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Comparative studies on the infection and colonization of maize leaves by *Fusarium graminearum, F. proliferatum* and *F. verticillioides*

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Abstract

Comparative studies on the infection and colonization of maize leaves by *Fusarium graminearum, F. proliferatum* and *F. verticillioides*

Infection of *Fusarium* species causes quantitative along with qualitative damage on small grains and maize plants. This is due to leaf damage together with contamination by formation of different mycotoxins. Because the vegetative as well as the reproductive plant parts of maize are used especially for animal feed and can be affected, information about the infection process and damage of the entire plants needed further elucidation.

The infection and colonization of maize leaves by the most important three *Fusarium* species provided insights in a role of the spread of *Fusarium* species from the different leaves into the cobs. Using microbiological assessments maize plants inoculated by *Fusarium* at the growth stage (GS) 15 reached higher infection rates than those inoculated at GS 35. Higher spore concentration and increased relative humidity resulted in more intensive colonization. Light regimes had no effect on the infection of different cultivars by *Fusarium*. The colonization of lower leaves was higher than the infection of upper leaves.

The lesion development of maize plants infected by *Fusarium* occurred especially on the immature leaves. Disease severity showed no difference among three species. Colonization was higher on symptom leaves than on symptomless leaves, but nevertheless even symptomless infections resulted in further propagation. Disease symptoms appeared on leaves inoculated by *F. graminearum* 4-5 days after inoculation (dai) and by *F. proliferatum* and *F. verticillioides* 7-8 dai. *F. graminearum* caused small water-soaked lesions and the lesions turned into yellow spots. *F. proliferatum* and *F. verticillioides* caused necrotic lesions, small holes and streaks.

The germination of conidia of all *Fusarium* species was present at 12 hours after inoculation. The penetration of all three *Fusarium* species was quite similar: All species were able to penetrate into the tissue through cuticles, epidermal cells, trichomes, but also via stomata. Forming appressoria, infection cushions or direct penetration demonstrated the broad host tissue these species resembled a high potential leading to symptomatic as well as asymptomatic infections.

All pathogens showed intercellular and intracellular infection of epidermal and mesophyll cells. Additionally, *F. graminearum* hyphae were found in sclerenchyma cells, xylem and the phloem vessels of detached leaves. The superficial hyphae and re-emerging hyphae of the three species produced conidia. Especially, macroconidia of *F. graminearum* produced secondary macroconidia and *F. proliferatum* formed microconidia inside tissues and sporulated through stomata and trichomes.

According to quantitative fungal DNA the biomass of *Fusarium* species increased until the 5^{th} dai but afterwards decreased from the 5^{th} dai to the 20^{th} dai and increased again until the 40^{th} dai. Disease severity and fungal biomass, disease severity and colonization of the 6^{th} and 7^{th} leaves were significantly positive correlation at 10 dai and 40 dai, respectively.

The infection of maize leaves by the three *Fusarium* species and their sporulation indicated an inoculum contribution to cob and kernel infection which may lead to reduce yield, quality and increase in potential mycotoxin contamination on maize.

Kurzfassung

Vergleichende Untersuchungen zur Infektion und Besiedlung von Maisblättern durch *Fusarium graminearum*, *F. proliferatum* und *F. verticillioides*

Infektionen von Fusarium Arten verursachen quantitative und qualitative Schäden an Getreide und Mais. Diese Beeinträchtigungen erfolgen durch Blatt- und Kolbenschäden, vor allem aber auch durch die Kontamination der Pflanzenteile mit sehr unterschiedlichen Mykotoxinen. Von Mais werden sowohl vegetative als auch reproduktive Pflanzenteile des Mais beslastet sein können und diese werden vor allem in Gänze in die Tiernahrung eingebracht werden. Daher galt es Informationen über den Blattbefall an Mais zu gewinnen und daher den Infektionsprozess und die Schadwirkung an Mais detailliert zu verfolgen.

Die Infektion und Besiedelung von Maisblättern wurde bezüglich der 3 bedeutendsten *Fusarium*-Arten an Mais verfolgt und ergaben wesentliche Rückschlüsse über die Ausbreitung von *Fusarium*-Arten an Maispflanzen von Blättern bis hin zum Kolben. Mit mikrobiologischen Erhebungen an Maisplanzen konnte nach Inokulationen geklärt werden, dass junge Maispflanzen (inokuliert im Stadium GS 15) deutlich anfälliger waren als im Stadium GS 35. Die Erhöhung der Inokulumdichte und eine erhöhte Luftfeuchte förderten die Blattinfektionen. Belichtungsbedingungen ließen keinen Einfluss auf die Infektionen erkennen. In allen Erhebungen waren die Befälle der unteren Blätter der Maispflanzen deutlich höher als die Infektionen der oberen Blätter.

Die Entwicklung von Läsionen auf durch *Fusarium* infizierten Maispflanzen trat vor allem auf den unreifen Blättern auf. Die Befallshäufigkeit und Befallsintensität zeigte keinen Unterschied zwischen den drei Arten. Auch wenn die Besiedelung auf Blättern mit Symptomausprägung höher war, führten auch die symptomlosen Infektionen zu einer weiteren Ausbreitung. Bei *Fusarium graminearum* traten die Symptome 4-5 Tage nach der Inokulation, bei *F. proliferatum* und *F. verticiolliodies* 7-8 Tage nach der Inokulation. *F. graminearum* verursachte Läsionen, die anfangs aussahen, wie Verbrennungen durch heißes Wasser und sich anschließend in gelbe Flecke verwandelten. *F. proliferatum* und *F. verticilloides* verursachten Nekrosen, die als kleine Löcher und Streifen erschienen.

Die Konidien aller *Fusarium*-Arten keimten im Zeitraum von 12 Stunden nach der Inokulation. Alle 3 zu vergleichenden Arten wiesen ein ähnliches Infektionsverhalten auf: Alle Arten konnten direkt in das Wirtsgewebe eindringen, penetriert wurden Cuticulen, Epidermiszellen, Trichome – gelegentlich erfolgte auch eine Eindringung über Spaltöffnungen. Dabei werden von den Pathogenen Appressorien gebildet, zudem Infektionskissen – aber dennoch kamen stets auch direkte Infektionen vor. Dies bestätigt das besonders breite Infektionsvermögen der Fusarien. Vor allem wurden aber symptomatische und asymptomatische Infektionen beobachtet.

Alle Pathogene zeigten ein inter- und intrazelluläres Wachstum in Epidermis und Mesophyll der Blätter. *Fusarium graminearum* besiedelte auch Gefässgewebe – sowohl Xylem- als auch Phloemgewebe. Die oberflächlichen Hyphen sporulierten stets auf dem Blattgewebe. *F. graminearum* bildete sekundäre Makrokonidien. *F. proliferatum* bildete Mikrokonidien im Gewebe und sporulierte als ubiquitärer Pathogen durch Stomata und Trichome.

Mittels quantitativer PCR wurde die pilzliche Biomasse erfasst. Bis zum 5. Tag nach der Inokulation stieg der Gehalt an – die symptomlose Infektion – in der Nekrotisierungsphase sank der Pilzgehalt um anschließend in der saprophytischen Phase der Infektion wieder anzusteigen.

Die Infektion von Maispflanzen und insbesondere Blättern durch 3 repräsentative *Fusarium* Arten und deren Sporulation sogar auf symptomlosen Blättern belegt die Bedeutung latenter Infektionen für die Kolben- und Körnerinfektion – dies gilt es zu vermeiden, um Ertragsbeeinträchtigungen und Einschränkungen der Qualität des Erntegut zu reduzieren.

Tóm tắt

Nghiên cứu sự xâm nhiễm và ký sinh của nấm *Fusarium graminearum, F. proliferatum* và *F. verticillioides* trên lá ngô

Nhiễm nấm *Fusarium* gây ra thiệt hại về năng suất và chất lượng ngũ cốc và ngô. Nhiều loại độc tố của nấm hình thành trong quá trình xâm nhiễm. Do ngô được sử dụng cho chăn nuôi nên nhiễm nấm có thể ảnh hưởng đến sức khỏe vật nuôi. Vì thế quá trình xâm nhiễm của nấm và sự thiệt hại cần được nghiên cứu.

Xâm nhiễm và ký sinh lá ngô bởi ba loài *Fusarium* dẫn đến phát tán nguồn bệnh từ lá đến các lá bên trên và lên quả. Sử dụng phương pháp phân lập nấm sau khi chủng bệnh cho thấy cây ngô được chủng bệnh bởi nấm *Fusarium ở* giai đoạn sinh trưởng 15 có mức nhiễm cao hơn chủng bệnh ở giai đoạn 35. Sự ký sinh xảy ra với tần suất cao hơn khi chủng nồng độ bào tử nấm cao và tăng ẩm độ tương đối. Chế độ ánh sáng đã không ảnh hưởng đến sự nhiễm nấm *Fusarium* trên hai giống ngô. Những lá bên dưới bị *Fusarium* ký sinh mạnh hơn lá trên.

Những vết bệnh xuất hiện trên lá ngô non, đặc biệt trên lá đang mọc. Tỉ lệ bệnh không khác biệt ý nghĩa giữa ba loài *Fusarium.* Tỉ lệ ký sinh cao hơn đối với lá có triệu chứng bệnh so với lá không có triêu chứng. Triệu chứng bệnh xuất hiện sớm trên lá ngô được chủng bởi *F. graminearum* 4-5 ngày sau khi chủng nấm và 7- 8 ngày sau khi chủng *F. proliferatum* và *F. verticillioides.* Triệu chứng bệnh gây ra bởi *F. graminearum* ban đầu là những đốm nhỏ sũng nước sau đó chuyển sang màu vàng nhạt với tâm xám trắng. *F. proliferatum* and *F. verticillioides* gây nên các đốm nhỏ liên tục và nối với nhau thành những sọc chạy dọc theo gân lá hoặc mô lá bị thiệt hại hình thành các lỗ thủng trên lá, thường là hình mắt én.

Bào tử nấm của 3 loài *Fusarium* bắt đầu nẩy mầm 12 giờ sau khi chủng. Ba loài *Fusarium* có khả năng xâm nhiễm mô lá ngô qua lớp cutin, tế nào biểu bì, lông và khí khổng. Nấm hình thành đĩa áp hoặc mô đệm hoặc xâm nhiễm trực tiếp vào lá ngô. Cách xâm nhiễm đa dạng của ba loài *Fusarium* cho thấy tiềm năng xâm nhiễm cao gây ra triệu chứng bệnh trên lá cũng như xâm nhiễm mà không gây ra triệu chứng. *Fusarium* species ký sinh trong tế bào hoặc giữa các tế bào của lá. Hơn nữa, nấm *F. graminearum* đã được tìm thấy trong tế bào cương mô và tế bào bó mạch khi chủng nấm trên lá ngô trong đĩa petri với ẩm độ cao.

Sợi nấm trên mặt lá và sợi nấm mọc ra từ mô lá bị nhiễm của cả ba loài nấm sinh bào tử. Đặc biệt, bào tử của *F. graminearum* hình thành thế hệ bào tử thứ hai và *F. proliferatum* hình thành bào tử bên trong mô lá và phóng thích ra ngoài thông qua khí khổng hoăc lông của lá.

Sử dụng qPCR để đánh giá sự phát triển của ba loài nấm trên lá ngô cho thấy sinh khối của nấm tăng từ lúc chủng cho đến 5 ngày sau khi chủng nhưng giảm từ sau 5 ngày đến 20 ngày và tăng trở lại sau đó, 40 ngày sau khi chủng. Có sự tương quan giữa tỉ lệ bệnh và sinh khối nấm, 10 ngày sau khi chủng bệnh, tỉ lệ bệnh và mức độ ký sinh, 40 ngày sau khi chủng bệnh.

Sự xâm nhiễm và ký sinh của 3 loài nấm *Fusarium* trên lá ngô và phóng thích bào tử đã cho thấy đây là nguồn gây bệnh đối với quả và hạt ngô và có thể dẫn đến giảm năng suất, chất lượng và tăng nguy cơ nhiễm độc tố của nấm trên ngô.

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Abbreviations

°C	Celsius
µg	Microgram
µl	Microliter
15-AcDON	15-Acetyldeoxynivalenol
3-AcDON	3- Acetyldeoxynivalenol
CZID-Agar	Czapek-Dox-Iprodione-Dichloran-Agar
Dai	Day after inoculation
DNA	Deoxyribonucleic acid
GS	Growth stage
Hai	Hour after inoculation
L	Liter
mg	Milligram
ml	Milliliter
MON	Moniliformin
NIV	Nivalenol
PCR	Polymerase Chain Reaction
PDA	Potato-Dextrose-Agar
pg	picogram
qPCR	TaqMan® real-time Polymerase-Chain-Reaction
RH	relative humidity
rpm	rotation per minute
Sec	second
SEM	Scanning electron microscopy
spp.	species
TEM	Transmission electron microscopy
T-2	T-2 Toxin

1. Introduction

Maize (*Zea mays L.*) plays an important role throughout the world. In 2011, the worldwide harvested area was 170.4 million hectares with a total production of 883 million tons (FAOSTAT, 2013). Maize is used as a staple food for more than 1.2 billion people (IITA, 2009) as well as for livestock feed and biogas production. However, maize is also known as one of the major host plants of *Fusarium* species. *Fusarium* infections not only reduce yield, but also lead to mycotoxin production in the grain and thereby contamination of food and feed products. These secondary metabolites of *Fusarium* are harmful to both humans and animals. In 1987, an epidemic outbreak of gastrointestinal symptoms occurred in India which was associated with the consumption of wheat contaminated with trichothecenes (Bhat et al., 1989). In 1995 symptoms of mycotoxin contamination was shown to be related to the consumption of sorghum and maize contaminated with Fumonisin B1 (Bhat et al., 1997). In China and Southern Africa, esophageal cancer was suspected to be associated with Trichothecenes and Fumonixins present in wheat and maize (Luo et al., 1990; Sydenham et al., 1990; Rheeder et al., 1992; Yoshizawa et al., 1994). T-2 toxin in rice infected with *Fusarium heterosporum* and *F. graminearum* was reported to cause nausea, dizziness, vomiting, chills, abdominal pain, and diarrhea in China (Wang et al., 1993). *Fusarium* mycotoxins have also been shown to affected health and productivity of hens, pigs and cattle (Bristol and Djurickovic, 1971; Pestka et al., 1987; Prathapkumar et al., 1997; Res., 1997; Pestka, 2007). Moreover, *Fusarium* reduced yield and quality of agricultural production caused severe economic loss (McMullen et al., 1997; Edwards, 2004). In the USA, 2.7 billion US dollars were lost due to Fusarium head blight between 1998 -2000 (Nganje et al., 2002).

Mycotoxins are also important for infection and development of plant diseases (Desjardins et al., 1998). For example, fumonisins produced by *F. verticillioides* are required for the development of foliar disease symptoms on maize seedlings (Glenn et al., 2008). DON was shown to assisted fungi in the infection process and spread of Fusarium head blight within the spike (Bai et al., 2002; Munkvold, 2003). Boenisch and Schäfer (2011) found that *F. graminearum* synthesized DON to stimulate the formation of infection structures. Since food and feed contamination by *Fusarium* mycotoxins have

been associated with human and animal toxicosis, the United States Food and Drug Administration (FDA, 2010) and The Commission of the European Communities (EU Commission, 2006) have recommended guideline values for mycotoxins levels in products used for animal feed.

In an attempt to understand the biodiversity of *Fusarium* species and their impact in plant health, investigations have been carried out in many cereal-producing countries. For instance, in China, 32 *Fusarium* isolates were isolated from 50 maize kernel samples. *Fusarium moniliforme, F. semitectum* and *F. scirpi* were identified in those samples (Hsia et al., 1988). In Western Kenya, *F. moniliforme* was isolated most frequently, followed by *F. subglutinans*, *F. graminearum, F. oxysporum, F. solani* in 1996 (Kedera et al., 1999). In Argentina, *F. moniliforme* and *F. nygamai* followed by *F. semitectum*, *F. subglutinans*, *F. proliferatum* were the most frequent *Fusarium* species isolated in 158 samples of poultry feeds between 1996-1998 (Magnoli et al., 1999). In Slovakia, *F. verticillioides*, followed by *F. proliferatum* were frequently isolated in 1996 while *F. subglutinans* dominated in 1998 (Srobarova et al., 2002). In Canada, 124 samples from 42 maize hybrids were collected in 2006, in which *F. subglutinans* was the most dominant species followed by *F. verticillioides, F. graminearum, F. poae, F. sporotrichiodes* and *F. proliferatum* (Schaafsma et al., 2008). Görtz et al. (2008) collected maize kernels in the major maize producing areas in Germany. They found 13 *Fusarium* spp. in kernels with an incidence ranging from 0.7 to 99.7 %. The predominant *Fusarium* spp. differed between years in a two year survey**.** *F. verticillioides, F. graminearum* and *F. proliferatum* dominated in 2006 while *F. graminearum* was mostly isolated in 2007. In Switzerland, investigations of infection of maize kernels and stems were carried out in 2005 and 2006. Dorn et al. (2009) isolated 16 *Fusarium* species from kernels and 15 from stem pieces. On kernels, *F. verticillioides, F. graminearum, F. proliferatum* and *F. crookwellense* dominated in the North while *F. verticillioides, F. subglutinans, F. proliferatum* and *F. graminearum* predominated in the South. On the stem, *F. equiseti,*

F. verticillioides, F. graminearum, F. crookwellense and *F. subglutinans* were frequently isolated.

A number of plant diseases such as blight of maize seedlings, stalk rot and ear rot are considered to be serious diseases affecting cereal productivity worldwide. Seedling blight is caused by *F. verticillioides, F. graminearum, F. proliferatum* and *F. subglutinans* on maize. However, disease symptoms may vary depending on the fungal species involved. For example, *F. graminearum* causes brownish-red lesions with a sunken center and/or rotting of the scutellum mesocotyl, roots, and nodes on maize seedlings (Hampton et al., 1997). *F. moniliforme* rot of maize seedlings causes black lesions on the mesocotyl but without any coloration on the seeds and roots (Pastirčák, 2004).

