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Fusarium verticillioides contamination patterns in Northern Italian maize during the growing season

GIOVANNI VENTURINI, GEMMA ASSANTE and ANNAMARIA VERCESI

Dipartimento di Produzione Vegetale sezione di Patologia Vegetale, Università degli Studi di Milano, via G. Celoria 2, 20133 Milano, Italy

Summary. *Fusarium verticillioides*, often found in maize kernels, is the major fungal colonizer of maize in Northern Italy. The fungus can cause plant diseases, or grow endophytically and synthetize mycotoxins. Ear rot caused by *F. verticillioides* may reduce crop yield. Fumonisins produced by the fungus may harm humans and animals. In order to gather information on contamination patterns of *F. verticillioides* under field conditions, the current study assessed the isolation frequency percentages (IFs) of the fungus during different growth stages (GS) of four maize hybrids (Arma, Costanza, Kubrick and Tucson) cultivated in Northern Italy. *Fusarium verticillioides* contamination was detected in all the examined plants and in maize crop residues, but IF levels varied depending on the GS. The fungus colonized all the residues of maize plant organs, and ear debris were the preferential survival sites. *Fusarium verticillioides* was the major fungal contaminant at GS 00, in all seed lots with the only exception of Tucson hybrid. At the seedling stage GS 13, a similar isolation pattern was observed, but with lower IFs than in the correspondent seedlings grown in aseptic conditions: roots and mesocotyls were more contaminated than leaves. In plants before silking (GS 53), *F. verticillioides* contamination was localized in the basal organs. At maturity (GS 89), however, a general increase of IFs was observed in all organs. Since glumes and husks were the most contaminated organs, silks can be considered the most important pathways for *F. verticillioides* infection. The present study analyzes the endemic presence of *F. verticillioides* in Northern Italian fields and suggests further research of resistance factors in silks and husks as to indicate possible mechanisms for reducing fungal contamination.

Key words: *Gibberella moniliformis*, isolation frequency, *Zea mays* L.

Introduction

Maize (*Zea mays* L.) is one of the most important crops in Northern Italy, where about 820,000 hectares are grown with a production of 7.2 million tonnes of silage and kernels (ISTAT, 2009). Maize in Northern Italy is often infected by several *Fusarium* species which can cause seedling blight, root, stalk and ear rot (Logrieco *et al*., 2002). *Fusarium* pink ear rot usually occurs on individual or groups of kernels that are often covered with white or light pink mycelium (Koehler, 1959; Moretti *et al*., 2002). The main etiological agent of pink ear rot is *Fusarium verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* Sheldon, teleomorph *Gibberella moniliformis* Wineland, syn. *G. fujikuroi* mating population A) (White, 1999; Nelson, 1992). Two other mating populations belonging to the *G. fujikuroi* species complex may occur in pink ear rot: *F. proliferatum* (Matsushima) Nirenberg (teleomorph *G. intermedia*, syn. *G. fujikuroi* mating population D, and *F. subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas, teleomorph *G. subglutinans*, syn. *G. fujikuroi* mating population E)

Corresponding author: G. Venturini
Fax: +39 02 50316781
E-mail: giovanni.venturini@guest.unimi.it

(Desjardins *et al*., 1994; Bottalico, 1998; Reynoso *et al*., 2006). Occasionally, other *Fusarium* species causing red ear rot can be associated with maize, namely *F. graminearum* Schwabe, *F. culmorum* (W. G. Smith) Saccardo and *F. crookwellense* Burgess, Nelson & Toussoun (Moretti *et al*., 2002). The relative presence of all these species depends on climatic conditions, genetic resistance of maize hybrids and cropping rotations (Moretti *et al*., 2002). *Fusarium* pink and red ear rots are the major causes of quality and yield losses in maize, especially in temperate areas characterized by dry periods before or during kernel filling coupled with high humidity and rainfall during maturity (Koehler, 1959; Shelby *et al*., 1994).

