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Effect of moisture on symptom development and colonization of *Fusarium* species on maize leaves

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Abstract

Maize plants, fifteen-day old, were inoculated with three *Fusarium* species on the 4th mature leaf and 6th immature emerging leaf. The plants were grown under (1) 50-60% and (2) 80-90% relative humidity (RH). The symptoms of *Fusarium* were found on immature emerging leaves at high and low RH. Symptoms of *F. graminearum* occurred the fourth day after inoculation (DAI), followed by *F. verticillioides* and *F. proliferatum* in the 7th and 8th DAI. The holes, necrotic lesion, streaks depended on which *Fusarium* species were involved. Humidity regimes had a significant effect on re-isolation frequency of leaves infected by *F. verticillioides* but did not influence by *F. proliferatum* and *F. graminearum*. The humidity regimes affected on DNA content of *F. graminearum* and *F. proliferatum* but there was no significant influence to *F. verticillioides* DNA. This study showed maize plants cultivated in dry or wet season may be affected by *Fusarium* species.

Keywords – disease incidence – *F. graminearum* – *F. proliferatum* – *F. verticillioides*

Introduction

One of the serious diseases on maize are the *Fusarium* induced infections like foot-rot, Fusarium head blight and seedling blight (Doohan et al. 2003, Fandohan et al. 2003, Qin et al. 2020). Such infections reduce yield, and remain the primary source of mycotoxin contamination in feed products and in food. Moreover, these mycotoxins lead to health problems to both humans and animals when consumed (Bacon & Nelson 1994, Qin et al. 2020). Therefore, the chances of mycotoxin contamination of maize increases, if *Fusarium* epidemics occur in the field. This reduces the safety and market value of the crop harvested (Nganje et al. 2002).

Many *Fusarium* species with mycotoxin producing ability have reported and been characterized. Among these, *F. verticillioides*, *F. proliferatum* and *F. graminearum* are frequently observed infecting maize (Leslie 1996). In most cases, these fungi exhibit both parasitic and saprophytic modes of nutrition (Bacon et al. 2008). The infection of *Fusarium* into the host plant, however, is influenced by several factors including environmental conditions, physiology of the host and spore condition amongst others (Pastirčák 2004, Nguyen & Dehne 2015, Schöneberg et al. 2019, Pfordt et al. 2020). Many reports showed humidity and temperature regimes influenced the infection process, development, dissemination and mycotoxin producing ability of *Fusarium* in stalk, kernel or ear of maize (Marin et al. 1995a, Dilkin et al. 2002, Etcheverry et al. 2002, Doohan

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et al. 2003, Murillo-Williams & Munkvold 2008, Aguín et al. 2014, Czembor et al. 2015, Gai et al. 2018) but there has been little research on maize leaves. This study estimated the effect of humidity on symptom and colonization of *F. graminearum* AG23d, *F. proliferatum* AG31g, *F. verticillioides* AG11i on maize leaves by using microbiological assays and quantitative PCR.

Materials & Methods

Preparing fungal pathogen and inoculum

Fusarium verticillioides AG11i, *F. proliferatum* AG31g, and *F. graminearum* AG23d were collected from maize kernels and identifyed by sequencing factor 1-alpha gene and by species-specific PCR (Görtz et al. 2010). These species were stored at the Institute of Crop Science and Resource Conservation, Bonn University, Germany for other researches.

Cultures were grown on Potato Dextrose Agar (PDA) then incubated at 22℃ for 7 days. Two pieces (Ø 1 cm) of fungi were cut from the 7-day old cultures, then added to Potato Dextrose Broth media. The cultures were incubated in darkness for 3-4 days and on a shaker at 120 rpm, 22℃. Then a 0.5 ml *Fusarium* spore suspension was spread on the surface of low-strength PDA media (12.5 g/l PDA, 19 g/l Agar- agar) and these dishes were dried under a laminar flow cabinet for 10-20 minutes (min) then incubated under conditions of near ultra violet light at 22℃ for 3-5 days (Moradi 2008). Conidia were flooded with sterile distilled water containing Tween 20 (0.075%) and harvested and sieved through cheesecloth. The concentration of the conidia was adjusted to $2x10^6$ spores per ml.