Fusarium stalk rot in maize is caused by *F. moniliforme*, *F. proliferatum*, and *F. subglutinans.* (Nelson, 1992; Agrios, 2005). *Fusarium* infections cause decay of the pith tissue in the lower stalk internodes and result in poor kernel fill and premature plant death. The decay of the maize stalk affects the structural integrity of the stalk, and the plant is more prone to lodging. Distinctive symptoms in the stalk are a tan-to-brown discoloration of the lower internodes and a pink-to-reddish discoloration of the pith tissue (Munkvold and Desjardins, 1997; Santiago et al., 2007). Like seedling rot disease, symptoms and severity of stalk rots are dependent on several factors including the species of *Fusarium*, type of crop, environment condition and origin of the fungal species (Dodd, 1980; Schneider, 1983; Gilbertson et al., 1985; Gilbertson, 1986; Skoglund and Brown, 1988; Osunlaja, 1990). In Colorado, for example, *F. graminearum* was noted to be more virulent than *F. moniliforme* and *F. subglutinans* in 1983. In Australia and in the United States, *F. graminearum* was capable of causing head blight of wheat, crown rot of wheat and stalk rot of maize. *F. culmorum* from foot rot of wheat and barley was also capable of causing stalk rot of maize (Purss, 1971). Western corn rootworm beetles (*Diabrotica virgifera*) were vectors of the *F. moniliforme* and *F. subglutinans* which caused maize stalk rot in eastern Colorado from 1982-1984 (Gilbertson, 1986).

Fusarium infection of maize ears and kernels are categorized into two distinct diseases such as pink ear rot or Fusarium ear rot and red ear rot or Gibberella ear rot. *F. verticillioides*, *F. proliferatum* and *F. subglutinans* are reported as the causal agents of pink ear rot while *F. graminearum*, *F. culmorum*, *F. cerealis* and *F. avenaceum* are often associated with red ear rot (Logrieco et al., 2002; Munkvold, 2003). However, the occurrence of these diseases often depends on environmental conditions. The pink ear rot, for instance, frequently occurs in temperate regions (Marin *et al.*, 1995b; Munkvold and Desjardins, 1997; Doohan *et al.*, 2003) while the red ear rot is often found in regions that experience high humidity (rainfall) and moderate temperatures. The optimum conditions for Gibberella ear rot are high levels of moisture around the silk as well as moderate temperatures and high rainfall during the maturation period (Sutton, 1982). Favorable conditions for Fusarium ear rot development are warm, dry weather during the grain filling period. The symptoms of Gibberella ear rot usually starts from the tip of the ear and spreads down the ear as a pink to reddish mold (Logrieco et al., 2002). The symptoms of Fusarium ear rot appears on scattered single kernels or groups of kernels, usually as tan to brown discoloration, which develops pink mycelium under moist conditions (Logrieco et al., 2002).

However, *Fusarium* spp. are also considered symptomless fungi. For example, *F. verticillioides* infected maize plants are often symptomless (Thomas, 1980; Bacon and Hinton, 1996; Desjardins et al., 1998; Vieira, 2000; Bakan et al., 2002; Bacon et al., 2008). Although this fungus infected without symptoms, mycotoxins were still produced during the infection process (Bacon and Hinton, 1996) as well as saprophytic growth (Desjardins et al., 1998; Bacon, 2001).

F. verticillioides (Sac) Nierenberg, synonyms *F. fujikuroi* Nierenberg, *F. moniliforme* Sheldon (W&R,B,J) and *F. proliferatum (*Masushima*)* Nierenberg are placed in the section *Liseola* of the genus *Fusarium.* They form abundant microconidia and rarely form macroconidia. Conidiophores of *F. verticillioides* are described as monophialides, while conidiophores of *F. proliferatum* are monophialides and polyphialides.

Microconidia of *F. verticillioides* are formed in long chains and false heads whereas microconidia of *F. proliferatum* are formed in short chains. Clamydiopores are absent in section *Liseola* of the genus *Fusarium (Nelson et al., 1983)*. The sexual stage of *F. verticillioides* is *Gibberella fujikujoi* (Sawada) (wollenw*)* mating population A and of *F. proliferatum* is mating population D (Kerényi et al., 1999). The optimal conditions for the germination of *F. verticillioides* microconidia are temperatures of 25–37 °C at 0.96–0.98 water activity (*a*w) or 30°C at 0.90–0.94 *a*w (Marín et al., 1996). Maximum sporulation occurred at 27°C, with an increase between 5°C and 27°C and then a rapid decline (Rossi et al., 2009). For *F. proliferatum*, the germination rate of microconidia is optimal at 30°C, regardless of *a*w (Marín et al., 1996).

Fusarium graminearum Schwabe belongs to the section *Discolor* of the genus *Fusarium.* This species forms only macroconidia. Chlamydiospores are formed in the macroconidia or in the mycelia (Nelson et al., 1983). The sexual stage is *Gibberella zeae* (SCHW) (Petch). It forms abundant perithecia and ascospores (Xu, 2003). The growth rate of *F. graminearum* increases between 10 and 25°C and the optimal temperature for growth is 25°C (Brennan et al., 2003).

Parry et al. (1994) described the life cycles of *Fusarium* spp. on small grain cereals. Sutton (1982) and Trail (2009) on the other hand described the life cycle of *F. graminearum*. Sutton (1982) reported that soil, seeds and host debris were inoculum sources of *F. graminearum.* However, the fungus survives in debris such as old stems and on cobs of maize. The straw and debris of wheat, barley and other cereal are the main reservoir of *F. graminearum*. Chlamydospores or perithecia of this fungus formed on debris can survive over winter and infect maize or wheat seedling during the following crop season. During crop growth, the macroconidia and ascospores produced from debris are dispersed in the air, then infect and colonize the wheat spikes, stems, leaf sheaths and ears of maize. At harvest, plant debris contaminated with the fungus are left on the field soil and the fungus then continues with a new life cycle (Sutton, 1982).

Munkvold and Desjardin (1997) outlined the disease cycle of *F. verticillioides* on maize. The authors noted that this fungus survived in crop residues which provided an inoculum source for root and leaf sheath infections. Wind and rain spread spores from the crop residue to the cob and from there the spores are spread to the silks and kernels. Insect vectors also can distribute the fungus to cobs or to stems. Fungi in infected seeds can be transmitted by systemic growth through the stalk into the kernels. Sporulation of the fungi on the tassels or from other the infected plants in a field may also lead to silk infection. The disease cycle of *F. proliferatum* was considered similar that of *F. verticillioides* (Munkvold and Desjardins, 1997). *Fusarium* infection through silks has been reported to play an important role in kernel infection (Reid, 1992; Chungu, 1996 ; Munkvold *et al.*, 1997b; Reid, 2002). Koehler (1942) reported that *F. moniliforme* originated in the region of the silks, spread to the kernels, pedicels, vascular cylinder, and finally to the shank. *Fusarium* also infected the root or mesocotyl epidermis by either direct penetration or through wounds or natural openings (Lawrence, 1981). The author noted that *F. moniliforme* infected the outer cortex of the root, collapsed parenchyma cells and ramified through the cortex. The hyphae then invaded xylem vessel elements of the stem and occluded the protoxylem vessel elements (Lawrence, 1981). *F. culmorum* hyphae were found to penetrate the different parts of wheat spikelets (Kang and Buchenauer, 2000a). *F. graminearum* was shown to form lobate appressoria and infection cushions (Boenisch and Schäfer, 2011). Murillo et al. (1999) reported that *F. moniliforme* directly penetrates the epidermal cells of the seedling and colonizes the host tissue by inter- and intracellular ways.

Sporulation occurred before cells collapsed by hyphae emerging through stomata or rupturing the epidermal cells (Lawrence, 1981). Dispersal of spores by rain splash or, wind plays an important role in the dissemination of fungal pathogens in the field (Fitt et al., 1989; Aylor, 1990; Jenkinson and Parry, 1994; Madden, 1997). In corn fields, spores of *F. moniliforme* were spread by wind and rain. Wind dispersed spores for long distances (300-400 km) and rain washed spores from leaf sheaths about 3 - 50 $x10^4$

propagules/mm (Ooka and Kommedahl, 1977). *F. verticillioides* produced conidia continuously and abundantly for a number of weeks, with an average of 1.59×10^7 conidia g^{-1} of stalk residues (Rossi et al., 2009). Ascospores of *Gibberella zeae* were released 600-9000 ascospores/ m^3 per hour. The release of ascospores was reduced on days with continuously high relative humidity (> 80%) and ascospores were rinsed off under heavy rain (>5 mm) condition (Paulitz, 1996). Under wind tunnel condition, the ascospore release of *Gibberella zeae* was greater under light than in complete darkness (Trail et al., 2002).

In the last few decades, the polymerase chain reaction (PCR) by Karry Mullis and Faloona (1987) has allowed numerous advances in our understanding of these fungi and improvement in the technology allow its use for more specific purposes. Heid. et al. (1996) described the method of real time quantitative PCR (qPCR). The qPCR provides precise and reproducible quantitation of DNA products. Typical application of real-time PCR includes pathogen detection, gene expression analysis, single nucleotide polymorphism analysis, analysis of chromosome aberrations, and most recently also protein detection (Kubista et al., 2006). In classical PCR, after amplification, the product is run on a gel for detection of this specific product but real-time PCR does not require post-PCR sample handling. It prevents potential contamination and results in much faster assays (Heid et al., 1996). Real-time PCR has been developed for the detection of bacterial, fungal, and viral plant pathogens (Schaad and Frederick, 2002) and particularly, used for quantification of *Fusarium* DNA in host tissue (Möller et al., 1999; Mulè et al., 2004; Strausbaugh et al., 2005; Sarlin et al., 2006; Vandemark and Ariss, 2007; Stephens et al., 2008; Yli-Mattila et al., 2008; Nicolaisen et al., 2009; Nutz et al., 2011; Obanor et al., 2012).

Fusarium infection is responsible for mycotoxin contamination, yield losses and quality reduction in the crop production and processing of food and feed productions. Particularly, green maize biomass is important for animal feed, animal production and

the most important substrate for biogas production in industrial countries. Feeding animals with *Fusarium* contaminated productions lead to threat of domestic animal and human heath all over the world. Therefore, the protection of cereal crops from mycotoxin producing species of *Fusarium* is needed for the production of healthier food and animal feed.

Research objectives

Although many studies have described the infection of *Fusarium* into host plants, most of these reports concentrated on the infection and the symptoms of *Fusarium* on kernels, seeds or crown. Conversely, only a few investigations have described *Fusarium* infection of host plants via the leaves. Additionally, it remains unknown if *Fusarium* spp. infected maize leaves is or is not followed by the formation of disease symptoms on leaves. In the current study, it was hypothesized that *F. graminearum, F. proliferatum* and *F. verticillioides* infect maize leaves and disseminate inoculum to upper leaves and to ears.

The specific objectives of the study were to:

i. study factors affecting the infection of *Fusarium* spp. into maize leaves.
ii. investigate the infection process of the three species of *Fusarium* on maize leaves and
iii. assess the development of the three *Fusarium* species on maize leaves using quantitative PCR and microbiological bioassays.

2. Factors affecting the infection of maize leaves by *Fusarium* species

2.1. Introduction

Throughout the world, maize plays an important role in the livelihood of humans. Apart from serving as a staple food and source of income for millions of people, maize also is used extensively as animal feed and as a substrate for biogas production. Today, intensive maize production is practiced in many parts of the world and the acreage under maize cultivation continues to enlarge. However, several production constraints including pests and diseases pose a threat to the productivity and availability of healthy and safe maize grain. Among the crucial diseases affecting maize are the *Fusarium* induced infections like ear rot of maize, seedling blight, foot-rot and Fusarium head blight (Doohan et al., 2003). Such infections not only reduce yield, but they also remain the primary source of mycotoxin contamination in food and feed products. Moreover, when consumed, these mycotoxins cause health problems to both humans and animals. Thus, when *Fusarium* epidemics occur in the field, the chances of mycotoxin contamination of maize increases and this reduces the safety and market value of the crop harvested.

To date, several *Fusarium* species with mycotoxin producing ability have been characterized. Among these, *Fusarium verticillioides* (*Gibberella moniliformis, G. fujikuroi* mating population A), *F. proliferatum* (*G. fujikuroi* mating population D), and *F. graminearum* Schwabe, (*Gibberella zeae*) are frequently observed infecting maize (Cole et al., 1973; Nelson, 1992; Nelson et al., 1993; Leslie, 1996; Doohan et al., 2003; Naef and Defago, 2006; Görtz et al., 2008; Patricia Marín, 2010). In most cases, these fungi exhibit both parasitic and saprophytic modes of nutrition (Ali and Francl, 2001; Bacon, 2001; Bacon et al., 2008). According to research on the life cycle of *Fusarium*, the fungus is believed to infect maize kernels either locally or systemically (Sutton, 1982; Parry et al., 1995; Munkvold and Desjardins, 1997). Although infection of maize kernels by *Fusarium* can occur through several routes, local infection through silks seems to play an important role in kernel infection (Munkvold and Desjardins, 1997). Most research reports indicate that *Fusarium* conidia are dispersed by wind and/or water. Upon

landing on the host, they infect silks and then kernels (Gulya et al., 1980; Nelson, 1992; Munkvold and Desjardins, 1997).

The infection of *Fusarium* into the host plant, however, is influenced by several factors including environmental conditions, physiology of the host and spore condition among others (Dodd, 1980; Magan and Lacey, 1984; Marin et al., 1995a; Doohan et al., 2003). Temperature and humidity conditions are believed to be determinants in the infection process, development, and dissemination as well as mycotoxin producing ability of *Fusarium* (Dilkin et al., 2002; Etcheverry et al., 2002; Murillo-Williams and Munkvold, 2008). Moreover, light conditions also influent pathogen infection of the host. For instance, plants grown under low light conditions were reported to exhibit symptoms of physiological weakening leading to severe rotting and high seedling mortality (Dodd, 1980; Oren et al., 2003). The physiological status of the plant and fungus also greatly affected the infection process of *Fusarium* (Yates and Jaworski, 2000). Additionally, the germination rate of *Fusarium* conidia was influenced by spore density, which in turn influenced disease development (Colhoun et al., 1968; Reid, 1995). On the other hand, the infection of kernels via silks depended on the development stages of the silks (King, 1981; Schaafsma, 1993; Yates and Jaworski, 2000; Reid, 2002).

Following infection, the infected plants showed disease symptoms or were symptomless depending on the biotic and abiotic surroundings of the plants (Bacon and Hinton, 1996; Wilke et al., 2007; Bacon et al., 2008). Although many studies have described the impact of biotic and abiotic factors on the infection of *Fusarium* into host plants, most of these reports concentrated on the infection and the symptoms of *Fusarium* on kernels, seeds or the crown. Conversely, some research reports described *Fusarium* infection of host plants via the leaves (Ali and Francl, 2001; Wagacha et al., 2012). In addition, it remains unknown if *Fusarium* infects maize leaves locally followed by the formation of disease symptoms on leaves or not. In order to provide additional insights on the interaction between *Fusarium* and maize as a host plant, this chapter aimed to identify

determinants affecting *Fusarium* infection into maize leaves. The specific objectives were to:

i. study the effects of plant age, leaf position and cultivar on the infection of *Fusarium* species into maize leaves.
ii. examine the effects of inoculum density on infection of maize leaves.
iii. evaluate the effects of light on *Fusarium* infection of maize leaves.

