is an important issue because of its significance in elucidating the association between fungus and host as well as in defining the population structure of the pathogen. The *F. verticillioides* strains considered in this study proved to be pathogen on maize although they induced different symptoms. *Fusarium verticillioides* did not have detrimental effects on seed germination even in presence of high SC I%Is, as showed by previous studies (Naik et al. 1982; Danielsen and Funck Jensen 1998; Venturini et al. 2010). These results demonstrate that seed decay caused by *F. verticillioides* occurs only in stressful conditions for maize, while plant development may even be enhanced by *F. verticillioides*-maize interaction (Yates et al. 1997; Oren et al. 2003). Depending on the amount and the type of fungal inoculum in soil and seed, *F. verticillioides* could be associated with post emergence diseases described by several symptoms such as SC, RD and CR.

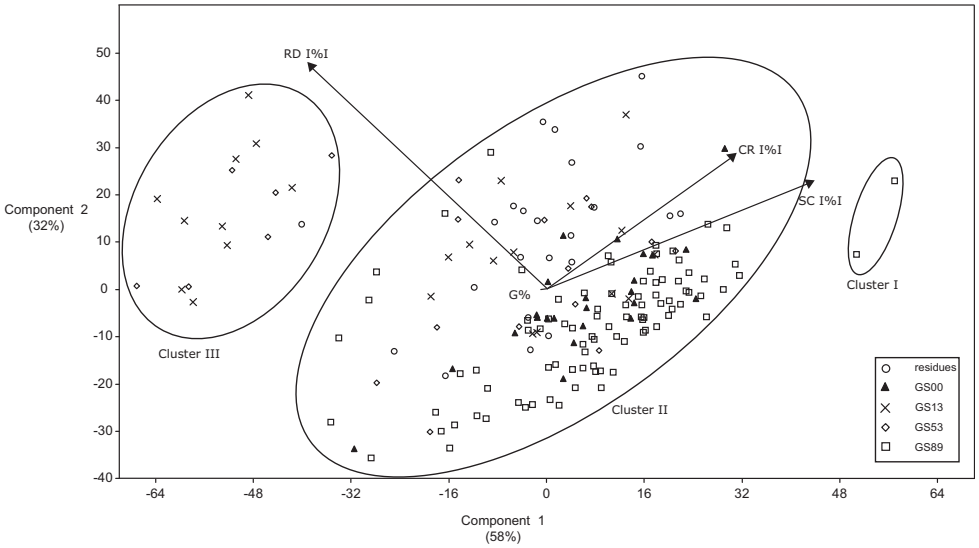

Fig. 2 – Bi-plot of principal component analysis computed from percentage infection index (I%I) of seed colonization (SC), radicle decay (RD) and coleoptile rot (CR) and germination percentages (G %) of 181 *F. verticillioides* strains. Maize growth stages (GS) at which *F. verticillioides* strains were isolated: maize residues (○); seeds (▲); seedlings with 3 unfolded leaves (×); plants with the tip of tassel visible (◇); fully ripe plants (□).

The CR severity distributions were similar for all the strains in the populations indicating that this trait is valueless in describing *F. verticillioides* population variability. The other quantitative traits measured, SC and RD abilities, showed evident fluctuations depending on the GS of strain isolation. The ability to colonize seed, expressed as SC, was widespread in all isolates although some differences were detected among groups. It seemed that *F. verticillioides* strains obtained from young (GS13) and flowering (GS53) plants were less pathogenic against seed tissues in comparison with the fungal individuals isolated at the beginning and at the end of the vegetative season. Also, the same *F. verticillioides* strains from young and flowering plants induced the highest disease severity considering RD symptoms. These results would suggest that the phenological stages of maize might operate as a selective pressure, thus selecting the isolates more able to colonize the seed or the radicle. In addition, such pathogenic variations could be explained by the multiple interactions occurring between *F. verticillioides* and the plant during maize's growth cycle. It is possible to speculate that colonizing ability of root tissues could represent the weak pathological response of endophytic *F. verticillioides* strains during seedling (GS13) and flowering (GS53) stages of the plant.

The large variation in pathogenicity was also explained by cluster analysis and PCA where the majority of the strains grouped in Cluster III were isolated during seedling and flowering stages of the plant showing high RD levels. On the other hand, Cluster I included two *F. verticillioides* strains, both isolated from ear during GS89, causing the most severe symptoms. The bi-plot allows to distinguish the isolates according to pathogenicity traits suggesting that pathogenicity might be a reliable marker for describing *F. verticillioides* population variability.

Neither the mating types nor the female fertility seems to influence the pathogenic attitude of *F. verticillioides* and consequently the average pathogenicity of the fungal population associated with maize in northern Italy. This study underlines the importance of careful characterization of *F. verticillioides* populations in order to clarify whether the phenological stages might also have an effect on the genetic diversity of fungal populations. The relative low frequency at which *F. verticillioides* sexual reproduction occurs in northern Italy (Venturini et al. 2011b) suggests that individuals within populations should not be genetically different. However, since the reproductive mode may be different among populations of the same fungal pathogen, the genetic diversity in several *F. verticillioides* populations from diverse geographic areas should be investigated.

Disclosure

The authors declare no conflict of interest. All the experiments undertaken in this study comply with the current laws of Japan.

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