Maize cultivation

Maize seeds (cv. Tassilo) were disinfected with hot water at 52℃ for 15 min (Rahman et al. 2008). Then these seeds were sown in trays in Klasmann potting substrate (Klasmann-Deilmann, Geeste, Germany) and uniform seedlings were transplanted, a plant per pot. The plants were kept in a growth chamber and watered once a day.

Experimental design and data collection

Maize plants, the growth stage 15 (fifteen-day old), were inoculated with *F. proliferatum*, *F. graminearum*, and *F. verticillioides* on the 4^{th} mature unfolded leaf and the 6^{th} immature emerging leaf by spraying fungal spore suspension. Maize plants were inoculated with each *Fusarium* species and incubated in chambers with relative humidity of 90-95% for 48 hours and then divided into two humidity conditions: (1) 50-60%, low humidity condition and (2) 80-90%, high humidity condition. A 15-hour photoperiod and 4000-5000 lux light intensity were applied for both humidity regimes (Nguyen 2014).

Disease incidence, disease severity and re-isolation frequency were estimated at 10, 20 and 40 DAI.

Fungal DNA extraction from leaf samples

Two plants per treatment with four replications were collected to analyze fungal biomass in the inoculated 4^{th} and 6^{th} leaf at 0, 5, 10, 20 and 40 DAI. Samples were stored in -20℃ and then freeze-dried. Lyophilized leaves were ground to a fine flour and 18-20 mg leaf flour was used for DNA extraction (Nguyen 2014).

Polymerase chain reaction

A TaqMan® real-time PCR on a StepOne plus real-time PCR system (Life technologies, Darmstadt, Germany) was used to quantification of genomic DNA of the three *Fusarium* species.

Primers and probes used for quantification of genomic DNA of *F. verticillioides* and *F. proliferatum*, fumonisins producing species were Taqfum-2F: ATGCAAGAGGCGAGGCAA, Vpgen-3R: GGCTCTCAGGAGCT TGGCAT and FUM-probe1: CAATGCCATCTTCTTG) (Waalwijk et al. (2008). Primers and probes: MGB-F: GGCGCTTCTCGTGA ACACA,

F. graminearum MGB-R: TGGCTAAACAGCACGAATGC and *F. graminearum* MGB-probe: AGATATGTCTCTTCAAGTCT were used for quantification of genomic DNA of *F. graminearum* (Waalwijk et al. 2004).

Reaction mixture for *F. graminearum:*

The reaction mixture for *F. graminearum* contained 0.5 µl of 6-FAM-labelled target probe (5µM), 1µl of each forward and reverse primer (10µM), 2µl of sample DNA, 15 µl of TaqMan® Universal PCR Master Mix (Roche Branchburg, New Jersey, USA) and 10.5 µl of distilled water. The amplification for *F. graminearum* consisted of a single cycle of two min at 50ºC and 10 min at 95ºC, followed by 40 cycles of 95ºC for 15 second and 60ºC for one min.

Reaction mixture for *F. proliferatum* and *F. verticillioides*

The PCR for the quantification of *F. proliferatum* and *F. verticillioides* DNA was performed according to the following protocol. The reaction mixtures for *F. verticillioides* and *F. proliferatum* DNA (20 µl) contained of 0.33 µl of target probe (5µM), 0.66 µl of each forward and reverse primer (10µM), 2µl of sample DNA, 10µl of Premix Ex Taq (perfect Real Time) (Takara Bio inc., Otsi, Shiga, Japan) 0.4 µl Rox II and 5.95 µl of distilled water. A single cycle of 20 second at 95ºC, followed by 40 cycles of 95ºC for one second and 60ºC for 20 second were set up for PCR reaction.

Quantification

The standard curve method was performed to quantification of fungal DNA. The linearity, the efficiency, the sensitivity and the reproducibility of assay were plotted on a standard curve by the Step One software version 2.2.2.