2.2. Materials and methods

2.2.1. Fungal pathogen and inoculum preparation

Fusarium proliferatum (Matsushima) Nirenberg, isolate AG31g and *F. verticillioides* (Sacc.) Nirenberg, isolate AG11i were utilized for examining the effects of the different factors on the infection of *Fusarium* into maize leaves. *F. graminearum* was included in the experiment on effect of inoculation sites of *Fusarium* infection and manifestation of symptoms on maize plants. These isolates were obtained from the culture collection of fungi preserved at -80 °C at INRES, University of Bonn. Originally the fungi were isolated from maize kernels harvested from Germany (Görtz et al., 2008). Depending on the objectives, the fungi were grown on different culture media. For the propagation of *Fusarium* conidia either full-strength (FS) or low-strength (LS) Potato Dextrose Agar (-PDA) or Potato Dextrose Broth (PDB) were used. Czapek-Dox-Iprodione-Dicloran Agar (CZID) was used to re-isolate *Fusarium* from leaves). Prior to utilization, all culture media except broth were prepared by suspending culture ingredients (i-iv) in distilled water followed by autoclaving at 121°C for 20 min. When the media had cooled to about 55 °C, LS-PDA or PDB or CZID were supplemented with 100 mg Penicillin, 100 mg Streptomycin and 10 mg of Chlotetracyclin antibiotics. In addition to the above antibiotics, 6mg of Rovral was added to the CZID media. Each medium was mixed with the antibiotics by swirling the bottle and then dispensed onto plastic Petri dishes (Ø 90 mm).

i. Full – strength Potato Dextrose Agar (PDA) (Merck, Darmstadt Germany)

Potato dextrose agar	39.0 g

ii. Potato Dextrose broth

Potato dextrose broth	24.0 g

iii. Low Strength Potato Dextrose Agar (LSPDA, Merck, Darmstadt Germany)

Potato dextrose agar	12.5 g
Agar	19.0 g

iv. Czapek-Dox-Iprodione-Dicloran Agar (CZID) (Abildgren et al., 1987)

Ingredient	Concentration (g/l)
Sacharose	30
Natriumnitrate	3
Magnesiumsulfat	0.5
Kaliumchlorid	0.5
Di-kaliumhydrogenphotphat	1
Ferroussulfate heptahydrate	0.001
CuSO4.5H2O	0.005
ZnSO4.7H2O	0.01
Chloramphenicol	0.05
Dicloran / ethanol 96%	0.002
Agar	21.33

For the production of fungal inoculum, cultures were prepared according Moradi (2008). The hyphae in cryo-culture were transferred onto PDA in Petri dishes and then incubated at 22 °C for at least 7 days. Then two fungal plugs (Ø1 cm) were cut from the 7-day old cultures and placed into the PDB media in 500 ml Erlenmeyer flasks containing 100ml of media. The cultures were incubated on a shaker at 120 rpm at 22 °C and total

darkness for 3-4 days. Then 0.5 ml of the fungal suspension was spread on the surface of LSPDA media. Inoculated Petri dishes were air-dried under a laminar flow cabinet for 10-20 min. The plates were then incubated under conditions of near ultra violet light at 22°C for 3 to 5 days. Conidia were harvested by flooding the plates with sterile distilled water containing Tween 20 (0.075%) followed by slight scraping with a spatula. The suspension was sieved through a double-layered cheesecloth. The concentration of conidia was determined using a Fuchs-Rosenthal chamber and then adjusted according to each experimental design.

2.2.2. Plant cultivation

External and seed-borne fungal disease contamination of maize were reduced by procedure of sterilization developed by Rahman (2008). Seeds were soaked in water for 4 hrs at room temperature and then treated in hot water at 50-52 ^{0}C for 15 minutes. Seeds were dried and stored at room temperature. The seeds were then sown in trays. After germination, uniform seedlings were selected and then transplanted into pots of different sizes depending on the experiment. For example, 4 l pots (Ø 20 cm) were used for research on the effect of plant growth stages on the infection of *Fusarium* into maize leaves, whereas small 0.6 l pots (8×8×10 cm) were used for the other trials. For all the experiments, Klasmann potting substrate (Klasmann-Deilmann, Geeste, Germany) was used. With the exception of the experiments on the effect of growth stages and inoculation positions on *Fusarium* infection in which only the cultivar cv. Tassilo was used, all the other trials were performed with the two cultivars cv. Tassilo and Ronaldinial. All the experiments were carried out inside growth chambers except for the experiment established to determine the effect of growth stages on infection of *Fusarium* into maize leaves that was conducted under greenhouse conditions. Plants in all experiments, in pots were fertilized with 1g of NPK (NPK: 20-15-15) at 10 days after emergence. Additional 2g of NPK was given 65 days after emergence to support plant establishment for assessing the influence of growth stages on infection of *Fusarium* into maize leaves. The plants were carefully water once a day over the soil surface but avoiding sprinkling of water on the foliage.

2.2.3. Experimental design

Five set of experiment was carried out in greenhouse and in growth chamber. The plants after inoculation in all experiments were incubated in high humidity chambers where plants were misted by hand spraying to keep continuous wetness for 48 hours after inoculation.

2.2.3.1. Impact of growth stage of maize plants on infection

The experiment was carried out under greenhouse conditions (temperature = 24.0 ± 4 °C, and photoperiod = 16h light) during the summer time. The experiment consisted of plants treated with *Fusarium proliferatum* and *F. verticillioides*. Maize cv Tassilo was used in the study. In total, four treatments were assessed. Each treatment comprised of 20 plants grown individually in pots. At 15 and 37 days after emergence (i.e. 5-6th leaf stage, BBCH 15 (GS15) and 11-12th leaf stage, BBCH 33-35 (GS35) (Meier, 1997) (Fig 2.1), the maize plants were inoculated by hand spraying the entire plant with a 5-10 mL fungal suspension containing 10^5 spores/mL. Control plants were treated with distilled water. Following inoculation, the plants were incubated in high humidity chambers and then were kept in the greenhouse until re-isolation assessment. The experiment was conducted two times.

Figure 2.1. Illustration of maize plants inoculated at different growth stages. A = at BBCH 15 and B= at BBCH 33-35. (Meier, 1997)

2.2.3.2. Impact of spore concentration on the infection of maize leaves

To assess the impact of spore concentration on *Fusarium* infection of different maize cultivars, maize plants were grown in 0.6 l pots in growth chambers. The experiment was organized with 3 levels of spore concentrations (10^5, 10^6, and $2*10^6$spore/mL), two varieties of maize cv. Ronaldinio and cv. Tassilo and *Fusarium proliferatum* and *F. verticillioides*. In total, twelve treatments were used, with each treatment replicated 6 times. Based on results of the above experiment, the more susceptible stage of maize growth was selected for the timing of inoculum application. The plants were sprayed with 5 mL of spore suspension at the 5-6 leaf stage as described above and maintained in the growth chamber at 18-20^{o}C and 22-24 ^{o}C, and 60 and 80%, relative humidity respectively and a day and night photoperiod of 15hours. Control plants were treated with distilled water and kept under similar growth conditions. Following inoculation, the plants were incubated in high humidity chambers and then were kept in the growth chambers for 10 days prior to re-isolation assessment. The experiment was repeated two times.

2.2.3.3. Impact of light on infection of maize leaves

To examine the impact of light on *Fusarium* infection, the experiment was carried out with two maize cultivars in growth chambers using 2 levels of light regimes: (1) 5800-6000lux, 9h/day and (2) 18000-20000lux, 15h/day. These light regimes were maintained during plant growth until inoculation time. The temperature and relative humidity of the growth chamber varied from 18-20 ^{o}C and 22-24 ^{o}C, and a relative humidity of 60 and 80%, respectively for the day and night phases. The plants were inoculated at the 5^{th}-6^{th} leaf stage by hand spraying the entire plants with a 5mL spore suspension containing 10^6 spore/mL. After inoculation, all plants were incubated in high humidity chambers and then were maintained under similar light conditions (18000-20000lux, 15h/day). In total, eight treatments were used, with each treatment comprised of 6 plants and the experiment was repeated two times.

2.2.3.4. Effect of inoculation site on infection and symptom manifestation on maize plants

Fusarium proliferatum, and the maize cv Tassilo were used to test the hypothesis that *Fusarium* produced symptoms on very young leaves i.e. emerging or immature leaves. The maize plants were grown inside growth chambers under similar growth conditions as described above (section 2.2.3.2). The experiment comprised of three treatments: dropping 750 µl suspension into the whorl with a pipette (W), coating the spore suspension on the 4^{th} leaf with a paintbrush (L), and a combination of dropping into the whorl plus coating with the spore suspension on the 4^{th} leaf (WL). Each treatment consisted of 16 plants (Fig. 2.2). The spore suspension contained $2x10^6$ spore/ ml. Control plants were treated with water. After inoculation, the plants were incubated in high humidity chambers and then were kept in the growth chambers for 10 days prior to data collection. Fungal re-isolation assessment was undertaken for only eight plants per treatment. The experiment was repeated once.

2.2.3.5. Effect of inoculation site on infection and symptom manifestation of different species

Fusarium graminearum, F. proliferatum and *F. verticillioides* inoculum were included in this experiment. Maize plants cv. Tassilo were grown in growth chambers. At the 5-6 leaf stage, the plants were inoculated with fungal suspensions of the three *Fusarium* species using both spraying and dropping. In total, three treatments were established with 16 plants per treatments. For each plant, the fungal suspension was sprayed on the fourth leaf until fully wet (≈2 mL) and then simultaneously 750 µl of the suspension dropped into the whorl of maize plants. (Fig. 2.2 B, C). For both treatments a spore concentration of $2x10^6$ spores/mL was used. Control plants were treated with water. Following inoculation, the plants were incubated in high humidity chambers and then were kept in the growth chambers for 10 days prior to data collection. The experiment was repeated two times. Data on disease incidence and severity were collected for all

the plants while fungal re-isolation was conducted for eight plants. Additionally, photographic techniques were used to record disease appearance.

2.2.4. Data collection

2.2.4.1. Re-isolation frequency

For the re-isolation of fungi from non-sterilized leaves, the maize leaves were cut into 1 cm^2 pieces. Then seven pieces were randomly selected and plated directly onto Petri plate containing 20 mL CZID-agar. For surface sterilized leaves, the remaining cut leaf pieces were placed into tea paper bags and then immersed in 1.3% NaOCl solution for two minutes. The leaves were rinsed twice in sterile distilled water for two minutes each and then dried on sterile tissue paper inside the laminar airflow cabinet. Seven pieces of leaf tissues per sample were then plated onto CZID-agar plates. To assess the effectiveness of the surface sterilization procedure, tissue imprints were made on CZID-agar plates prior to plating (Schulz and Boyle, 2005). All plates were incubated at room temperature (22 ± 3 ^oC) for 5-7 days before colonization assessment was carried out. Re-isolation frequency was calculated as number of pieces exhibiting the outgrowth of *Fusarium* per total number plated pieces multiplied by 100.

2.2.4.2. Disease incidence and disease severity

Data on disease incidence was measured as the proportion of plants that were diseased. Disease severity was estimated as the percentage of the leaf areas showing symptoms out of the total leaf area. Disease incidence and disease severity were scored at 10 days after inoculation.

2.2.5. Data analysis

All data were tested for normality and homogeneity of variance using Kolmogorov or Shapiro-Wilk tests prior to subjecting them to analysis of variance (ANOVA). IRRISTAT statistical package (version 5.0, International Rice Research Institute) was used to analyze the data. Data on disease incidence were arcsine square root transformed before carrying out ANOVA. Where significant differences occurred across treatments, mean comparisons were performed using Duncan's test or LSD at 5% significant level.

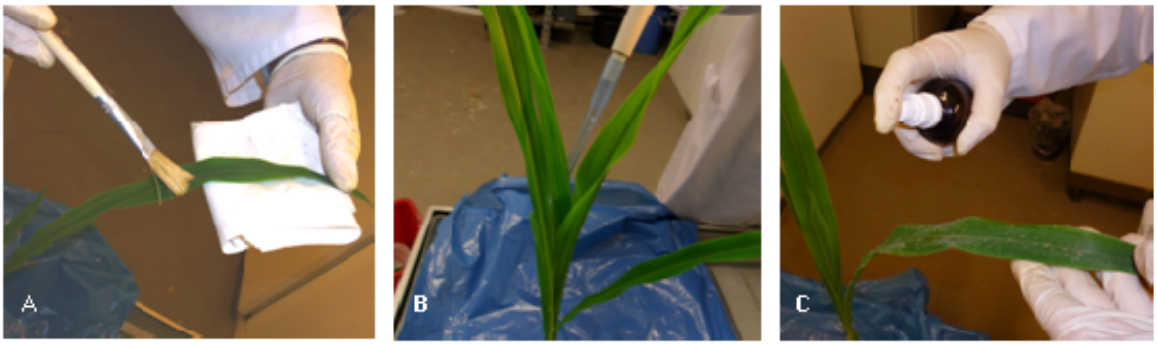


Figure 2.2. Description of inoculation of maize plant with *Fusarium* species. A= coating suspension on the 4th leaf. B= dropping suspension into whorl. C= spraying suspension on the 4th leaf.

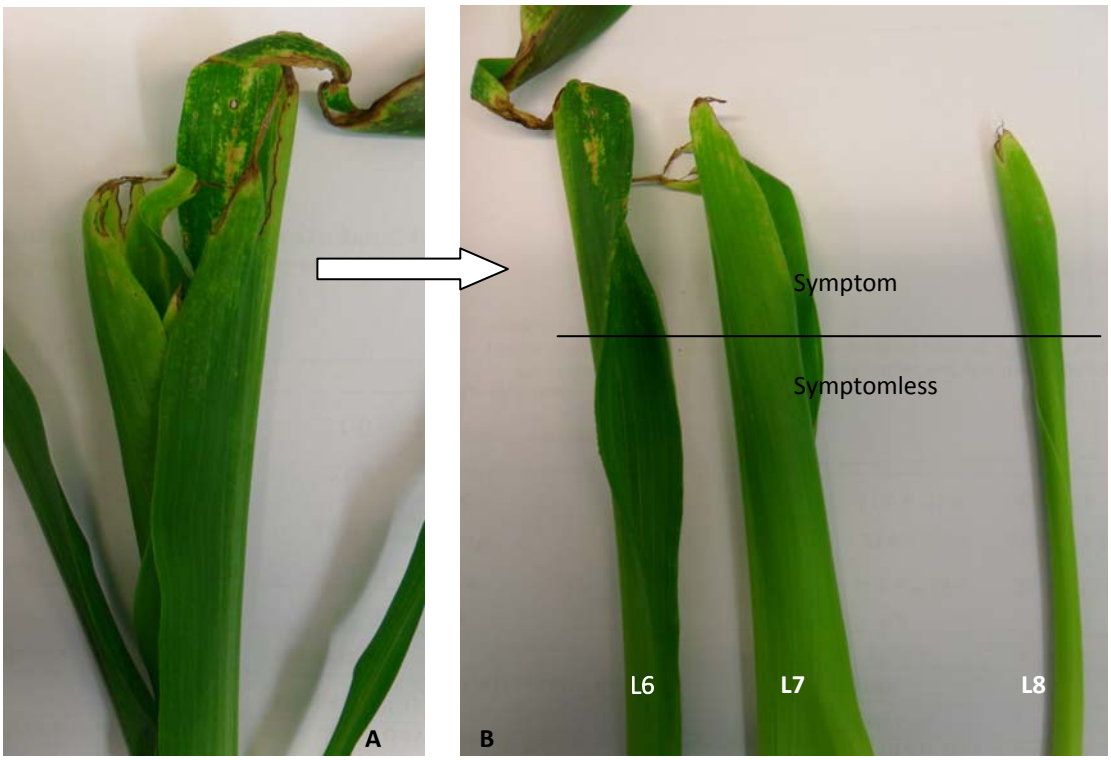


Figure 2.3. Description of symptom and symptomless parts of maize leaves used for the re-isolation of *Fusarium proliferatum*. A= symptoms on emerging leaves. B= symptom leaves in A separated into the 6th, 7th and 8th leaf (L6, L7 and L8).

2.3. Results

2.3.1. Impact of growth stage of maize plants on infection

Results of the re-isolation frequency revealed that the growth stage of the plant had a significant effect on *Fusarium* infection of maize leaves. Among the non-sterilized leaf samples, infection ranged between 60.4 and 70.8% and was not affected by growth stage ($P > 0.05$). However, for surface-sterilized leaf samples, the re-isolation frequency was influenced significantly by the growth stage of the plant ($P < 0.05$). The re-isolation frequency of leaves collected when inoculation was applied at the growth stage GS 15 was significantly higher than that performed at GS 35 ($P = 0.03$) only at 13 days after inoculation (dai). Nonetheless, this effect on *Fusarium* infection of maize leaves was not significantly different 26 and 39 dai (Fig. 2.4).

The re-isolation frequency depended on fungal species and assessment time. Although the re-isolation frequency of *F. proliferatum* and *F. verticillioides* for non-sterilized leaves was not significantly different at 13 dai (*F. proliferatum*: 67.7% and *F. verticillioides*: 61.6%), significant differences between the two species were noted at 26 dai ($P = 0.02$) and at 39 dai ($P = 0.003$). However, for sterilized leaves, significant differences were noted in colonization between the two fungal species at 26 dai. The re-isolation frequency of *F. proliferatum* (37.9%) was significantly higher than that of *F. verticillioides* (28.8%) (Fig. 2.5).