Fusarium pink ear rot can be associated with the accumulation of mycotoxins such as fumonisins, which are a group of at least 28 long-chain amino polyalcohols characterized from 1988 onwards (Bezuidenhout *et al*., 1988; Gelderblom *et al*., 1988). The fumonisin most frequently found in maize is fumonsin B_1 (FB_1), which is involved in animal and human diseases by interfering with sphingolipid metabolism (Marasas, 1995). Moreover, fumonisins have been associated with esophageal cancer of humans in some regions of the world (Marasas, 2001) and are subjected to severe regulation in many countries. Among the *Fusarium* species able to produce fumonisins, only those belonging to the *G. fujikuroi* clade, and especially *F. verticillioides* and *F. proliferatum*, have been associated with fumonisin contamination of agricultural commodities like maize and its derived products (Marìn *et al*., 2004).

Effective management of fumonisin contamination in maize relies on the control of the fungal infection and requires a better understanding of *F. verticillioides* biology and epidemiology. A regression model simulating the life cycle of *F. verticillioides* in both controlled and field conditions has been recently developed (Rossi *et al*., 2009), but the contamination dynamics of *F. verticillioides* during different maize growth stages and the host–fungus interactions require further elucidation. Previous studies have shown that under ordinary plant growth conditions, *F. verticillioides* grows endophytically within maize plants causing asymptomatic infection (Bacon and Hinton, 1996) which may benefit plants by increasing their size and productivity (Yates *et al*., 2005). However, under abiotic and/or biotic stress conditions, this relationship may convert to a disease and/or fumonisin–producing interaction (Bacon and Nelson, 1994; Abbas *et al*., 2006). Both symptomatic and asymptomatic kernel infections by *F. verticillioides* can result in decreased grain quality and economic losses due to contamination by FB_1 (Glenn *et al*., 2004). The primary source of inoculum for kernel infection resides in plant residues or in the soil. *Fusarium verticillioides* can produce thickened hyphae which also have survival capabilities (Nyvall and Kommedahl, 1968) and can colonize senescent host tissues (Cotten and Munkvold, 1998). Seedborne fungus is an additional inoculum source, although its role is controversial. Systemic infection can be due to conidia or mycelia located inside seeds. The fungus develops inside young plants, moving from the roots to the stalks and finally to the cobs and kernels (Sumner, 1968; Kedera *et al*., 1994; Munkvold *et al*., 1997; Oren *et al*., 2003).

Besides systemic transmission, insect injury and silk infection have also been identified as important ear infection pathways for *F. verticillioides* (Munkvold *et al*., 1997). In temperate areas, the occurrence of both *Fusarium* ear rot and symptomless infection are closely related to insect injuries, primarily due to *Ostrinia nubilalis* (Munkvold *et al*., 1999). Infection through silks was demonstrated as the most important pathway for *F. verticillioides* infection (Munkvold *et al*., 1997; Desjardins *et al*., 2002) by experimental inoculations carried out with *nit* mutants, but not tested with natural inoculum under field conditions. For this reason the objectives of the present work were: i) to assess the natural occurrence of *F. verticillioides* in several plant organs during different maize growth stages (GS) in three maize fields located in Regione Lombardia and sowed with four different hybrids; and ii) to investigate *F. verticillioides* population dynamics by evaluating its isolation frequency percentages (IFs).

Materials and methods

Sites and samplings

Samplings were carried out in three maize fields located in Lombardia (Italy), during the 2008 cropping season, in Sant'Angelo Lodigiano

(45°14' N, 9°24' E; altitude 73 m), Pontevico (45°16' N, 10°5' E; altitude 55 m) and Pieve d'Olmi (45°5' N, 10°7' E; altitude 36 m). All the fields were in a maize-maize rotation; standard soil fertilization and weed management practices were applied at all the sampling sites. Right and left diagonal sampling design was adopted (Delp *et al*., 1986). The hybrids cultivated in 2007 and 2008 in the three experimental fields, together with their FAO rating, are listed in Table 1. A first sampling was carried out in February 2008 on maize residues cultivated at the three sites during the 2007 cropping season. Since the sowing date, maize samples were collected at the following growth stages (GS): seeds (GS 00, Biologische Bundesanstalt, Bundessortenamt and Chemical Industry [BBCH] scale; Lancashire *et al*., 1991), seedlings with 3 unfolded leaves (GS 13), plants with the tip of tassel visible (GS 53), and fully ripe plants (GS 89).