Microscopic observation

Symptom and fungal development on the maize leaves were examined by a Leica MZ16 F stereo and Leitz DMR photomicroscope.

Data analysis

Kolmogorov tests were used to test for normality and homogeneity of variance of all data before subjecting them to analysis of variance at the 5% significant level of Duncan's test. IRRISTAT, version 5.0 statistical package, was used to analyze the data.

Results

Symptom description on maize leaf

The humidity regimes did not significantly influence on the formation of the disease symptom. Symptoms of three *Fusarium* species only appeared on the immature emerging leaves.

The symptoms were observed on leaves treated with *F. graminearum* at 4-5 days after inoculation. Firstly, the lesions formed water-soaked and turned into yellow spots with shades of brown or grey in the center (Fig. 1B). In cases where the lesions appeared small (< 1mm), the yellowish lesions appeared greenish or similar to that of mature leaf tissues leading to inconspicuous symptoms (Fig. 1C). The extensive symptoms were brown spots with yellow boundaries on leaves or on main veins or small holes with brownish edges (Fig. 1A).

Typical symptoms of *F. proliferatum* were necrotic lesions (holes) and streaks that were different in size (approximately 5-60 mm in length and 1-10mm in width) appeared on specific parts of the leaves at 6^{th} – 8^{th} DAI. Symptoms appeared at the distal end of the leaf or on the upper leaf tips of the immediate leaf emerging after inoculation. A dark brown and yellowish boundary line appeared between the holes and the green interior of the leaf (Fig. 1E). Mild symptoms such as the small streaks coalesced to create a line between leaf veins were observed (Fig. 1F). Heavy symptoms of *F. proliferatum* were deformation, unopened leaves with symptoms of "deadhearts" (Fig. 1D).

Heavy infected leaves of *F. verticillioides* showed symptoms of yellow necrotic lesions, streaks and small holes (approximately 1-5 mm in length) (Fig. 1G). Typical disease symptom included the coalescing of many small streaks to form light green-yellowish lines along the leaf blades (Fig. 1H). Mild symptoms were similar typical symptom but the streaks and light green-yellow lines were smaller (Fig. 1I).

Fig. 1 – Symptomatic maize leaves infected by: *F.graminearum* (A-C): A Heavy, B typical, C mild. *F. proliferatum* (D-F): D Heavy, E typical, F mild. *F. verticilliodes* (G-I): G Heavy, H typical, I mild.

Disease incidence, disease severity

The incidence of disease varied from 50% to 87.5% but were not significantly different among the *Fusarium* species, humidity regims, and time course (Table 1).

The influence of humidity condition on disease severity of three *Fusarium* species was not significantly different ($P>0.05$, Table 1). However, under low humidity condition, the lesions caused by *F. graminearum* were grey in the centre, brown spots and had edges between healthy and unhealthy tissue (Fig. 2A). And under high humidity condition, lesions appeared greyish in the center and no edges between healthy and unhealthy region (Fig. 2B, 2C) and mycelia densely grew (Fig. 2D, 2C). A dark brown boundary line between the hole and the green interior of the leaf were seen by *F. proliferatum* (Fig. 2E) under low humidity condition. However, in high humidity condition, the boundary line was only a yellowish line appearing between the holes and the green interior of the leaf (Fig. 2 F). Yellow necrotic lesions with brown edges (Fig. 2G) were seen by *F. verticillioides* in low RH, but in high RH, the necrotic lesions or streaks appeared without limiting edges, transparent (Fig. 2H).