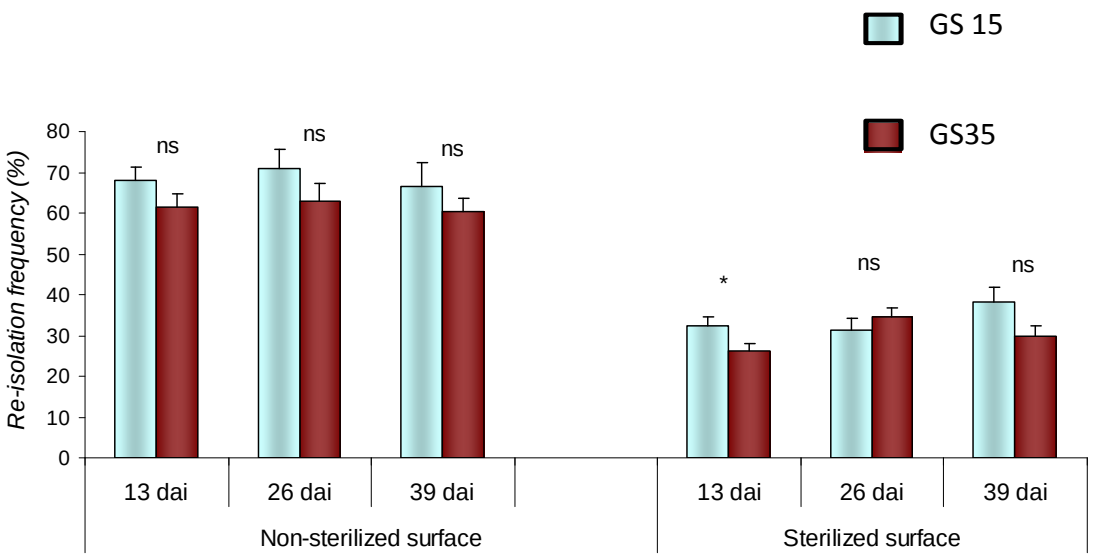


Figure 2.4. Colonization of maize leaves inoculated at growth stage (GS) GS15 and GS35 with *Fusarium proliferatum* and *F. verticillioides*.
Ns: non-significant and *: significant differences between two inoculated stages, P ≤ 0.05. Error bars represent the standard error of the mean.

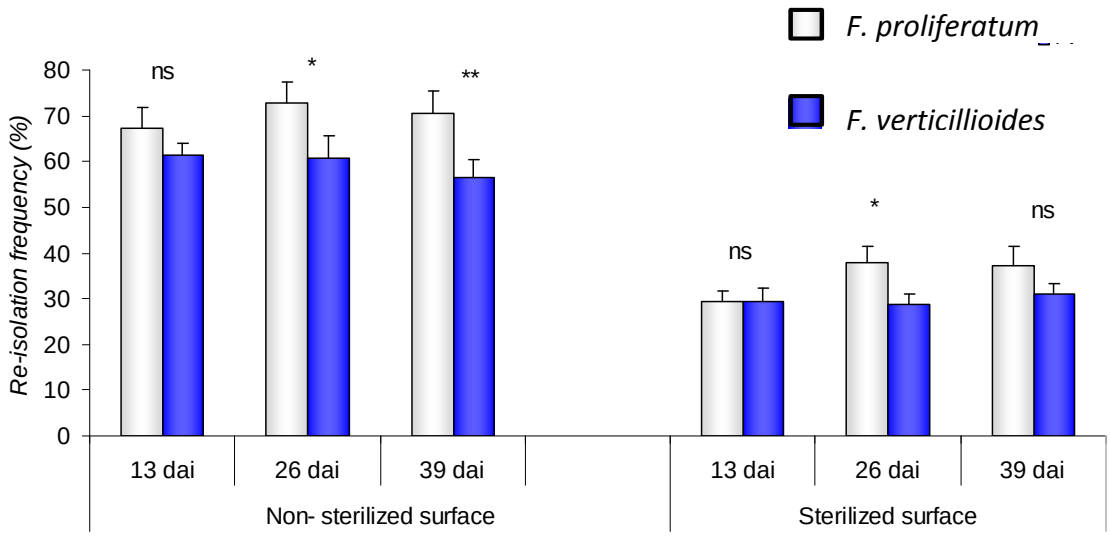


Figure 2.5. Colonization of maize leaves by *Fusarium proliferatum and F. verticillioides* at GS15 and GS35.
Ns: non-significant and *: significant differences between two *Fusarium* species, P ≤ 0.05. **: significant differences between two Fusarium species, P ≤ 0.01. Error bars represent the standard error of the mean.

2.3.2. Impact of spore concentration on the infection of maize leaves

The frequency of re-isolation on non-sterilized leaves differed significantly across treatments and depended on spore concentration (P=0.001). Higher spore concentrations resulted into higher levels of infection. Hence, re-isolation of the fungus of non-sterilized leaves obtained from samples inoculated with 10^5spore/mL was significantly lower than that inoculated with 10^6 and $2x10^6$spore/mL. Percentage colonization assessment of surface sterilized leaves showed that re-isolation frequency depended on spore concentration and was affected by the interaction between maize cultivar and spore concentration (P = 0.001). Percentage colonization of the maize cv Ronaldinio at a spore concentration of 10^6 spore/mL was significantly higher and lower than that with 10^5 spore/mL and the $2x10^6$ spore/mL, respectively. On the other hand, percentage colonization of cv Tassilo was significantly higher among plants inoculated with 10^6 and $2x10^6$ spore/mL in comparison to those treated with a suspension of 10^5 spore/mL (Table 2.1).

Percentage colonization of lower leaves was significantly higher than that of the upper leaves (Table 2.2, P = 0.001). Moreover, a three-way interaction occurred among the treatments i.e. among spore concentrations, *Fusarium* species and position of leaves (P=0.04). For lower leaves, percentage colonization for the 10^6 and $2x10^6$ spore/mL was significantly higher than that with 10^5 spore/mL for plants inoculated with *F. proliferatum* and *F. verticillioides* and similar for upper leaves inoculated with *F. proliferatum*. On the other hand, percentage colonization of upper leaves inoculated with $2x10^6$ spore/mL of *F. verticillioides* was significantly higher among plants inoculated with 10^5 and 10^6 spore/mL (Table 2.2).

Table 2.1. Effect of spore concentration of *Fusarium proliferatum* and *F. verticillioides* on the infection of maize cultivars assessed from non-sterilized and sterilized leaf surfaces (re-isolation frequency, %), 10 days after inoculation.

Spore conc./mL	Fungi (1)	Non-sterilized surface				Sterilized surface			
		Ron		Tas		Ron		Tas	
10^5	Fp	94.0	ab	89.3	b	13.6	c	20.0	bc
10^6	Fp	94.0	ab	98.8	a	40.7	b	52.1	a
$2*10^6$	Fp	97.6	a	98.8	a	55.7	a	46.7	ab
10^5	Fv	89.3	b	95.2	ab	19.2	c	17.5	c
10^6	Fv	94.0	ab	98.8	a	33.2	bc	51.4	a
$2*10^6$	Fv	100	a	100.0	a	49.6	ab	51.1	a

(1) Fp: *F. proliferatum* isolate AG31g and Fv: *F. verticillioides* isolate AG11i.
For each treated surface, mean values followed by the same letters are not significantly different at $P<0.05$.
Ron= cv. Ronaldinio, Tas= cv. Tassilo

Disease symptoms were observed on both maize cultivars Ronaldinio and Tassilo, but among plants inoculated with higher spore concentrations of 10^6 and $2x10^6$ spore/mL disease incidence did not differ between the two cultivars nor was it influenced by the spore concentration. Disease levels of 0, 8.4 and 8.4% corresponding to spore concentrations of 10^5, 10^6 and $2x10^6$ spore/mL (Table 2.3), respectively were recorded. Disease severity on the other hand was rather low and appeared mostly on very young leaves.

Table 2.2. Effect of inoculum concentration of *Fusarium* on the infection of lower and upper leaves assessed from non-sterilized and sterilized leaf surfaces (re-isolation frequency, %), 10 days after inoculation.

Spore conc./ mL	Fungi [(1)]	Non-sterilized surface				Sterilized surface			
		Lower leaves		Upper leaves		Lower leaves		Upper leaves	
10^5	Fp	96.4	ab	86.9	c	22.9	cd	10.7	d
10^6	Fp	100	a	92.8	bc	53.6	a	39.3	b
$2x10^6$	Fp	98.8	ab	97.6	ab	65.5	a	35.3	bc
10^5	Fv	94.0	ab	90.4	bc	21.1	cd	17.1	cd
10^6	Fv	100	a	92.8	bc	57.5	a	27.2	c
$2x10^6$	Fv	100	a	100	a	59.3	a	42.3	b

[(1)] Fp: *F. proliferatum* isolate AG31g and Fv: *F. verticillioides* isolate AG11i.
For each treated surface, mean values followed by the same letters are not significantly different (multivariate analysis, Duncan's test, $P \leq 0.05$).

Table 2.3. Disease incidence (%) of maize plants inoculated with different inoculum concentrations of *Fusarium,* 10 days after inoculation.

Spore concentration/mL	Fungi[(1)]	Ronaldinio	Tassilo	Mean
10^5	Fp	0	0	0
10^6	Fp	16.7	0	8.4
$2*10^6$	Fp	0	16.7	8.4
10^5	Fv	0	0	0
10^6	Fv	16.7	0	8.4
$2*10^6$	Fv	0	16.7	8.4

(1) Fp: *F. proliferatum* isolate AG31g and Fv: *F. verticillioides* isolate AG11i.

2.3.3. Effect of light regimes on infection of maize leaves

There was no effect of light on the infection of maize cultivars by *Fusarium* species (P > 0.05) . Hence, results of the re-isolation frequency for both non-sterilized and surface-sterilized leaves were similar across treatments (Table 2.4). However, re-isolation frequency was affected by leaf surfaces. Colonization frequencies differed significantly between the lower and upper leaves (P = 0.005 for non-sterilized; and P = 0.0001 for sterilized surface). Overall, the mean of re-isolation frequency for the lower leaves (71.9%) was significantly higher than for the upper leaves (42.1%). Similarly disease incidence of both maize cultivars was not affected by light regimes (Table 2.5). Disease severity also was very low and if any disease occurred in was only on very few young leaves.

Table 2.4. Effect of light on *Fusarium* infection of maize cultivars and leaf position assessed from non-sterilized and sterilized leaf surfaces (re-isolation frequency, %), 10 days after inoculation.

Factors [a]	Non-sterilized surface			Sterilized surface		
	Light [b] 1	Light 2	Mean	Light 1	Light 2	Mean
Ron	92.8	96.4	94.6 a	60.3	53.5	56.9 a
Tas	97.9	98.6	98.3 a	57.8	56.4	57.1 a
Fp	95.7	99.3	97.5 a	63.6	54.6	59.1 a
Fv	95.0	95.7	95.4 a	54.6	55.3	55.0 a
Lower leaves	100	100	100 a	71.8	72.1	72.0 a
Upper leaves	90.7	95.0	92.9 b	46.4	37.9	42.2 b

(a): Ron, cv. Ronaldinio; Tas, cv. Tassilo. Fp: *F. proliferatum* isolate AG31g and Fv*: F. verticillioides* isolate AG11i.
(b) Light 1: 5800-6000lux, 9h/day; light 2: 18000-20000lux, 15h/day.
For each factor, mean values followed by the same letters are not significantly different (P ≤ 0.05, Duncan's test).

Table 2.5. Disease incidence (%) on the cultivars Ronaldinio and Tassilo inoculated two species of *Fusarium* under different light conditions, 10 days after inoculation.

F. species	Cultivar	Light [a] 1	Light 2	Mean
F. proliferatum	Ronaldinio	4.8	11.4	8.1
F. proliferatum	Tasillo	19.1	0.0	9.6
F. verticillioides	Ronaldinio	13.3	5.6	9.5
F. verticillioides	Tasillo	4.8	5.6	5.2

[(a)] Light 1: 5800-6000lux, 9h/day; light 2: 18000-20000lux, 15h/day.

2.3.4. Effect of inoculation site on *Fusarium* infection and symptom manifestation

Only one species, *F. proliferatum*, was used to test the hypothesis that *Fusarium* could induce disease symptoms on very young emerging and immature leaves. Comparison analysis of different inoculation positions: i.e. Dropping 750µL suspension into the whorl (W), coating suspension on leaf 4th (L), and a combination of the two (WL) revealed pronounced symptoms of *Fusarium* infection in all the treatments except for the leaf coating. Disease incidence was high (90%) for both W and LW treatments. Disease severity of W and WL treatments ranged between 23.8 and 26.6%, but did not differ significantly between the two treatments (Table 2.6 and Fig. 2.6). No symptoms were detected for the 4th leaf inoculated by coating.

Table 2.6. Effect of inoculation site on disease incidence and disease severity following inoculation with *Fusarium proliferatum,* 10 days after inoculation.

Treatments [(1)]	Disease incidence (%)	Disease severity (%)	
		4th leaf	Emerging leaves
L	0.0 b	0	0.0 b
W	90 a	0	23.8 ± 4.3[(2)] a
LW	90 a	0	26.6 ± 3.1 a

[(1)] L, coating inoculum on the leaf 4th ; W, dropping 750µl inoculum into the whorl; LW, coating inoculum on the leaf 4th and dropping 750µl inoculum into the whorl.
[(2)] The standard error of the mean. Mean values in column followed by the same letters or no letter are not significantly different at P≤ 0.05, LSD test.

On the 4th leaf, re-isolation frequency was not significantly different between L (10.7%) and LW (15.5%) treatments (Table 2.8). On the 6th leaf (emerging leaf), re-isolation frequency was not significantly different between W and LW. However, colonization on the 4th leaf was significantly lower than for the emerging leaves. All sampled symptomatic tissues had very high colonization rates (98-100%). Conversely, symptomless leaf parts and/or the non-emerging leaf parts had no or little infection (Table 2.8).

Table 2.7. Re-isolation frequency (%) of *Fusarium proliferatum* on the 4th leaf and emerging leaves, 10 days after inoculation.

Treatments[1]	Non-sterilized surface		Sterilized surface	
	4th leaf	Emerging leaves	4th leaf	Emerging leaves
L	92.9 a [2]	14.3 b	10.7 a	0.0 b
W	21.5 b	82.3 a	0.0 b	80.4 a
LW	100 a	100 a	15.5 a	87.9 a

[1] L, coating inoculum on the leaf 4th ; W, dropping 750µl inoculum into the whorl; LW, coating inoculum on the leaf 4th and dropping 750µl inoculum into the whorl.
Mean values in column followed by the same letters are not significantly different at P≤ 0.05 (Duncan's test).

Table 2.8. Re-isolation frequency (%) of *Fusarium proliferatum* in symptomatic tissues and non-emerged tissues, 10 days after inoculation.

Treatments[1]	Leaves 6th, 7th		Leaf 8th
	Symptom	Symptomless	Non- emerged leaf part
W	97.8	2.0	0
LW	100	0	0

[1]: W, dropping 750µl inoculum into the whorl; LW, coating inoculum on the leaf 4th and dropping 750µl inoculum into the whorl.

2.3.5. Effect of site of inoculation on infection and symptom manifestation of different species

Results of the comparative analysis of different inoculation sites on the infection of maize plants by three *Fusarium* species revealed that the incidence of disease was very high for all treatments (86.4-90%). Disease severity was not significantly affected by the species of *Fusarium* (P = 0.073) (Table. 2.9).

No symptoms appeared on the 4th leaf. However, *Fusarium* colonization of maize leaves by *F. graminearum* (26%) was significantly lower than for *F. verticillioides* (56.9%). Conversely to infection of the 4th leaf, percentage colonization of maize leaves inoculated with *F. graminearum* was higher on the emerging symptomatic leaves (i.e. the 6th and 7th leaf samples) than for *F. proliferatum* or *F. verticillioides*. The results were illustrated by re-isolation frequency of 68% for *F. graminearum*, 58% for *F. proliferatum* and 57% for *F. verticillioides* (Fig. 2.7).

Table 2.9. Disease incidence and disease severity (%) in maize plants inoculated with *Fusarium graminearum, F. proliferatum* and *F. verticillioides* at 10 days after inoculation.