At each sampling date, 30 plants or seeds at GS 00 were collected, placed in a paper bag at 4°C and processed within 24 h. Different kinds of plant organs, depending on the sampling growth stage, were analyzed as following:

1. maize residues: roots, I-II-III internodes, I-II-III nodes, cobs, husks;
2. seeds (GS 00) grown in aseptic conditions until seedlings developed three unfolded leaves;
3. seedlings (GS 13) with three unfolded leaves withdrawn from fields;
4. plants before silking (GS 53): roots, brace roots, I internodes, II nodes, VI leaves, tassels;
5. fully ripe plants/ears (GS 89): roots, brace roots, I internodes, I-II-III-V-VII nodes, kernels, glumes, cobs, internal husks, external husks.

Isolation and identification of *Fusarium* strains

Strains belonging to the *G. fujikuroi* clade were isolated from symptomatic and asymptomatic plants and ears. Asymptomatic organs of maize plants collected at all different GS were rinsed in tap water to remove soil particles, surface treated with NaOCl (7%) for 1 min, and rinsed in sterile deionized water for 3 min. After drying, five fragments (5×5 mm) were obtained from each tissue sample and placed on potato dextrose agar (PDA, Difco™) acidified to pH 4.5–5.0 with a lactic acid solution (50%) (APDA). At GS 00, the seeds collected in the fields were sterilized, grown in aseptic conditions: the corresponding seedlings characterized by three unfolded leaves were split with a sterile knife and analyzed as described above. At GS 89, six samples each consisting of a kernel, its respective glume, and a portion of the cob tissue bearing the glume, were randomly selected from each collected ear. Symptomatic tissue samples were directly plated. After 4 days incubation at 25°C, all the APDA Petri plates were microscopically examined (×10 magnification), and at least one *Fusarium* spp. strain per organ was isolated. Isolates belonging to the *G. fujikuroi* clade (Leslie and Summerell, 2006a) were subcultured as single spores and then identified as biological species using the criteria recommended by Leslie and Summerell (2006b). In order to identify *F. verticillioides,* sexual crosses were made twice on carrot agar following standard protocols (Leslie and Summerell, 2006b) with the *G. moniliformis* standard tester strains FGSC7600 (*MAT–1*) and FGSC7603 (*MAT–2*) as female parents (Fungal Genetics Stock Center, Kansas City, MO, USA) and

Table 1. Maize hybrids tested in 2007 and 2008.

Site	Hybrid	
	2007 Maize residues	2008 Maize plant/ears
S. Angelo Lodigiano	DCK6666 (FAO rating 600)[a]	Tucson (FAO rating 700) Kubrick (FAO rating 600)
Pieve d'Olmi	Costanza (FAO rating 600)	Costanza (FAO rating 600)
Pontevico	Helen (FAO rating 600)	Arma (FAO rating 700)

[a] FAO rating is the maize relative maturity system adopted in Europe, based on a three digit code ranging from 100 to 900, calculated on the basis of the number of days required to reach a grain moisture content lower than 20%.

field *Fusarium* isolates as male parents. A set-up with tester strains alone was used as an experimental control. Before sexual crosses were made, cultural and microscopic features were utilized in order to divide all the isolates belonging to the *G. fujikuroi* clade into several homogenous groups. Sexual crosses were performed on at least 50% of the isolates belonging to each group. A cross was considered fertile when a cirrhus of ascospores oozing from a mature perithecium was observed 2–6 weeks after fertilization. Field isolates producing sterile crosses with *G. moniliformis* tester strains were crossed, following the same procedure, with standard tester strains of the mating population D (*G. intermedia* FGSC7615 [*MAT–1*] and FGSC7614 [*MAT–2*]) and E (*G. subglutinans* FGSC7616 [*MAT–1*] and FGSC7617 [*MAT–2*]) in the *G. fujikuroi* clade. Unfertile strains were considered belonging to unassigned species within the *G. fujikuroi* species complex.