Table 1 Disease incidence and severity (%) on the 6th maize leaf under low and high humidity

Humidity	Fungi	Disease incidence (%)			Disease severity (%)		
		10 DAI	20 DAI	40 DAI	10 DAI	20 DAI	40 DAI
Low RH	*F. graminearum*	62.5	75.0	50.0	2.39	2.25	2.50
	F. proliferatum	75.0	75.0	62.5	2.44	3.06	4.69
	F. verticillioides	75.0	75.0	50.0	2.00	2.50	2.19
High RH	*F. graminearum*	75.0	62.5	87.5	5.69	6.44	5.94
	F. proliferatum	50.0	87.5	50.0	2.63	2.75	3.19
	F. verticillioides	75.0	87.5	75.0	6.69	4.13	4.00

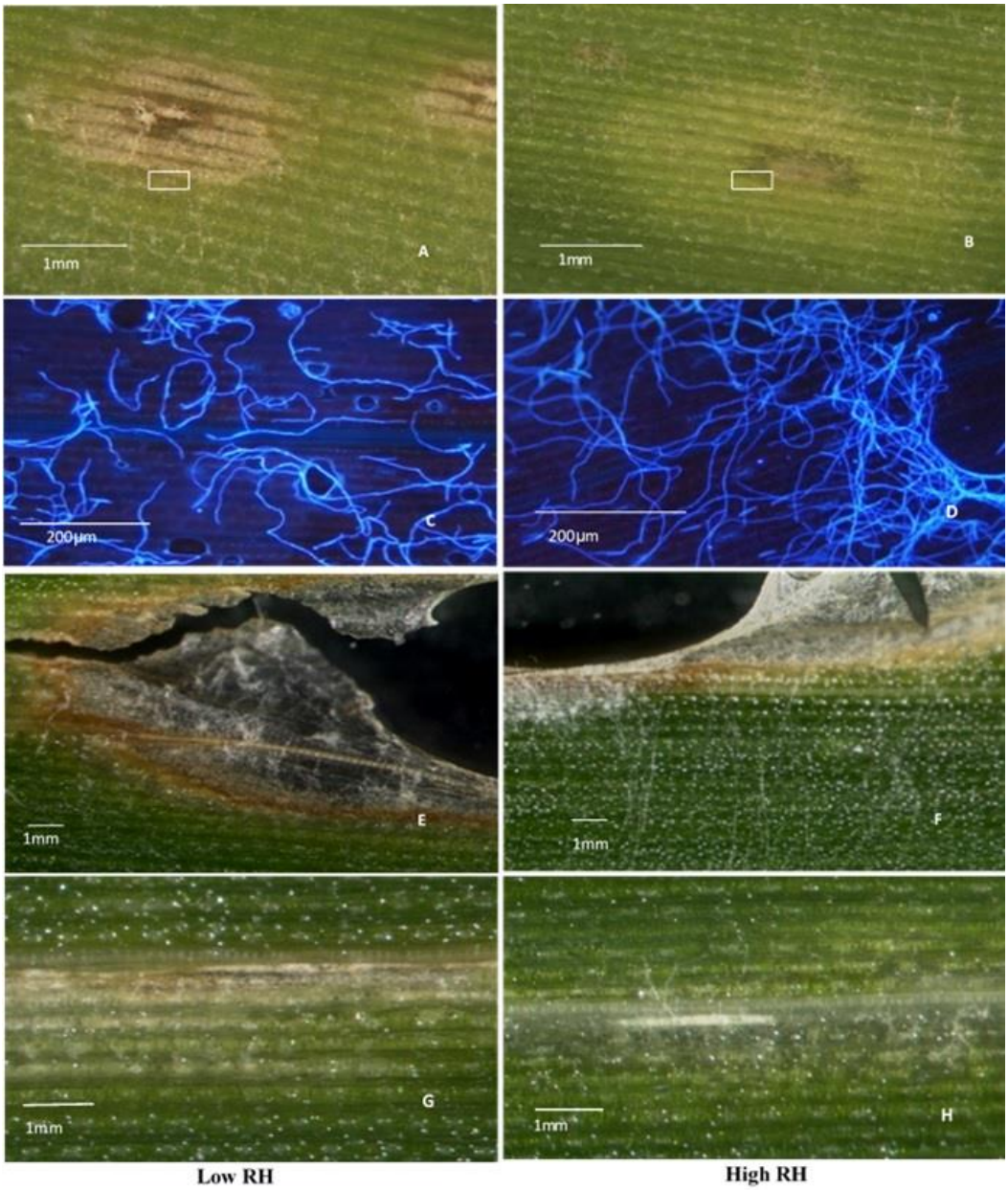

Fig. 2 – Symptoms and Fusarium mycelium growth on maize leaves. A, B symptoms of *F. graminearum.* C, D close-up box area in A and B. E, F symptoms of *F. proliferatum.* G, H symptoms of *F. verticillioides.*