Fuasrium species	Disease incidence (%)	Disease severity (%)
F. graminearum	93.7	7.3 ± 0.9
F. proliferatum	93.7	10.0 ± 1.2
F. verticillioides	86.5	7.1 ± 0.7

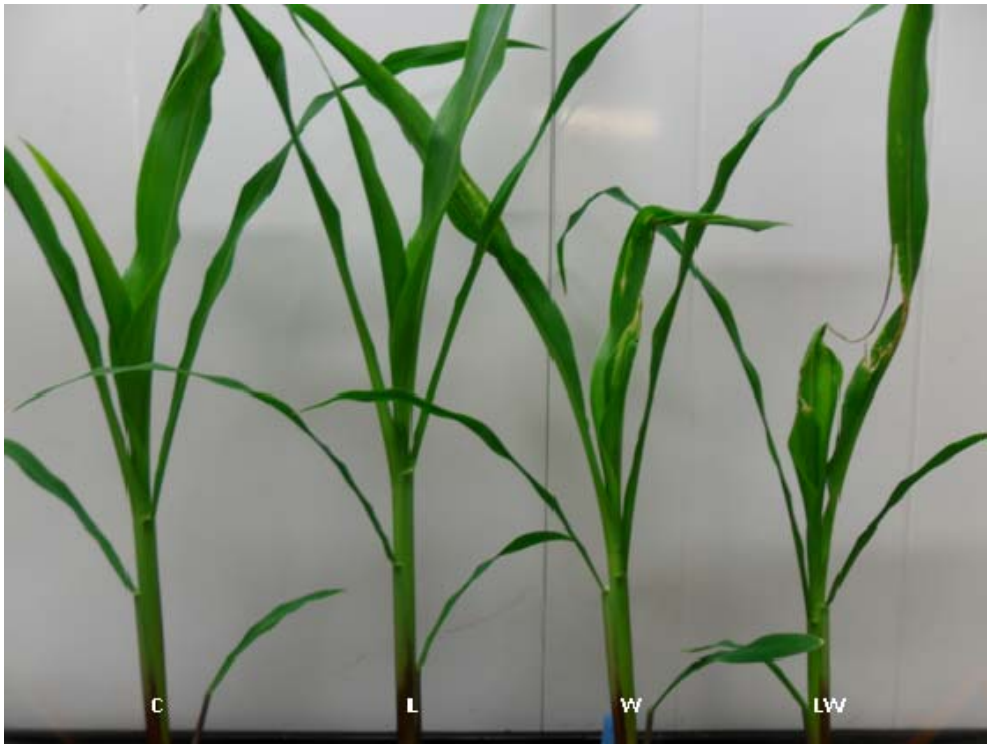


Figure 2.6. Growth and symptoms on maize plants inoculated with *Fusarium proliferatum*, 10 dai. Lesions, curling and dead leaf blade on treatments W and LW.
C= control, treated water, L= coating fungal inoculum on the 4th leaf, W= dropping 750 µl of inoculum into the whorl. LW= coating fungal inoculum on the 4th leaf and dropping 750 µl of inoculum into the whorl.

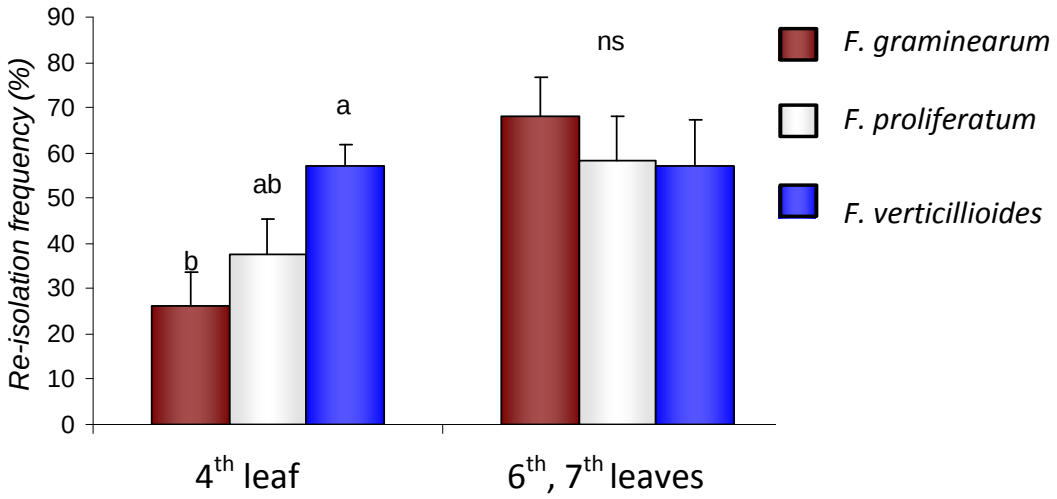


Figure 2.7. Re-isolation frequency of *Fusarium graminearum, F. proliferatum* and *F. verticillioides* in different aged maize leaves, cv. Tassilo, 10 dai.
For each parameter, the bars followed by the same letters or no letter are not significantly different at P ≤ 0.05 (Duncan's test). Ns: the means are not significantly different at P ≤0.05. Error bars represent the standard error of the mean.

The earliest macroscopic symptoms were observed on leaves treated with *F. graminearum* 4-5 dai. The symptoms first appeared as very small lesions 1 - 5mm in length. At first the lesions appeared water-soaked (Fig 2.8 D). However, overtime, the lesions turned into yellow spots with shades of brown or grey in the center (Fig. 2.8 B). 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. 2.8 C). Brown spots with yellow boundaries on leaves or on midribs/main veins or small holes with brownish edges were also observed on leaves with extensive symptoms (Fig. 2.8 A). Lesions or spots were also seen at the positions where fungal inoculum was dropped into the maize whorl. Mycelia were observed on the surface of both symptomic and asymptomic tissues (Fig. 2.8 E).

Disease symptoms of *F. proliferatum* were observed on immature emerging leaves 6th – 8th dai. Typical symptoms like necrotic lesion (holes) and streaks that were different in size appeared on specific parts of the leaves. They were observed at the distal end of the leaf where inoculum was dropped or on the upper leaf tips of the immediate leaf emerging after inoculation. The holes/streaks were approximately 5-60 mm in length and 1-10mm in width. A dark brown and yellowish boundary line appeared between the holes and the green interior of the leaf (Fig 2.9B). Mild symptoms such as slight chlorosis were also observed on the leaves. Many of the small streaks coalesced to create a line between leaf veins (Fig. 2.9C). Heavily infected leaves showed symptoms of deformation. For example, the distal end of the 6th leaf was observed to have severe chlorosis, particularly along the margins as well as at the tip of the leaf, leading to deformed, unopened leaves with symptoms of “deadhearts” (Fig. 2.9A). Under high relative humidity, dense fungal mycelia were observed on most dead and unopened leaves (Fig. 2.9 D and E).

Similarly, disease symptoms of *F. verticillioides* were also observed on emerging leaves, but this occurred a day earlier than for *F. proliferatum.* Heavy symptoms of infection mostly appeared at the distal end of the 6th leaf. Yellow necrotic lesions, streaks and small holes (1- 5 mm in length) were observed on the leaves with heavy symptom (Fig.

2.10A). Typical disease symptom also included the coalescing of many small streaks to form light green-yellowish lines along the leaf blades. Mild symptoms were similar except that the streaks and light green-yellow lines were smaller.

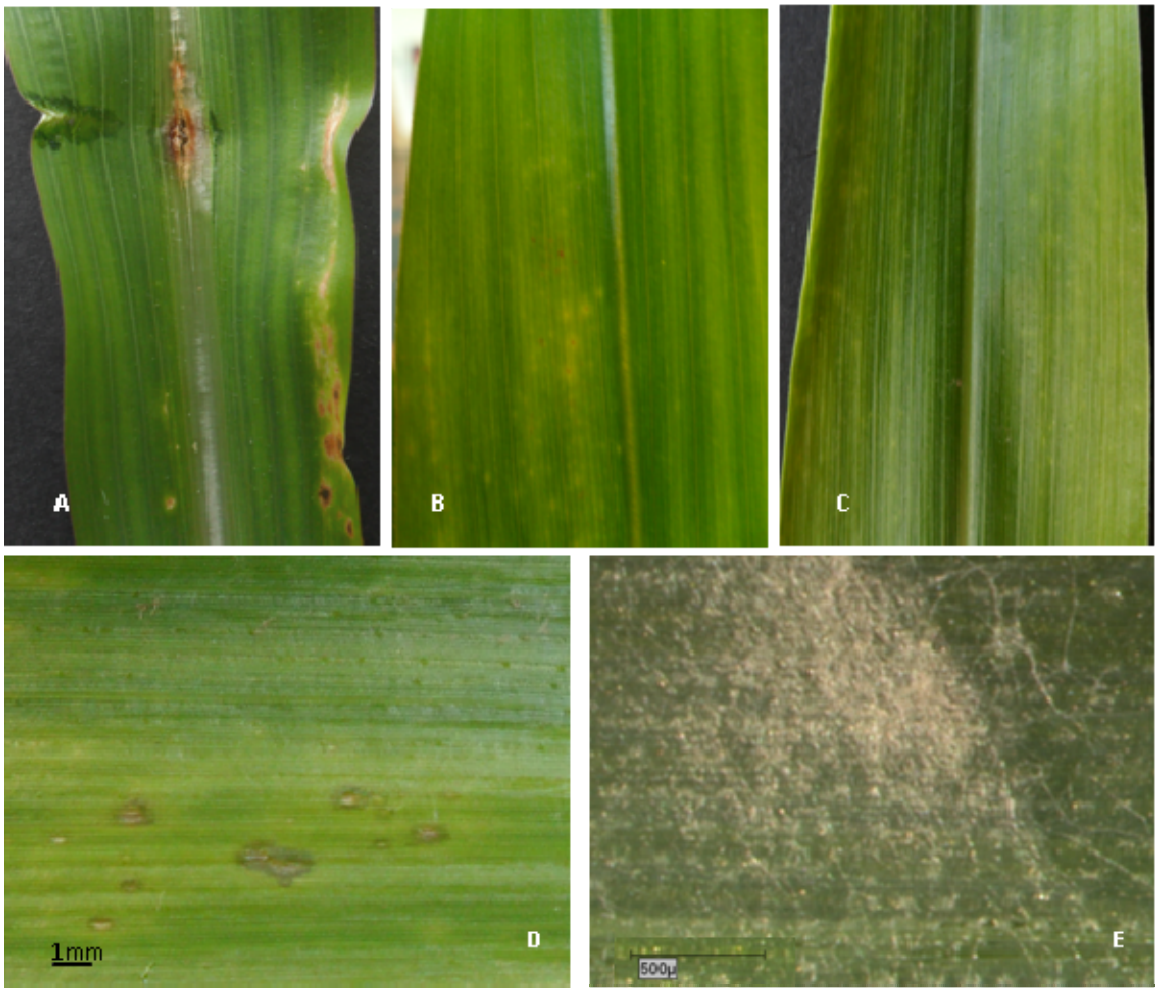


Figure 2.8. Symptomatic maize leaves infected with *Fusarium graminearum*. A = Heavy symptom, brown spots, 0.5-15mm in length; B = typical symptom, small yellow spots with some form of brown at the center; C = mild symptom, chlorotic spots; D = initiation of lesion as water soaked leaves during initial development of the fungus; and E = fungal mycelia on the leaf surface. A, B, C and F: 10 dai, D:5 dai.

Figure 2.9. Symptomatic maize leaves infected with *Fusarium proliferatum*, 10 dai. A = Heavy symptom, curling/ unopened and dead leaf blades; B = typical symptom, Yellow necrotic lesions, holes with dark brown border and 5-60mm in length, C = mild symptom, small chlorotic spots or streaks; D = infected leaf sample following incubation on wet filter paper over night; and E = Mycelia on unopened, atrophied and rotten leaf.

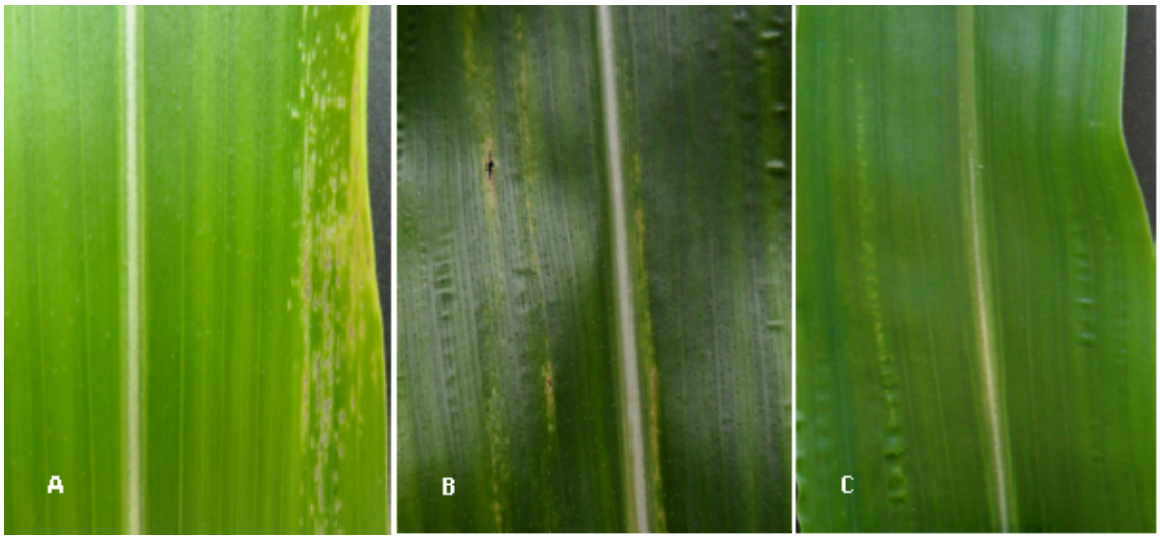


Figure 2.10. Symptomatic maize leaves infected with *Fusarium verticillioides,* 10 dai. A = Heavy symptom, yellow necrotic lesions or small holes (1-5 mm in length) along the leaf blade; B =typical symptom, streaks along the leaf blade; and C = mild symptom, small chlorotic streaks.

2.4. Discussions

Many investigations on infection and disease development of *Fusarium* species on or inside its host plants have been carried out with the kernels, crown or stalk. The present study showed that some species of *Fusarium* have the ability to infect maize plants via the leaves. The results also demonstrated that infection is influenced by plant age, inoculum concentration or site of inoculation. Plant age was observed to play a significant role in *Fusarium* infection of maize leaves. At 13 dai, for example, plants inoculated at BBCH 15 appeared more susceptible to *Fusarium* infection than the plants inoculated at BBCH 33-35. This finding is in line with previous research in which younger castor (*Ricinus communis*) plants were reported to succumb more to leaf blight disease caused by *Fusarium pallidoroseum* compared to mature plants (Wamza et al., 2008). Similarly, Reid (1996a) and Ullstrup (1970) noted that disease severity of *F. graminearum* on maize ears decreased with increasing silk age.

Furthermore, the results from the present study demonstrate that both *F. proliferatum* and *F. verticillioides* have the ability to survive for long periods on leaf surfaces without losing their ability to infect the plant. This observation was supported by the high success rates of maize leaf colonization attained at the two growth stages (BBCH 15,

BBCH 35), 26 and 39 days after inoculation. Earlier studies conducted by Wagacha (2008) revealed similar findings in which species of *Fusarium* were observed to infect wheat plants of different growth stages. The author found that *Fusarium* could infect all parts of both wheat seedlings and mature wheat plants. The colonization ability of different parts of maize plants by species of *Fusarium* is not new and has been reported for roots (Thomas, 1980; Williams et al., 2007), crown (Miller, 2007), stem (Lawrence, 1981; Kedera, 1994; Bacon and Hinton, 1996; Munkvold et al., 1997b), leaves (Williams et al., 2007) and kernels (Blandino et al., 2008) of maize plants. These results and the current findings of *Fusarium* infection of maize leaves shows clearly that *F. proliferatum* and *F. verticillioides* have the ability to establish infection and disease within maize via the leaf system. This form of infection may act as a locus and bridge for the later spread of inoculum to maize ears during the course of the growing season.

Irrespective of the maize cultivar, increasing inoculum concentration caused a significant increase in the rate of infection, disease incidence and symptom manifestation of *Fusarium* infection of the leaves. Research conducted by Reid (1995) revealed that disease severity of *F. graminearum* on maize increased with increasing spore concentration when the spore concentrations were varied between 10^4 to $2*10^6$ spore/mL. Equally, Colhoun et al. (1968) reported that high spore concentration of *F. culmorum* on wheat seeds led to severe attack of wheat seedling under wide range of environmental conditions. In other *in situ* studies on wheat, Dill-Macky et al.(2001) showed that increasing inoculum concentrations led to increasing levels of disease severity regardless of cultivar or environmental conditions of Fusarium head blight.

Light intensity and duration are two factors believed to have significant effects on the survival and pathogenesis of fungi. Solar irradiation, for example, was shown to influence germination of conidia of *Venturia inaequalis* (Aylor and Sanogo, 1997) as well as survival of airborne fungi (Ulevičius et al., 2004). Spore germination and mycelium growth of powdery mildew *Uncinula necato*r on grapevine leaves were also noted to

decrease when exposed to ultraviolet B radiation or sun light (Willocquet et al., 1996). On the other hand, the quantity of light, defined as the product of light duration and intensity, was noted to increase the efficiency of *Puccinia striiformis* infection of wheat by up to 36 % following pre-inoculation exposure to 30.1mol quanta per square meter (de Vallavieille-Pope et al., 2002). In the present study, the influence of pre-light treatment before inoculation on *Fusarium* infection of maize leaves was distinct. Though non-significant, low quantities of light were observed to cause a slightly higher level of disease incidence and colonization rates than high light intensity over all the treatments. This indicated that under the current study conditions, light played a non significant role on *Fusarium* colonization of maize leaves. Conversely to the results obtained in the current study, *F. verticillioides* was reported to cause intensive rotting and high seedling mortality when the seeds or the soil substrate were inoculated with the fungus and then maintained under low light intensity of 20 microeistein/m^2 (Oren et al., 2003). The authors noted that under low light intensity, *Fusarium* rapidly developed inside plant tissues leading to severe rotting and/ or plant death. However, when plants were grown under higher light intensity, mycelia only developed along the cell walls and did not cause rotting or other disease symptoms. Probably, the observed differences may have been attributed to differences in the inoculation methods used in the respective studies.