Statistical analysis

The isolation frequencies (IFs) were assessed only for *F. verticillioides*, and were calculated according to the following formula (Sahashi *et al*., 2000):

$$IF = N_i / N_t \times 100$$

where N_i and N_t are the number of fragments contaminated by the fungus and the total number of fragments cultured on APDA plates, respectively.

Frequency data were transformed (arcsine square root) prior to analysis of variance (ANOVA), with analyses carried out using PAST software (ver. 1.95; Hammer *et al*., 2001). Multiple comparisons of IF means were carried out using Tukey's test ($\alpha = 0.05$).

Results

A total of 1,428 *G. fujikuroi* clade strains were isolated from maize plant and ear organs. Of these, 891 isolates were cross–fertile with mating type tester strains FGSC7600 (*MAT–1*) or FGSC7603 (*MAT–2*). They were assigned to *F. verticillioides,* while 464 *G. fujikuroi* clade strains were assigned to *F. proliferatum* and *F. subglutinans*. The remaining 73 *Fusarium* spp. isolates could not be assigned, but morphological features and cultural characteristics indicated that they belonged to other species of the *G. fujikuroi* clade.

***Fusarium verticillioides* isolation frequencies in maize residues**

From the 900 tissue fragments obtained from maize residues, 321 *G. fujikuroi* clade strains were isolated and sexual crosses confirmed that 149 isolates belonged to *F. verticillioides*. With variable IFs, *F. verticillioides* contaminated all types of tissues, with the only exception of the II node of hybrid DKC6666 (Table 2A). In DKC6666 and Helen hybrids, the lowest IF values detected on root fragments were significantly different from those assessed in the majority of the other organs. Only in Helen hybrid *F. verticillioides* contamination did increase from roots to the I node and remained almost unchanged in the other stalk tissues. Cobs were also the most frequently contaminated organs in hybrid DKC6666. The highest IF in Helen hybrid was found in cobs and was similar to that detected in the corresponding husks (Table 2A). In contrast, Costanza hybrid was homogenously contaminated by *F. verticillioides,* since IF values assessed on its various organs did not show statistically significant differences (Table 2A).

***Fusarium verticillioides* isolation frequencies in seedlings grown in aseptic conditions**

In order to investigate *F. verticillioides* contamination in maize seeds and seedlings, seeds were grown under aseptic conditions till seedlings developed three unfolded leaves (GS 13). High seed germination rates, up to 85%, were observed for all hybrids except for Kubrick, which showed the lowest germination percentage (56%). All the seeds unable to germinate were completely colonized by *Fusarium* spp. mycelia. At GS13 whole seedlings, divided in suitable fragments, were plated on APDA, and 221 *G. fujikuroi* clade strains were isolated. Identification assays indicated that all the 221 isolates belonged to *F. verticillioides*. All the healthy seedlings were contaminated by *F. verticillioides* except for Tucson seedlings which showed a lower *F. verticillioides* incidence (45%). *Fusarium verticillioides* contamination did not cause any disease in maize seedlings, such as root rot, damping off or seedling blight. IFs assessed in seedling fragments (Table 2B) obtained from Kubrick and Costanza hybrids were very high, ranging from 89 to 100%, in all the seed-sorrounding organs and in the I leaves. In the same hybrids, IF values significantly decreased in II and III leaves. A similar contamination pattern was detected in

Table 2. *Fusarium verticillioides* isolation frequencies (IFs) in different plant organs during different maize growing stages: IFs in crop residue organs; IFs in seedlings grown in aseptic conditions; IFs in seedlings grown in the field; IFs in plants before silking; IFs in fully ripe plants and ears. Each value represents the average IFs of 30 plants. Within each hybrid and within each growth stage (GS), values with different letters indicate significantly different values at $P \leq 0.05$ (Tukey's test). Absence of letters indicates no significant differences.