Re-isolation frequency

The frequency of re-isolation from the 4th leaf was lower than that of the 6th leaf. Humidity regimes had a significant effect on re-isolation frequency of the 6th leaf infected by

F. verticillioides but did not influence by *F. proliferatum* and *F. graminearum* (P = 000). Colonization of *F. proliferatum* and *F. verticillioides* were higher than *F. graminearum* at 10 DAI (P = 0.002). At 20 DAI, the frequency of re-isolation of *F. verticillioides* was significantly higher (46%) under high RH than under low RH (26%). At 40 DAI, the frequency of re-isolation by *F. graminearum* and *F. proliferatum* increased under high RH (Table 2).

Biomass of Fusarium species

Fusarium DNA in the 4th leaf was higher than that on the 6th leaves at 10, 20 and 40 DAI. *F. graminearum* DNA was significantly higher than *F. proliferatum* and *F. verticillioides* in both low and high RH at 5 DAI. At 10 DAI, *F. graminearum* DNA was high under high RH and significantly different to the others. At 40 DAI, *F. graminearum* DNA and *F. proliferatum* DNA in the 4th leaf was very high under high RH (210,680 and 94,024 pg/mg DW, respectively. Table 3).

Table 2 Re-isolation frequency (%) of *Fusarium* infection of maize leaves

Humidity	Fungi[1]	10 DAI		20 DAI		40 DAI	
Low RH	*F. gra,* 4th [2]	10.7	e	12.5	d	5.4	f
	F. gra. 6th	25.0	de	46.4	ab	32.1	cd
	F. pro, 4th	37.5	bcd	30.4	bcd	40.4	cd
	F. pro, 6th	44.6	bcd	25.0	bcd	39.7	cd
	F. ver, 4th	28.6	cde	35.7	bc	50.3	bc
	*F. ver,*6th	25.5	de	16.1	cd	19.6	ef
High RH	*F. gra,* 4th	25.0	de	26.8	de	87.5	a
	F. gra. 6th	48.2	bcd	46.4	ab	83.9	a
	F. pro, 4th	50.0	abc	28.6	bcd	83.9	a
	F. pro, 6th	42.9	bcd	66.1	a	53.6	bc
	F. ver, 4th	71.4	a	44.6	ab	58.9	ab
	F. ver, 6th	55.4	abc	48.2	ab	62.5	b

[1] *F. gra: F. graminearum, F. pro: F. proliferatum* and *F. ver: F. verticillioides.* [2] Inoculated the 4 mature leaf and the 6th immature emerging leaf. Values in a column followed by different letters are significantly different at $P \leq 0.05$.

Table 3 Biomass (pg/mg DW) of *F. graminearum, F. verticillioides* and *F. proliferatum* inoculated maize leaves

Humidity	Fungi[1]	0 DAI		5 DAI		10 DAI		20 DAI		40 DAI	
Low RH	*F. gra,* 4th [2]	5,070	a	6,741	b	2,124	c	578	b	4,045	c
	F. gra, 6th	7,241	a	6,843	b	2,073	c	1,021	b	2,128	c
	F. pro, 4th	2,671	b	3,238	c	1,411	c	484	b	4,577	c
	F. pro, 6th	3,210	b	3,045	c	1,157	c	400	b	5,146	c
	F. ver, 4th	2,388	b	1,672	c	1,127	c	1,015	b	1,924	c
	*F. ver,*6th	2,174	b	1,168	c	633	c	492	b	406	c
High RH	*F. gra,* 4th	5,068	a	11,173	a	9,889	a	7,911	a	210,680	a
	F. gra, 6th	7,243	a	9,336	a	5,251	b	1,592	b	8,291	c
	F. pro, 4th	2,671	b	3,592	c	1,953	c	1,946	b	94,024	b
	F. pro, 6th	3,211	b	2,039	c	1,215	c	1,220	b	6,977	c
	F. ver, 4th	2,390	b	2,338	c	1,385	c	1,846	b	7,451	c
	F. ver, 6th	2,169	b	1,304	c	1,206	c	719	b	2,150	c