A number of asymptomatic leaves were detected in the current study in inoculated plants even though the frequency of re-isolation demonstrated fungal infection of internal leaf tissues. The endophytic association of many fungi including *F. verticillioides* with cereal crops is known to exist at most growth stages of the host plant (Schulz et al., 1999; Bacon, 2001). Endophytic fungi also can produce mycotoxins like the fumonisins inside plants (Bacon, 2001). Beside producing mycotoxin, symptomless infections reduced the photosynthetic ability and yield of crops and changed seedling morphology or histology (Yates et al., 1997).

In this study, symptomless, endophytic infection was observed when mature leaves were inoculated. The levels of the symptomless infection tended to be higher for *F.*

verticillioides and *F. proliferatum* and lower for *F. graminearum*. This indicates that dissimilarities exist in the endophytic nature of different parasite fungi on maize. Nonetheless, symptoms appeared when maize leaves were inoculated into the whorl of immature leaves. According to Oren et al. (2003) disease symptoms may occur as a result of imbalanced interactions between the host plant and the fungus. Additionally, symptoms can manifest depending on the different structures of young leaves or developmental stage of the leaves infested. For example, unemerged young leaves may lack preformed defense mechanisms such as wax and cuticle, making them more susceptible to infection compared to mature leaves. Symptoms of *F. graminearum* was noted to occur quickly (4-5 dai) while symptoms of *F. proliferatum* and *F. verticillioides* appeared later (7 dai). The variability in disease manifestation between the *Fusarium* isolates may have been related to the level of individual fungal virulence. These findings are in agreement with previous research findings in which *F*. *oxysporum* was found to be very aggressive and virulent in terms of being able to change from symptomless to the symptom phase within a few days. Previously, *F. verticillioides* was also found to be less aggressive and to grow slowly (Oren et al., 2003). Consequently, the host has time respond to the slow growing pathogen by accumulating pathogenesis-related protein (Murillo et al., 1999), increasing the formation of phenolic compounds (Siranidou et al., 2002) as well as lignin deposition (Yates et al., 1997). Cell walls of cotton, for example, are known to synthesize more callose when infected by *F. oxysporum* (Rodriguez-Galvez and Mendgen, 1995). Moreover, the appearance of disease symptoms is believed to be related to mycotoxin formation (Reid, 1996b; Desjardins et al., 1998; Kedera et al., 1999). The main mycotoxins produced by *F. proliferatum* and *F. verticillioides* are Fumonisins (Bacon and Nelson, 1994; Bush et al., 2004; Patricia Marín, 2010) while *F. graminearum* produces deoxynivalenol (DON) (Reid, 1996b; Munkvold, 2003; Yoshida and Nakajima, 2010), and this may additionally explain the differences observed in disease severity among the fungal species.

Higher frequencies of re-isolation frequency of the *Fusarium* species was obtained from non-surface sterilized leaf samples compared to the surface-sterilized samples. This is

characteristic behavior of *Fusarium* on cereal leaves, where it can cause superficial infection of leaf surfaces or internal infection and colonization of inner plant tissues. Similar results were reported on wheat leaves by Ali and Francl (2001) whereby *F. graminearum*, *F. sporotrichioides, F. avenaceum*, and *F. poae* were observed to survive parasitically and saprophytically on wheat leaves throughout the season. Wagacha (2008) attributed such behaviors of *Fusarium* species i.e *F. avenaseum*, *F. culmorum*, *F. graminearum*, *F. poae* and *F. tricinctum* to superficial infection without colonization of inner host tissues and to the role of vegetative wheat parts in spreading *Fusarium* species to wheat ears.

The adherence and colonization of *Fusarium* on or in maize leaves differed between the lower and upper leaves. These differences could be explained by: (i) morphological features of the leaf, (ii) senescence status of the leaf, and (iii) microclimatic conditions. For example, the upper leaves are positioned almost erectly, so conidia may run-off along the leaf surface; while the lower leaves are positioned obtusely or at right angles to the stem and can therefore hold more conidia due to less loss through run–off. High humidity is another condition that facilitates disease development. For instance, without suitable relative humidity, *Mycosphaerella* development on pea plants have been reported to be nearly impossible (Le May et al., 2009). Other studies have similarly reported that vines with extensive canopy growth favor higher humidity, that in turn supports the grey mould (*Botrytis cinerea*) development (Valdés-Gómez et al., 2008).

The present study showed a number of factors affect *Fusarium* infection of maize leaves, which in turn influenced the levels of infection and severity of *Fusarium* in maize leaves. *F. proliferatum and F. verticillioides* infected and colonized both growth stages of maize plants (BBCH 15 and BBCH 35). Light regimes had limited influence on *Fusarium* colonization and appearance of disease symptoms on maize leaves. However, increased in spore concentration was observed to enhance the colonization of maize plants by *Fusarium* regardless of the cultivar. Interestingly, the symptoms caused by *F. proliferatum and F. verticillioides* and *F. graminearum* appeared only when the fungal

inoculum came into direct contact with immature leaf parts, while symptomless infections were observed on mature leaves.

3. Histopathological assessment of the infection of maize leaves by *Fusarium* species

3.1. Introduction

Several fungal species belonging to the genus *Fusarium* are known to constrain cereal production in many regions of the world. Among the economically important diseases of cereal crops caused by *Fusarium* spp. are the root, stem and ear rot of maize, *Fusarium* head blight (FHB) and the crown rot (Koehler, 1942; Burgess et al., 1981; Parry et al., 1995; Doohan et al., 2003; Williams et al., 2007; Görtz et al., 2010). The infection of cereals by such fungi cause significant yield losses, both in quantity and grain quality (Klein et al., 1991; Southwell et al., 2003). In general, many fungi including species of *Fusarium* are mycotoxin producers. Apart from causing a variety of health problems in humans and animals, some of the mycotoxins have been reported to play a role in pathogen virulence during infection of the plant (Lamprecht et al., 1994; Desjardins et al., 1998). Moreover, yield losses and the reduction in grain quality have been observed to be related to the amount of mycotoxin produced in a particular grain by such fungi (McMullen et al., 1997).

Many studies have been conducted worldwide to distinguish between the different types of diseases caused by *Fusarium* spp. For instance, Logrieco et al. (2002) concluded that maize ear rots caused by *Fusarium* spp. can be categorized as pink ear rot and red ear rot. The authors further showed that *F. verticillioides*, *F. proliferatum* and *F. subglutinans* were the causative agents of pink ear rot, while *F. graminearum*, *F. culmorum*, *F. cerealis* and *F. avenaceum were* often associated with red ear rot (Logrieco et al., 2002). However, the occurrence and appearance of these diseases often depend on environmental conditions. The pink ear rot, for instance, occurred frequently in temperate regions with cooler climates (Munkvold and Desjardins, 1997) while the red ear rot was often found in regions that experience high humidity or rainfall and moderate temperatures (Logrieco et al., 2002; Munkvold, 2003). However, species of *Fusarium* have also been considered symptomless endophyte of maize (Thomas, 1980; Bacon and Hinton, 1996; Munkvold et al., 1997a; Bacon et al., 2008).

Like many other fungi, *Fusarium* can infect hosts in various ways. In maize, *Fusarium*

infection can take place systemically or locally (Sutton, 1982; Parry et al., 1995; Munkvold and Desjardins, 1997). In systemic infection, fungal hyphae usually grow from infected seeds, colonize the stalk and then the kernel (Lawrence, 1981). Local infection through silks has been reported to play an important role in kernel infection (Reid, 1992; Chungu, 1996 ; Munkvold et al., 1997b; Reid, 2002). In most cases, the fungal hyphae play a significant role in host attack and infection due to their ability to produce enzymes that degrade host cell walls (Kang and Buchenauer, 2000b). In addition, Lawrence (1981) demonstrated that *F. verticillioides* hyphae entered through the xylem of leaf and stem tissues. The infection of floral organs of wheat by *F. graminearum* have also been reported, and infection began with the formation of foot structures, lobate appressoria and infection cushions (Boenisch and Schäfer, 2011). On the other hand, *F. culmorum* hyphae was noted to penetrate different parts of wheat spikelets and sometimes via stomata (Kang and Buchenauer, 2000a).

The causative agents of FHB and ear rot produce many airborne conidia that aid in dispersal and host invasion. The conidia can be windblown or rain splashed on to the silks or spikelets prior to the infection of the kernels (Sutton, 1982; Munkvold and Desjardins, 1997; Trail, 2009). Only a few studies have examined the infection and colonization of maize plants by species of *Fusarium* through the leaves. Thus far, no information exists on the process associated with the spread of *Fusarium* conidia to the silks and then to the cobs of maize following leaf colonization. Such information is important in assessing plant health and safety during the early stages of growth.

This study, therefore, aimed at investigating the histopathological processes involved in the infection of maize leaves by *Fusarium graminearum, F. proliferatum* and *F. verticillioides*. The specific objectives of this study were to:

i. examine the conidial characteristics of the species during the germination stage.
ii. investigate the process by which they infect maize leaves.
iii. assess the ability of the fungi to sporulate on maize leaves after infection.

3.2. Materials and methods

3.2.1. Fungal pathogen and inoculum preparation

Fusarium proliferatum (Matsushima) Nirenberg, isolate AG31g, *F. verticillioides* (Sacc.) Nirenberg, isolate AG11i and *F. graminearum* isolate AG 23d were used in the study. The isolates were originally obtained from maize kernels collected in Germany (Görtz, 2008).

For the propagation of *Fusarium* conidia either full-strength or low-strength Potato Dextrose Agar (FSPDA, LSPDA) or Potato Dextrose Broth (PDB) were used. Ingredients of culture media are described in section 2.2.2. To start generating fungal inoculum, cultures were prepared according Moradi (2008). Briefly, the hyphae in cryo-culture were transferred onto PDA in Petri dishes and then incubated at 22 °C for at least 7 days. Then two fungal plugs (Ø1 cm) were cut from the 7-day old cultures and added to PDB media in 500 ml Erlenmeyer flasks containing 100ml of media. The cultures were incubated on a shaker at 120 rpm, 22°C and total darkness for 3-4 days. Thereafter, 0.5 ml of the fungal suspension was spread on the surface of LSPDA media. Inoculated Petri dishes were air-dried under a laminar flow cabinet for 10-20 min. The plates were then incubated under conditions of near ultra violet light at 22°C for 3-5 days. Conidia were harvested by flooding the plates with sterile distilled water containing Tween 20 (0.075%) followed by slight scraping with a spatula. The suspension was sieved through double-layered cheesecloth. The concentration of the conidia was determined using a Fuchs-Rosenthal chamber and then adjusted to 10^6 and $2*10^6$ spore/mL.

3.2.2. Cultivation of plant

Maize cv. Tassilo was used in the study. The seeds were disinfected with hot water (Rahman et al., 2008) and grown as described in section 2.2.2.

3.2.3. Inoculation and sampling collection

A detailed study of the growth pattern of *F. graminearum, F. proliferatum and F. verticilioides* was carried out on the attached 4^{th}, 6^{th}, and 7^{th} leaves. Leaves exhibiting symptoms and symptommless leaves were examined. An in vitro bioassay was also

performed for both the 4th and 6th leaves to continue microscopic evaluation on detached leaves by *F. graminearum*.

3.2.3.1. Attached leaves

Fifteen day old maize seedlings were inoculated by: i) hand spraying fungal suspension on the 4th leaf and ii) adding a droplet (750µl) of fungal suspension into the whorl of the 6th emerging leaf (immature leaves). Inoculated plants were incubated in growth chambers under high humidity condition (90-95%) for 48 h and then maintained at a temperature ranging from 18-20 ^{o}C and 22-24 ^{o}C, and a relative humidity from 45-55% and 75-83%, respectively during the day and night. A photoperiod of 15hours for 30 days was applied. For the hand sprayed plants, four specimens (~ 1cm^2) were collected from the 4th leaf, of each inoculated plant at 8, 12, 24 and 48 hour intervals after inoculation (hai). Thereafter, leaf samples were collected every day until the 30th day after inoculation (dai). For the plants that were inoculated in the whorl leaf samples were collected from the 6th leaf, on the 3rd dai. The sampling took place at one day intervals until the 30th dai. Depending on the purpose of the tests to be conducted, the leaf samples were processed differently (section 3.2.5) prior to leaf examination under the microscope.

3.2.3.2. Detached leaves

For the detached leaves, the 4th and 6th leaves were cut off from the plant. The leaves were laid on moistened *tissue* paper on a wire screen in plastic humidity chambers and inoculated by coating the suspension on the abaxial (upper side). The moisture chamber was then taken to an environmentally controlled growth chamber and incubated as mention above.

The tissues were collected from day 1 until 7 dai. After collection, the samples were observed fresh under the fluorescent light microscope. In addition, leaf tissues were collected from the *F. graminearum* inoculated leaves at 7 dai. These samples were processed for the transmission electron microscopy work.

3.2.4. Measurement of conidia

The test was carried out on diagnostic microscope slides (Erie Scientific Company, Braunschweig, Germany). Each slide contained three wells. Twenty five microliters of conidial suspension containing 10^6 spore/mL of each species was pipetted into each well of the diagnostic microscope slides. For each species, a total of 14 slides were used. Each slide was laid on a wire screen in the humidity chambers and kept at room temperature for 24 h. To determine conidial characteristics, two sets of slides were randomly picked for assessment at 2, 4, 6, 8, 10, 12, and 24 h after incubation. At each sampling, the size of conidia, number of germinated conidia, and the number and length of germ tubes were assessed. The counting and measurements were done using a Leitz DMR photomicroscope (Leica, Wetzlar, Germany). A conidium was considered to have germinated if the germ tube was longer than the width of the conidium. In total, 120 conidia were assessed per well.

3.2.5. Microscopy

In order to describe the development of the fungal structures on the leaf surface or inside the leaf, microscopic examinations were made under light, fluorescence, scanning electron and transmission electron microscope.

3.2.5.1. Light microscopy

Light microscope (Leica Microsystems, Wetzlar, Germany) was used to observe conidia germination, growth, colonization and sporulation of the species of *Fusarium* on the maize leaves. Procedures of leaf samples for light microscope work were as follows.

3.2.5.1.1. Fresh specimen

For the observation of germ tube and fungal pre-penetration fungal structures on the surface of the inoculated leaves, 1 cm^2 fresh leaf sections were cut out of the leaves at 24, 48 and 72 hai. The section were mounted on a microscopic slide in diethanol (0.01%) and covered with cover slip. The specimens were observed with the BP 340-380/FT 400/LP 430 filter combination using the Leitz microscope in the fluorescence mode.

3.2.5.1.2. Whole specimen

Clearing specimens*:*

Leaf pieces of 1 cm^2 were cut and soaked immediately in saturated chloral hydrate solution (250 g/100 mL H_2O). To reduced washing off of conidia from the leaf surface, 0.01% formaldehyde was added to the chloral hydrate solution before using. For full clearance of the chlorophyll, leaf pieces were left in the clearing solution for 7 days at room temperature. After the sections were cleared of chlorophyll, they were stained and examined with a light microscopy (Leitz DMR photomicroscope from Leica). Images were photographed with a fitted digital camera and saved using the program "Discus" (Technisches Büro Hilgers, Koenigswinter, Germany).

Staining:

For staining of whole specimens, the cleared leaf samples were immediately immersed in solution according to Bruzzese and Hasan (1983) (95% ethanol, 300 mL; Chloroform, 150 mL; acid lactic (90%), 125 mL; Chloral hydrate, 450 g and Alinine blue, 0.6 g) or acid fuchsin (Phenol, 10 mL; Glycerin, 10 ml; acid lactic, 10 ml; and acid Fuchsin 3mg) for 24 h and 24-48 h, respectively. Fungal structures were stained blue when stained with the solution by Bruzzese and Hasan and pink with Fuchsin solution.

Observation:

Stained samples were mounted on microscope slides and then covered with a cover slip and observed with the Nomarski-interference-contrast filter of the Leitz DMR photomicroscope from Leica. Images were photographed with a fitted digital camera and saved using the program "Discus" (Technisches Büro Hilgers, Koenigswinter, Germany).

3.2.5.2. Scanning electron microscopy

The leaf tissues were mounted on stubs and coated with gold by an automatic sputter coater MSC1 S/N 201106 (Ingbuero Peter Liebscher) and then observed with a Phenom World SEM (Fei, Eindhoven, Netherlands).