Growth stage (GS)[a]	Plant organs	S. Angelo Lodigiano	Pieve d'Olmi	Pontevico	Mean
		DKC6666 [b]	Costanza	Helen	
Maize residues	Roots	8 c	9	4 d	7 [c]
	I internode	11 bc	12	20 bc	14
	I node	9 bc	16	30 b	19
	II internode	14 b	10	19 bc	14
	II node	0	13	17 bc	10
	III internode	n.a.[d]	12	15 cd	13
	III node	n.a.	9	16 bc	13
	Cobs	47 a	4	36 a	29
	Husks	9 bc	25	33 a	22
		$P=10^{-7}$	$P=0.483$	$P=0.002$	$P=0.395$

Growth stage (GS)[a]	Plant organs	S. Angelo Lodigiano		Pieve d'Olmi	Pontevico	Mean
		Tucson [e]	Kubrick	Costanza	Arma	
GS 00[f]	Roots	14	97 a	89 b	91 a	73 [c]
	Seed	25	100 a	100 a	94 a	80
	Mesocotyl	29	100 a	100 a	84 bc	77
	Coleoptile	12	100 a	100 a	87 b	94
	I leaf	4	100 a	91 b	82 bc	69
	II leaf	6	71 b	82 c	80 cd	60
	III leaf	5	68 c	74 d	72 d	55
		$P=0.147$	$P=0.01$	$P=10^{-8}$	$P=0.004$	$P=0.825$
GS 13	Roots	11	37	27	23 b	25 b [c]
	Seed	12	23	40	20 bc	24 b
	Mesocotyl	27	38	36	39 a	35 a
	Coleoptile	12	31	36	22 b	25 b
	I leaf	7	14	27	15 bcd	16 c
	II leaf	2	6	16	12 cd	9 d
	III leaf	0	0	18	9 d	7 e
		$P=0.193$	$P=0.288$	$P=0.131$	$P=0.024$	$P=0.003$
GS 53	Roots	11	4	5	3	6 b [c]
	Brace roots	16	10	7	11	11 a
	I internode	6	2	0	6	4 c
	II node	12	2	0	0	3 d
	VI leaf	0	0	3	0	1 e
	Tassel	0	0	0	0	0
		$P=0.736$	$P=0.581$	$P=0.111$	$P=0.416$	$P=0.015$

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Table 2. *continue*

Growth stage (GS)[a]	Plant organs	S. Angelo Lodigiano		Pieve d'Olmi	Pontevico	Mean
		Tucson [e]	Kubrick	Costanza	Arma	
GS 89	Roots	7	1	11 cd	3	5 h [c]
	Brace roots	13	7	5 cde	21	12 efg
	I internode	17	15	2 e	10	11 fg
	I node	17	13	7 cde	22	15 defg
	II node	15	22	17 c	15	17 cde
	III node	15	35	17 c	14	20 bcd
	V node	12	22	30 b	11	19 cd
	VII node	3	10	13 cde	10	9 gh
	Kernels	12	28	33 b	19	23 bc
	Glumes	24	31	53 a	18	32 a
	Cobs	18	31	44 ab	15	27 ab
	Internal husk	33	33	39 ab	25	33 a
	External husk	18	22	6 cde	20	16 cdef
		P=0.534	P=0.097	P=10^{-7}	P=0.281	P=0.002

[a] GSs are the phenological growth stages listed in the BBCH scale of maize (Lancashire *et al*., 1991).
[b] Maize hybrids cultivated in the experimental fields during 2007 cropping season; C, D, E.
[c] Values are means of IFs in each row; C, D, E.
[d] n.a., Plant organs not available; C, D, E.
[e] Maize hybrids cultivated in the experimental fields during 2008 cropping season; C, D, E.
[f] GS00 represents seeds, sterilized and grown in aseptic conditions until seedlings with three unfolded leaves.

Arma hybrid, with an IF decrease in the leaves, and also in mesocotyls and coleoptiles. Hybrid Arma showed the highest IF values in seeds, comparable to those detected in roots. Fragments obtained from the different seedling organs of Tucson hybrid were clearly less contaminated than those obtained from other hybrids and showed statistically similar IF values (Table 2B).