[1] *F. gra: F. graminearum, F. pro: F. proliferatum* and *F. ver: F. verticillioides.* [2] Inoculated the 4 mature leaf and the 6th immature emerging leaf. DW: dry weight. Values in a column followed by different letters are significantly different at $P \leq 0.05$.

Discussion

Disease symptoms occurred as a result of imbalanced interactions between the fungus and the host plant (Oren et al. 2003). Additionally, symptoms manifested themselves depending on the

structures of young leaves or developmental stages of the leaves infested. Young leaves (immature emerging leaves) lacked defense mechanisms such as wax and cuticle, making them more susceptible to infection compared to mature leaves (Nguyen et al. 2016a). Symptoms of *F. graminearum* formed early while symptoms of *F. verticillioides* and *F. proliferatum* appeared later. This variability depended on the level of fungal virulence and the pathways of infection. Gordon & Martyn (1997) reported that *F. oxysporum* was very virulent in terms of changing from symptomless to the symptom stage within a few days. *F. verticillioides* was reported to grow slowly and less aggressive (Oren et al. 2003). *F. graminearum* mostly formed mild symptoms because it infected via trichomes (Nguyen et al. 2016b) which scattered distribution on the leaves.

F. graminearum DNA production under high RH was high, while the frequency of re-isolation was low. In contrast, *F. proliferatum* and *F. verticillioides* had higher re-isolation frequencies but lower DNA content. Our results were comparable with study reported by Moradi et al. (2010) that using microbiological and real-time PCR assays gave different results for *Fusarium* species. The results demonstrated that *F. graminearum* not only grew endophytically but also grew densely over the leaf surface, while *F. proliferatum* and *F. verticillioides* infected the tissue with a lower amount of mycelia on the leaf surface than *F. graminearum* (Nguyen 2014). Some reports showed that in high humidity condition, *F. graminearum* grew well and producing large amounts of dense mycelia (Nelson et al. 1983, Bottalico 1998, Miller 2001, Manstretta & Rossi 2016). Our study found that the biomass of *F. proliferatum* and *F. graminearum* in the 4th leaf increased rapidly at 40 DAI. Most of the 4th leaf became senescent at 40 DAI and leaf senescence and high humidity conditions were favorable condition for fungal growth (Leonard & Bushnell 2003, Trail 2009). Numerous studies shown that humidity differently influenced to infection and growth of *Fusarium* species (Sutton 1982, Bottalico 1998, Miller 2001, Bottalico & Perrone 2002, De Wolf et al. 2003). *F. graminearum* was known favor under high level of moisture, *F. verticillioides* and *F. proliferatum* referred 0.97 water activity (Marin et al. 1995b, Reid et al. 1999).

Disease incidence and severity of maize leaf by three *Fusarium* species was similar under low and high RH, but the colonization was high under high RH. These findings suggest that the effect of moisture on disease symptom of maize leaves was on the development of *Fusarium* in the host tissue. These results were similar to report of Beddis & Burgess (1992) that the incidence of *F. graminearum* on wheat seedlings effected under stressed and unstressed water condition.

The results confirm that moisture differently influence on colonization of *Fusarium* species on maize leaves. High moisture favor the infection by *F. verticillioides* and the development of biomass of *F. graminearum.* The disease symptoms of *F.graminearum, F. proliferatum* and *F. verticillioides* form on immature emerging leaves in both low- and high- moisture.

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