3.2.5.3. Transmission electron microscopy

Fixing specimens:

Leaf tissues showing lesion symptoms were cut into small pieces of 2 x 2 mm^2. These pieces were immediately fixed for 4 hours at room temperature. The fixative was prepared by mixing 2 *%* paraformaldehyde, 2 % glutaraldehyde and 0.03% calcium chloride in 0.2 M cacodylic acid sodium salt trihydrate buffer, pH 7.3-7.5 (Karnovsky, 1965). After fixing, the samples were kept overnight at 4°C. The samples were then washed by placing them in cacodylic acid sodium salt trihydrate buffer for 10 min. The washing was repeated nine times and thereafter the tissues were post-fixed in 1% Osmium tetraoxide (OsO_4) for 1-2 h. The tissues were then rinsed eight times in cacodylic acid sodium salt trihydrate buffer (pH 7.35). For each rinsing, the tissues were left in the wash buffer for 15 min. Rinsed samples were dehydrated in increasing concentrations of ethanol as follows:

- Tissues placed in 15, 30 and 50% ethanol for 15 min each.
- Tissues transferred to 70% ethanol for 30 min and the process repeated one more time.
- Tissues transferred to 80% ethanol for 15min, followed by a wash in 90% ethanol for the same period of time.
- Tissues transferred to 100% ethanol for 30 min and the process repeated one more time.

The dehydrated samples were washed in 2 changes of propylene oxide of 10 min each and infiltrated with different ratios of agar low viscosity resin (Agar Scientific Ltd.) and propylene oxide. The agar low viscosity resin comprised of LV resin (48 g), VH1 hardener (16 g), VH2 hardener (36 g) and LV accelerator (2.5 g). The concentrations of agar low viscosity resin in relation to propylene oxide were: 1:3, 1:1, 3:1 and 1:0. For each concentration, the infiltration process lasted 22 h. The samples were subsequently polymerized in 100% agar low viscosity resin in flat embedding trays (Agar-Aids) at 60 °C for 24 h.

Sectioning and observing

Semi-thin sections: Semi thin sections (500 nm thick) were cut by using a 45° glass knife and directly suspended in distilled water. The sections were subsequently transferred onto a glass slide and dried on an electric plate at 70 °C. Dried leaf sections on the glass slides were stained in 0.5% toluidine blue (w/v) in 0.01 M phosphate buffer (pH 7.4). The sections were washed in tap water and in distilled water to removed excess stain and dried on an electric plate at 70 °C. The slides containing stained specimens were placed in xylene for 5 min, mounted and sealed in an entellan rapid mounting media (Merck), and then air-dried overnight in a fume chamber before being viewed under light microscope.

Ultra-thin sections and contrasting: If the desired fungal structures were found in the semi-thin sections, ultra-thin sections were continuously cut out of the same block. The ultra-thin sections were cut with a Reichert-Jung Ultramicrotome Ultracut E to a thickness of 70-72 nm using a diamond knife. Ultra-thin sections were placed on copper or nickel grids followed by the contrasting process (Geyer, 1973). The grids were laid out in drops of saturated 2% uranyl acetate for 8 min, rinsed twice in aqua bi-distilled water and then placed in drops of lead acetate solution (1.33 g $Pb(NO_3)_2$, 1.76 g $Na_3(C_6H_5O_7).2H_2O$ and 30 ml aqua bi-distilled water) for 2 min. Prior to using the lead acetate solution, the ingredients were thoroughly mixed and stored in darkness for 30 min. The pH was adjusted to 12 and then topped up to 50 ml with aqua bi-distilled water). The uranyl acetate and lead acetate were centrifuged before use. After use, the grids were rinsed in 2 changes of aqua bi-distilled water (Reynolds, 1963), air dried at room condition before being stored in a grid box.

The ultra-thin sections were observed with a Zeiss EM 109 transmission electron microscope (Carl Zeiss, Wetzlar, Germany) and images were photographed with a K-Frametransfer CCD camera for EM 109 and saved using the program images Sys Prog (Tröndle Restlichtverstarstärkersysteme, Germany).

3.2.6. Data analysis

All data were tested for normality and homogeneity of variance using Kolmogorov or Shapiro-Wilk tests prior to subjecting them to analysis of variance (ANOVA). Where significant differences occurred across treatments, the mean comparisons were performed by using Duncan's test or LSD at the 5% significant level. IRRISTAT statistical package (version 5.0, International Rice Research Institute) was used to analyze the data. Z-test two samples for the mean were used to analyze the size of hyphae in the tissue and on the leaf surface.

3.3. Results

3.3.1. Morphology of maize leaves

The examination of the maize leaf under the microscope revealed that the leaf was covered by epidermal layers (i.e. upper and lower). Both epidermal layers include the cuticle, stomata (Fig. 3.1 A). The epidermal cells were also observed to consist of other types of cells, i.e. long cells, short cells (silica and cork cells), and bulliform cells and trichomes. Macro hairs or macro trichomes, prickle hairs or prickle trichomes (single cells) and bi-cellular microhairs or bi-cellular microtrichomes (two-celled) were observed on the 6^{th} maize leaf (Fig. 3.1 B, C, D). The trichomes, however, were not uniformly distributed on the leaf area. The prickle trichomes and bi-cellular trichomes were more numerous than the macro trichomes. In total, there were 1900 ± 540 trichomes/cm^2 leaf area. Waxes were not observed on the trichomes (Fig. 3.2 A, B). In contrast with the 6^{th} leaf, the 4^{th} leaf had no trichomes. The structure of a stoma was an aperture and two guard cells (Fig. 3.1 A, 3.2 C). The stomata were arranged in parallel rows. The number of stomata varied from 4200 ± 520 in the 4^{th} leaf to 7200 ± 2000 in the 6^{th} leaf (cv. Tassilo). The substomatal cavity was a big space underneath the stoma (Fig. 3.1 A). Bulliform cells were often observed with limited deposition of waxes (Fig. 3.2 D). Between the epidermal layers were mesophyll cells and air spaces. Xylem, phloem and sclerenchyma cells were also observed (Fig. 3.1 A).

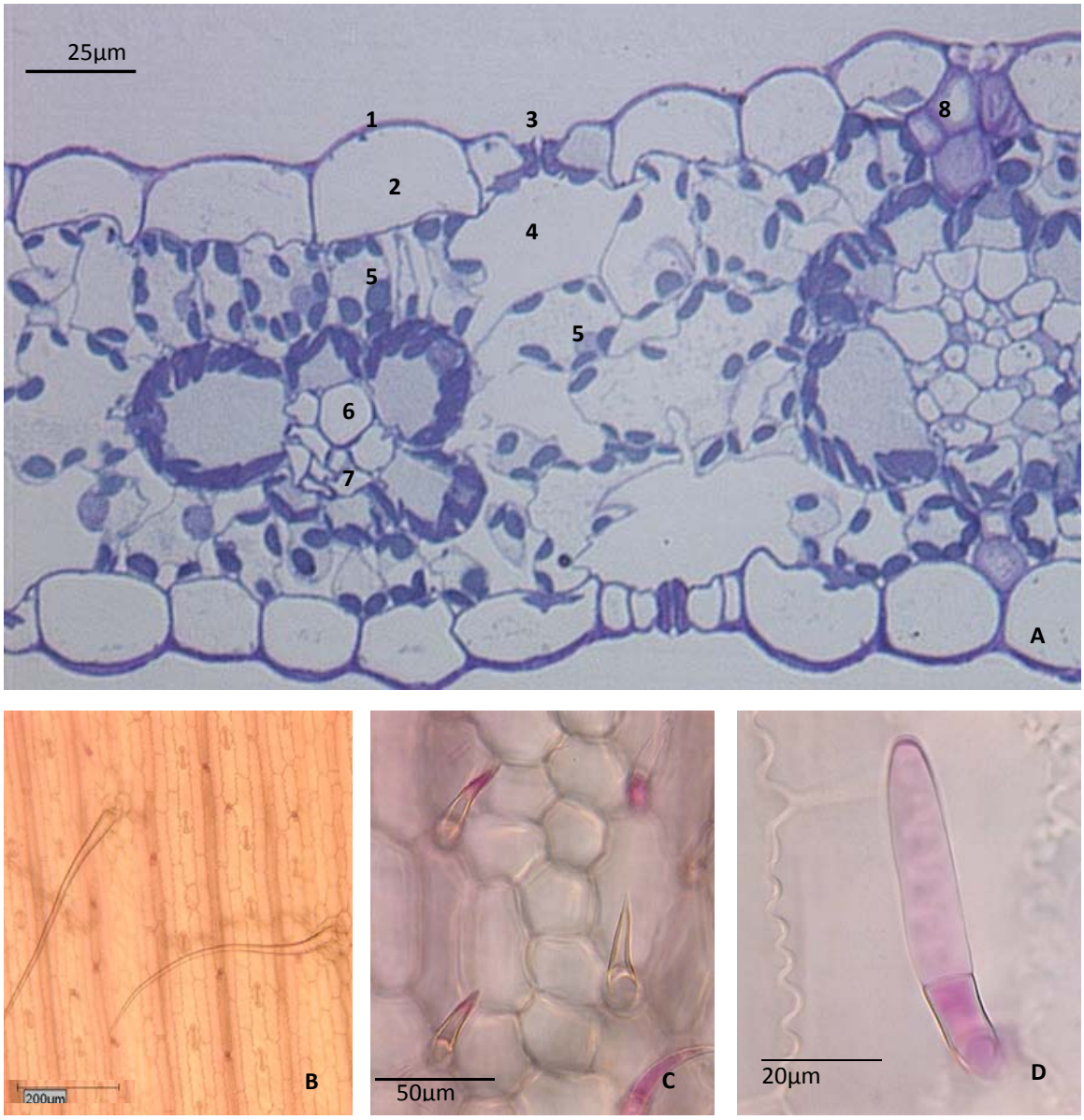


Figure 3.1. Vertical section of maize leaves and different kinds of trichomes on the 6th leaf. A= vertical section: (1) cuticle, (2) epidermal cell, (3) stoma, (4) substomal cavity, (5) mesophyll cells, (6) xylem, (7) phloem, (8) sclerenchyma cell, B= macro trichomes. C= prickle trichomes. D= bi-cellular microtrichomes. Light microscope photographs.

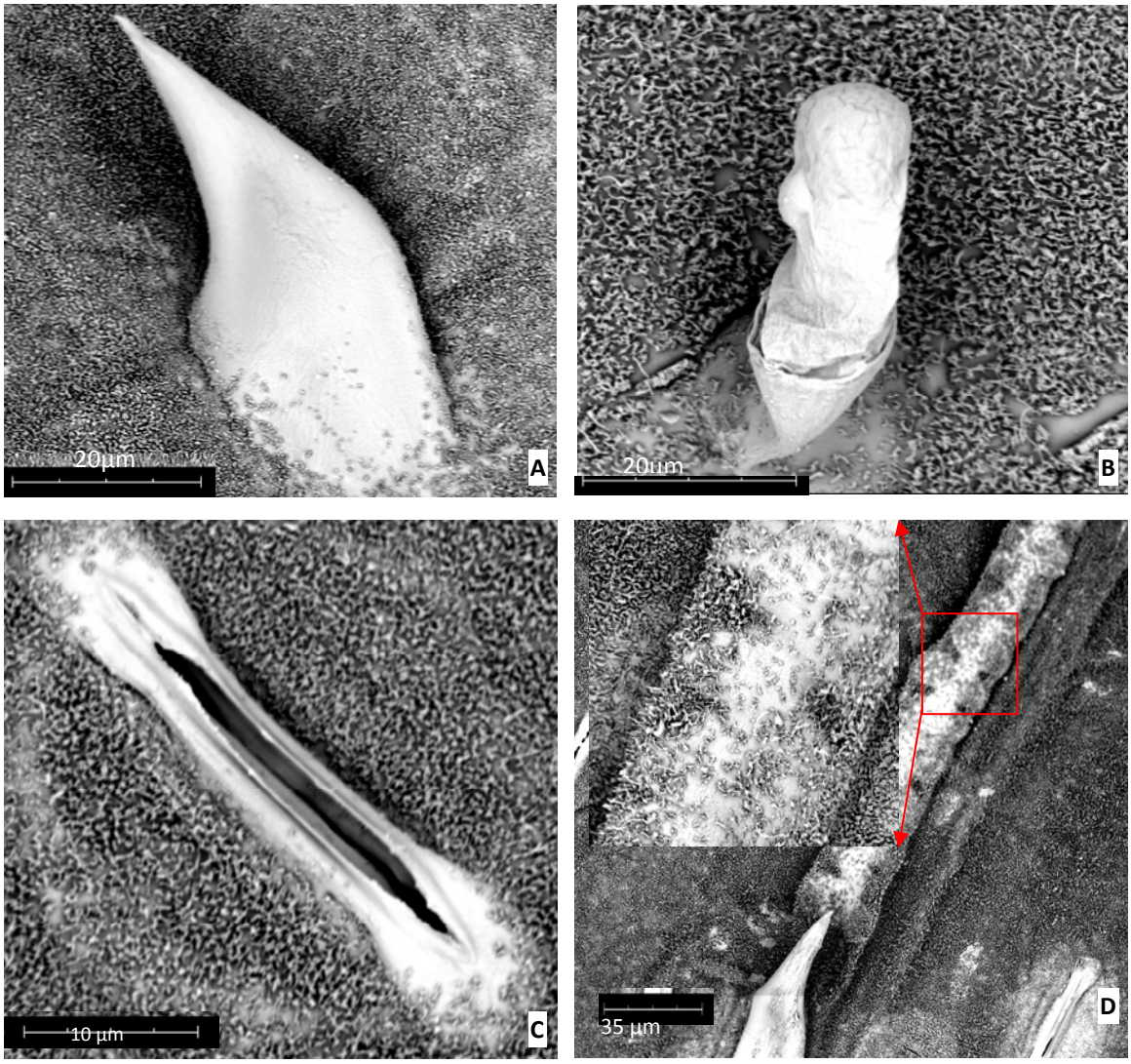


Figure 3.2. Scanning electron photograph of the surface of maize leaves. A= prickle trichomes with no waxes. B= bi-cellular microtrichomes with no waxes, C= stomatal aperture and around aperture with no wax. D= bulliform cell and close -up of bulliform cell (boxed region with less wax covering)

3.3.2. Conidial characteristics

3.3.2.1. Size and number of conidia

On low strength PDA, *F. graminearum* formed macroconidia while *F. proliferatum* and *F. verticillioides* produced abundant microconidia. Whereas most macroconidia had 5-6 cells, the microconidia were single-celled, i.e. not septate. The macroconidia were 4-5 times longer than the microconidia and about 1.5 time wider (Table 3.1).

3.3.2.2. Germination and germ tube formation

Eight hours after plating the conidial suspension on diagnostic slides, the rate of germination was faster for *F. graminearum* (8.3%) followed by *F. verticillioides* (4.7 %) and *F. proliferatum* (4.2 %). However, germination of *F. proliferatum* and *F. verticillioides* increased and exceeded that of *F. graminearum* 10, 12 and 24 hai. For example, 24 hai, the germination rate of *F. proliferatum* and *F. verticillioides* reached 66 and 63%, respectively compared to only a 45% for *F. graminearum*. Within the 24 h period following inoculation, germinated conidia were noted to produce one or more germ tubes; however, the number of germ tubes depended on the fungal species. Approximately 77% germinated conidia of *F. graminearum* and *F. proliferatum* formed one germ tube verses 44% for *F. verticillioides* (Table 3.2).

3.3.3. Conidial characteristics of *Fusarium* species on maize leaves

Conidial germination was also observed on the surface of attached maize leaves. At the 8^{th} hai, no conidia had germinated. At 24 hai, the germination rate of fungal conidia was low (< 50%) on the leaf surface. Amongst the germinated conidia, variability in the number of germ tubes was noted. The number of germ tubes ranged between 1 and more than three depending on the species. For example, >90 % of *F. proliferatum* and *F. verticillioides* conidia formed one germ tube compared to a < 50% observed in *F. graminearum.* The remaining germinated *F. graminearum* conidia had variable numbers of germ tubes, i.e. two germ tubes (36.3%), three germ tubes (19%) and between four and six germ tubes (Table 3.3). Similarly, the lengths of the germ tubes were significantly different among species. The germ tubes of *F. graminearum* conidia were significantly longer than that of *F. proliferatum* and *F. verticillioides* (Table 3.3).

Table 3.1. Size and number of cells per conidium of *Fusarium* species at 3 days after inoculation on LSPDA.

Fusarium species	Length (µm)	Width (µm)	Number of cells/conidium
F. graminearum	45.90 ± 0.54 [(1)]	4.63 ± 0.03	4 – 7 (5, 6)
F. proliferatum	9.56 ± 0.22	3.04 ± 0.07	1 – 2 (1)
F. verticillioides	10.30 ± 0.27	3.23 ± 0.05	1 – 2 (1)

[(1)] Standard error of mean (n=120). The numbers presented in brackets represent the most frequently observed number of cells/conidium.