***Fusarium verticillioides* isolation frequencies in seedlings grown in the field**

A total of 143 *G. fujikuroi* clade strains were isolated from asymptomatic seedlings collected in the field at GS13 and directly plated on APDA. Eighty-four *F. verticillioides* isolates out of 143 were identified on the basis of biological criteria. Therefore a lower number of *F. verticillioides* strains amongst *G. fujikuroi* clade isolates was obtained from seedlings grown in the field (59%) than from the seedlings grown in aseptic conditions (100%). Likewise, the *F. verticillioides* IFs assessed in seedlings grown in the field were lower than those detected in the corresponding seedlings grown in aseptic conditions in all the seedling organs except the I leaf in Tucson hybrid (Table 2C). Only in Arma hybrid were significant differences detected between IFs from different seedling organs: in particular IFs detected in mesocotyls were significantly higher than those in all the other organ fragments. In addition, IF values concerning II and III leaves were significantly lower than those obtained for the other fragments, with the only exception of the IFs detected in the seed and in the I leaf. This trend was also confirmed by comparing the IF average values concerning each seedling organ of the four hybrids. In Tucson and Kubrick hybrids, grown in Sant'Angelo Lodigiano field, *F. verticillioides* did not contaminate the III leaf (Table 2C).

***Fusarium verticillioides* isolation frequencies in plants before silking**

At GS 53, the 120 plants collected in the field did not show any root, stalk or foliar disease

symptoms. No visible insect damage was detected on the plant organs. Among the 153 *G. fujikuroi* clade strains isolated from the 3,600 tissue fragments plated on APDA, 47 isolates belonged to *F. verticillioides*. Very low and not significantly different IF values were detected on plant organs (Table 2D). *Fusarium verticillioides*, however, contaminated roots and brace roots of the four examined hybrids. In Arma hybrid, *F. verticillioides* colonization extended from roots to the I internodes and did not exceed the II nodes in Tucson and Kubrick hybrids. In hybrid Costanza, *F. verticillioides* was isolated from roots, brace roots and VI leaf segments. Tassels did not show any fungal contamination. Significant differences were found between the IF mean values for the various plant organs: roots and brace roots both showed significantly higher IFs than stalk organs and leaves. *Fusarium verticillioides* IFs progressively decreased from the I internodes to the VI leaves (Table 2D).

***Fusarium verticillioides* isolation frequencies in fully ripe plants and ears.**

At maturity, GS 89, 120 plants and ears were collected from the experimental sites. The plants of Tucson and Kubrick hybrids collected from the Sant'Angelo Lodigiano field showed very high rates of insect damage, respectively 100 and 93%, both on stalks and ears. *Fusarium* ear rot, diagnosed by the presence of mycelia both on the tips and groups of kernels randomly distributed in different ear parts, was detected in Tucson and Kubrick hybrids on respectively 13 and 40% of the collected ears. Moreover, in Kubrick hybrid ear rot symptoms and insect damage were simultaneously observed in 33% of the ears. In Costanza hybrid, 27% of stalks and 53% of ears showed insect damage, while *Fusarium* ear rot was associated with insect damage only in 3% of the symptomatic ears. In Arma hybrid, 90% of stalks and ears were damaged by insects. *Fusarium* ear rot symptoms were observed on 60% of ear tips and on 10% of randomly distributed kernels. In addition, 55% of ears were affected both by ear rot and injured by insects.

Symptomatic organs were directly plated on APDA and 26 *G. fujikuroi* clade strains were isolated. Amongst these strains, 20 isolates belonged to *F. verticillioides*. 8,160 asymptomatic tissue fragments were plated on APDA. Among the 590 *G. fujikuroi* clade strains isolated from plant tissues, 390 belonged to *F. verticillioides*. No significant differences were found between the IFs detected in the tissue fragments collected from the Sant'Angelo Lodigiano field (Table 2E). In Tucson and Kubrick hybrids, IF values did not exceed 40% in all the investigated organs: the highest IF (35%) was detected in the III nodes of Kubrick hybrid and the lowest (1%) in the root fragments of the same hybrid. In Tucson hybrid, IF values were higher than those obtained for Kubrick hybrid only in the basal parts of the maize plants. *F. verticillioides* contamination in Arma hybrid was similar in all tissue fragments, while a peculiar *F. verticillioides* contamination pattern was observed in Costanza hybrid (Table 2E). The highest IF values were detected in hybrid Costanza ear tissues and in particular in the glumes. The other tissue fragments, except for the V nodes, were significantly less contaminated than the reproductive plant organs apart from the external husks. Kernels showed IFs lower than glumes, but similar to those found on cobs, internal husks and the V nodes. *Fusarium verticillioides* contamination in stalk tissues was similar in all the nodes between I and VII, but not in the V nodes. IF values observed for roots and brace roots were similar to those detected in the majority of stalk fragments. A slightly different pattern was observed in the mean values. Roots showed IFs comparable only to the VII nodes, which was significantly less contaminated than ear and stalk tissues. The I internodes and the I nodes, however, were highly contaminated. IFs observed on kernels and those calculated for stalk tissues were similar except for the I, the VII nodes, and the I internodes. External husks were less contaminated than other ear tissues, while kernels were frequently infected by *F. verticillioides* (Table 2E).

No significant correlations were found between *Fusarium* ear rot incidence, insect injury incidence and IFs.

Discussion

The present study is the first report of *F. verticillioides* isolation frequencies (IFs) during dif-

ferent maize growth stages under field conditions in Italy, and aimed to evaluate the variation of *F. verticillioides* contamination in field crops during the growing season. The long term goal was to better understand the fungal life cycle and the most important infection pathways of the pathogen. A comparison with earlier studies is affected by the uncertainty arising from the revision of the species previously named *F. moniliforme*, which prevents the assumption of a complete equivalence between this name and *F. verticillioides*. Moreover, the only study, carried out by Kommedahl *et al*. (1979), on natural *Fusarium* spp. contamination in symptomless maize plants during the growing season, dealt uniquely with IFs in roots and I internodes. Kommedahl *et al*. (1979) detected *F. moniliforme* infection in maize stalks only after silking, while the roots had already been contaminated in previous growing stages. A similar contamination pattern was observed in North Italian maize on a major number of plant organs. *Fusarium verticillioides* colonized all the examined maize residues and growing plants with a great variation depending on the GS.

During the first sampling on debris from the previous maize crop, *F. verticillioides* was found in all residues of maize organs. Because of their high IFs, ear debris seemed to be the preferential site for survival. *Fusarium verticillioides* populations on maize debris derived both from previous season contamination and populations in the soil that can colonize senescent maize tissues (Cotten and Munkvold, 1998). In winter, *F. verticillioides* usually survives on maize stubble, forming quiescent structures that resemble chlamydospores, but which are actually thickened hyphae (Nyvall and Kommedhal, 1968).

All tissues of seedlings derived from seeds previously sterilized and grown in aseptic conditions showed high contamination levels due to *F. verticillioides*. All the isolated strains derived exclusively from seedborne *F. verticillioides*, which had no detrimental effects, however, on seed germination and seedling growth, as has been reported in other studies (Naik *et al*., 1982; Danielsen and Funck Jensen, 1998). Aseptic conditions were probably conductive for *F. verticillioides* colonization of seedlings which was not hampered by competition with other microorgansims. In addition, Rheeder *et al*. (1990) demonstrated that *F. verticillioides* could act as a deterrent to kernel invasion by other seed-infecting fungi including *F. graminearum* and *Diplodia maydis*. With the only exception of Tucson hybrid, high IFs were detected in all the seedling tissues, even if significant decreases were observed in II and III leaves. These data demonstrated that seedborne *F. verticillioides* could asymptomatically colonize maize seedlings, confirming that seeds are potential *F. verticillioides* inoculum sources for the growing maize plants, although the presence of *F. verticillioides* seed-inoculated strains into ears seemed to be dependent on several environmental and host factors (Munkvold *et al*., 1997).

Seedlings collected in the field at GS13, apart from those belonging to the Tucson hybrid, were characterized by lower IFs than the corresponding seedlings grown in aseptic conditions, even if the contamination pattern detected in the several plant organs was similar. Oren *et al*. (2003) have demonstrated that roots and mesocotyl are the most contaminated plant organs, and suggested that the small amount of fungal biomass involved in systemic seedling colonization and localized in root and mesocotyl tissue did not cause rot or other diseases symptoms.

The *G. fujikuroi* clade population associated with seedlings in the field included other species besides *F. verticillioides,* which was contrary to this species dominating in aseptic conditions. The presence of *F. proliferatum* and *F. subglutinans* in seedlings collected in the field was probably due to an external contamination source, but seed transmission mechanisms similar to those described for *F. verticillioides* can not be excluded for these other fungi (Logrieco and Bottalico, 1988; Wilke *et al*., 2001).

Fusarium verticillioides contamination decreased at GS 53, resulting in very low IFs in all the examined plant organs. The species was constantly detected only in the basal organs of the plants, as also observed by Wilke *et al*. (2007). They reported that inoculated *F. verticillioides* resided in roots and crown tissues at the early maize growth stages. Moreover, the present results were similar to those obtained by Lawrence *et al*. (1981), who found that *F. verticillioides* colonized entire plants only after tasseling. The isolation data confirmed that seed–to–kernel transmission involved several growing stages: seed–to–seedling

transmission was just the first step, while seedling–to–stalk transmission in the following stages (GS 53) was probably limited by the host and by environmental conditions.

At GS 89 *Fusarium* ear rot was observed on collected ears, indicating that *F. verticillioides* infection often evolved from asymptomatic to symptomatic at plant maturity. The plants appeared to outgrow the fungus until silking. On the contrary, after silking the entire plant was colonized by *F. verticillioides*. At the end of the growing season, *F. verticillioides* contamination in maize underwent rapid changes, and multiple infection pathways took place through silk colonization, insect lesions, and systemic transmission. Beyond fungus internal growth in the plant stalks, *F. verticillioides* is able to colonize ears through wounds caused by insects, birds or hail (Bakan *et al*., 2002). Our results indicated that an important *F. verticillioides* infection court was represented by silks. Airborne *F. verticillioides* conidia, abundantly produced and well-adapted for wind, rain and vector dispersal, are likely to land on maize silks and to grow down to the developing kernels (Munkvold and Desjardins, 1997). At GS 89 asymptomatic ear tissues, except external husks, were significantly more contaminated by *F. verticillioides* than roots and stalks. The V nodes, however, were contaminated as well as the ear tissues. In addition, glumes showed higher IFs than asymptomatic kernels This agrees with the observation of Koehler (1942) who indicated that the fungus entered through the silks and spread into the internal husks and glumes. The precise pathway of fungal colonization in intact kernels has not yet been identified. Probably, after glume colonization, *F. verticillioides* uses the stylar canal as the only route to enter the pericarp cells of the kernel in the absence of injuries (Duncan and Howard, 2010). This result could be inferred by the significantly higher *F. verticillioides* IFs observed in glumes and internal husks in comparison with the IFs assessed in kernels. Finally, contamination data indicated that in the examined sampling sites infection through the silks could be considered the most important pathway for *F. verticillioides*. Other infection pathways, such as stalk lesions or systemic growth, may be relevant only under favorable temperature regimes. Although not new, this result further supports the predominance of silk infection pathway leading to ear contamination, and emphasises the importance of pursuing resistance factors in silks and husks.

In conclusion, different levels of *F. verticillioides* contamination were detected in maize plants growing under field conditions depending on their growth stage. *Fusarium verticillioides* contamination levels during vegetative growth till the pre-silking stage indicated that the fungus endophytically colonizes plants and, according to recent studies, protects the host against more devastating pathogens (Knop *et al*., 2007; Lee *et al*., 2009). *Fusarium verticillioides* conversion from asymptomatic to symptomatic lifestyle is probably due to numerous interacting factors and particularly to senescence processes occurring in the host, favourable climatic conditions and increasing fungal biomass derived from endophytic growth and from airborne inoculum. The generally high *F. verticillioides* contamination found in seeds suggested that seedborne populations should be investigated in order to better understand the role of migration in the *F. verticillioides* population genetics.

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