Table 3.2. Germination rate of *Fusarium* spp. conidia and variability in germ tube formation on a glass surface.

F. spp.[(2)]	Incubation time (hours)				% germinated conidia with germ tubes (GT)	
	8	10	12	24	One GT	> one GT
Fg	8.3 ± 1.1[(1)]	22.4 ± 3.7	34.5 ± 4.4	45.0 ± 2.1	76.6 ± 2.0	20.9 ± 1.6
Fp	4.2 ± 1.4	27.7 ± 4.6	39.4 ± 5.5	66.6 ± 2.4	77.1 ± 4.4	22.9 ± 4.4
Fv	4.7 ± 2.2	18.5 ± 3.8	35.5 ± 7.5	63.0 ± 2.6	44.2 ± 4.0	55.8 ± 4.0

[(1)]: Standard error of mean (n=120).
[(2):] Fg, Fp and Fv stands for the fungal isolates *Fusarium graminearum* isolate AG23d, *F. proliferatum* isolate AG31g and *F. verticillioides* isolate AG11i, respectively.

Table 3.3. Variability in the rate of germination, number of germ tubes and length of germ tubes exhibited by different *Fusarium* species 24 hours after inoculation on the leaf surface of maize.

F. spp.[(2)]	Germination rate (%)	Germ tube (GT) formation by germinated conidia (%)				Length of germ tube (µm)
		One GT	Two GT	Three GT	> three GT	
Fg	41.4	43.0	36.3	19.0	1.7	58.3 ± 5.9 [(1)] a
Fp	35.2	99.0	1.0	0		14.5 ± 0.8 b
Fv	39.8	97.8	2.2	0		12.1 ± 0.6 b

[(1)]: Standard error of mean (n=120).
[(2):] Fg, Fp and Fv stands for the fungal isolates *Fusarium graminearum* isolate AG23d, *F. proliferatum* isolate AG31g and *F. verticillioides* isolate AG11i, respectively. SE= standard error of mean. Mean values followed by the same letters are not significantly different at P<0.05.

3.3.4. Infection process on maize leaves

The mature 4th leaf, was symptomless whereas the immature 6th, and 7th leaves, exhibited clear symptoms of infection. In addition, the detached leaves were also infected by *F. graminearum*.

3.3.4.1. Infection of maize leaves by *Fusarium graminearum* and fungal sporulation

3.3.4.1.1. Germination of macroconidia and mycelia growth

The macroconidia of *F. graminearum* started germ tube formation at 12 hai (Fig. 3.3 A). By the 24th hai, one or two germ tubes originated from the tips or middle cells of the macroconidia (Fig. 3.3 B) and started branching (Fig. 3.3 C). From the 48th hai, three to six germ tubes were observed, most often on macroconidia that had five or more cells (Fig. 3.3 D, E, F). Anastomosis between two macroconidia or amongst three macroconidia was observed 72 hai (Fig. 3.4 A, C). After germination, the hyphae elongated very fast and formed dense mycelia on the leaf surface (Fig. 3.4 B, D).

3.3.4.1.2. Infection of asymptomatic mature leaves

Penetration and colonization of epidermal cells

F. graminearum was observed to form appressoria- like structures, i.e. the hyphal tips became enlarged and rounded off (Fig. 3.5 B, C) from the 3rd dai. However, some hyphae penetrated the cuticle directly without appressoria formation. Penetration points were usually detected at the groove where a cell was in contact with an adjacent cell wall and at the cell corners (Fig. 3.5 B). Near the penetration point, subcuticular hyphae (Fig. 3.5 C) and the invasion of epidermal cells was observed at 7 dai. The hyphae grew along epidermal cells or grew in a fascicle of parallel hyphae (Fig. 3.5 D, E). Some epidermal cells were seen to be filled with the hyphae (Fig. 3.5F). Penetration of fungal hyphae through stomatal aperture although less frequent, was observed in the 4th leaf. Infected epidermal cells exhibited brown cell walls (Fig. 3.5 A).

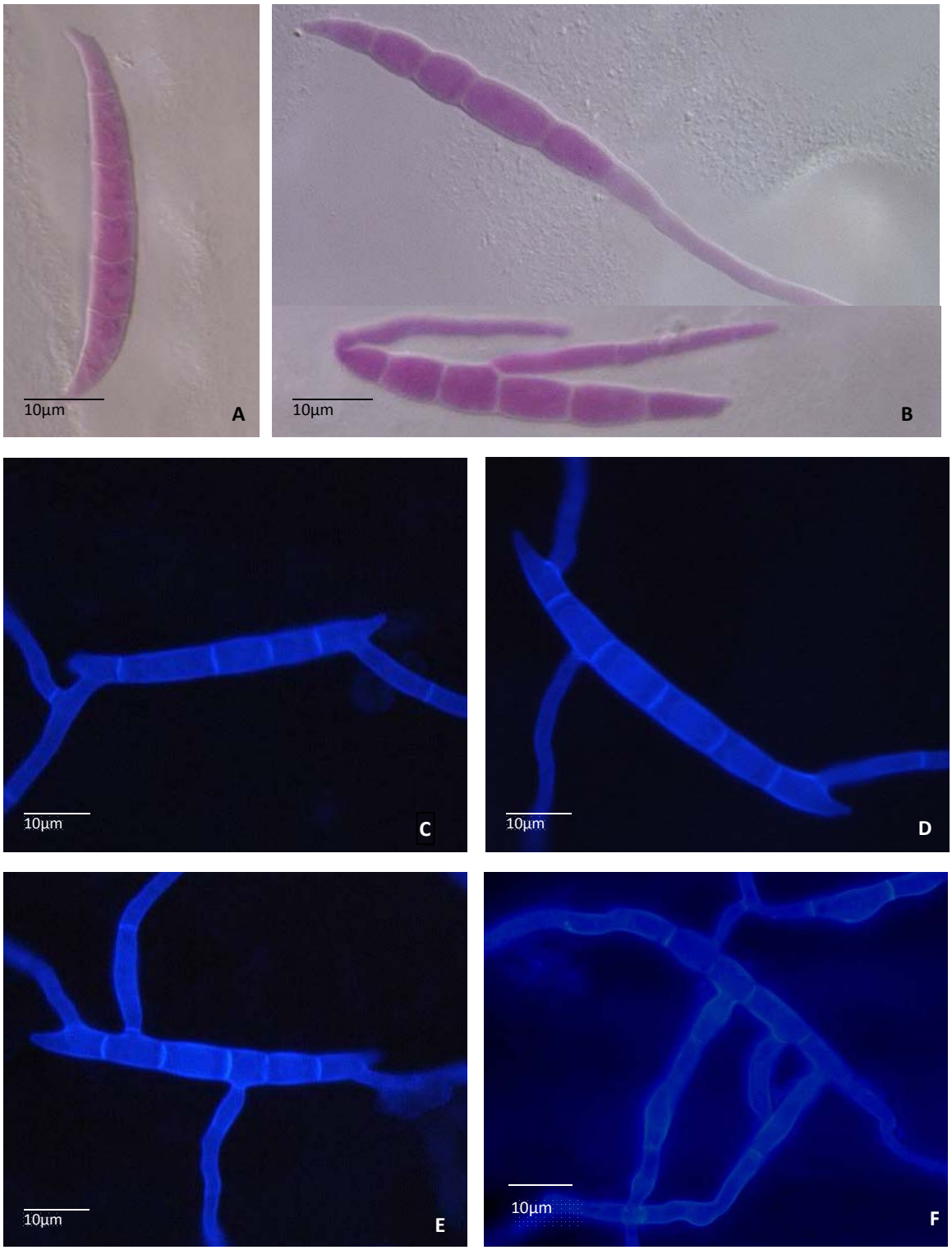


Figure 3.3. Conidia of *Fusarium graminearum* on the maize leaves. A= macroconidia, eight hours after inoculation (hai). B=macroconidia germinating with one, two germ tubes, 24 hai. C= macroconidia germinating with two germ tubes and branching. D, E and F= macroconidia germinating with three, four and six germ tubes at 48 hai, respectively. Light microscope photographs.

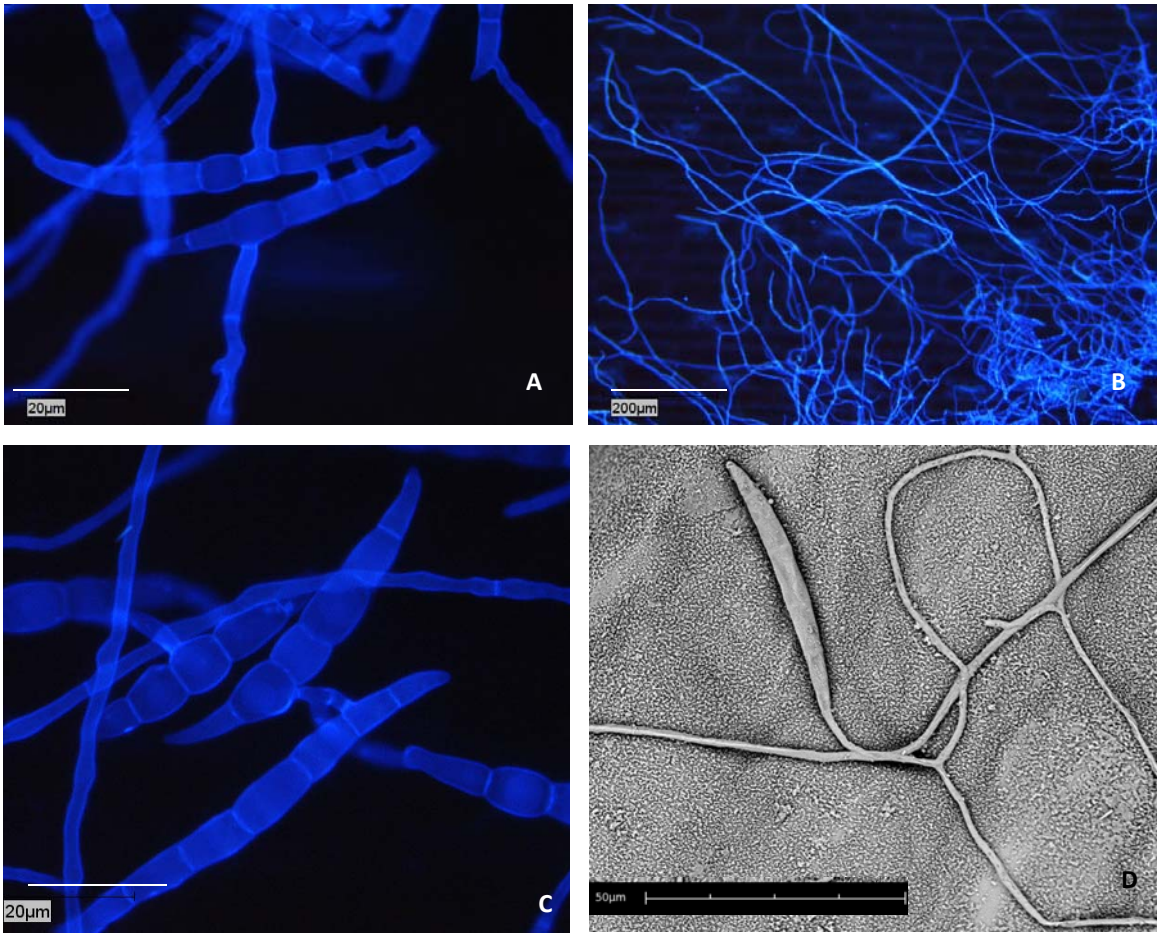


Figure 3.4. *Fusarium graminearum* on the maize leaves, 72 hours after inoculation: A, C= fusion (anastomosis) of two and three macroconidia. B= dense mycelia on leaf surface. D= hypha growing from macroconidium. A-C: Light microscope photographs, D: Scanning electron microscope photograph.

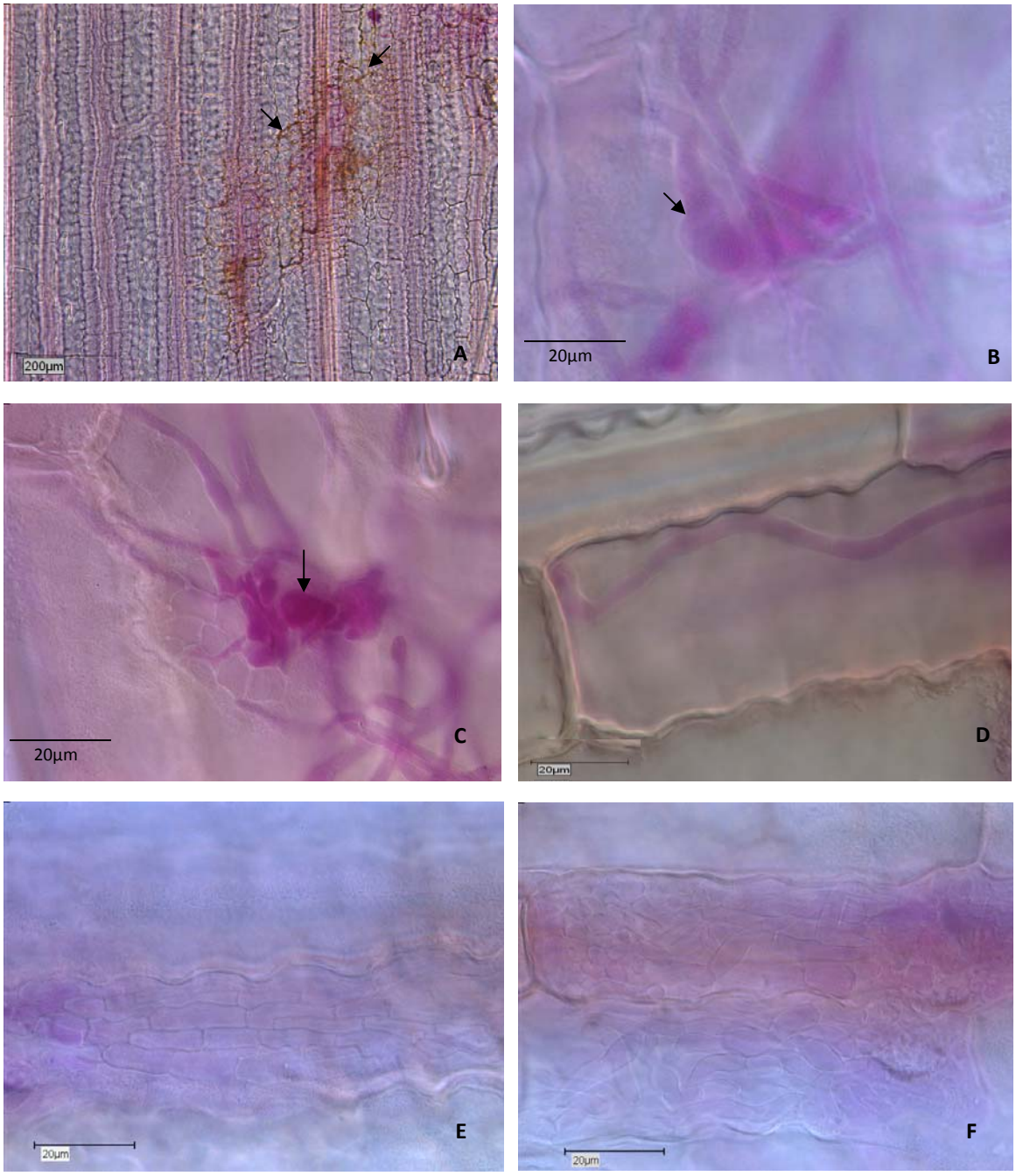


Figure 3.5. Infection of asymptomatic maize leaves (the 4th leaf) by *Fusarium graminearum*. A= infected cells (arrow). B, C = swelling at a hyphal tip occurring over infected sites (arrow), 3dai. D= a hypha within epidermal cell and swelling at a hyphal tip forming at crossing wall. E= hyphae growing in fascicle of parallel hyphae, 7dai. F= dense mycelia growing in epidermal cells, 7dai. Light microscope photographs.

3.3.4.1.3. Infection of immature leaves with symptoms

Subcuticular infection

Different growth patterns were observed (Fig. 3.6). In the first growth pattern, *F. graminearum* hyphae were observed to grow as a hand-shaped subcuticular structure upon bulliform cells (Fig. 3.6 A). The hyphae originated from the groove between prickle trichome and adjacent cells, and grew in a radial pattern. Another infection pattern was observed in which the hyphae grew along the cell walls of the bulliform cells. The hyphae were septate and had many short cells (Fig. 3.6 B). *F. graminearum* hyphae also colonized the short cells of the epidermis before spreading to adjacent cells (Fig. 3.6 C). Subcuticular hyphae continued to grow and colonize adjacent cells (Fig. 3.6 D). Fungal hyphae also invaded the cuticle through the corner of cell walls and then spread along the cell walls (Fig. 3.6 E, black arrow). Parallel subcuticular growth of *F. graminearum* hyphae on silica and cock cells was also observed (Fig. 3.6 E, F).

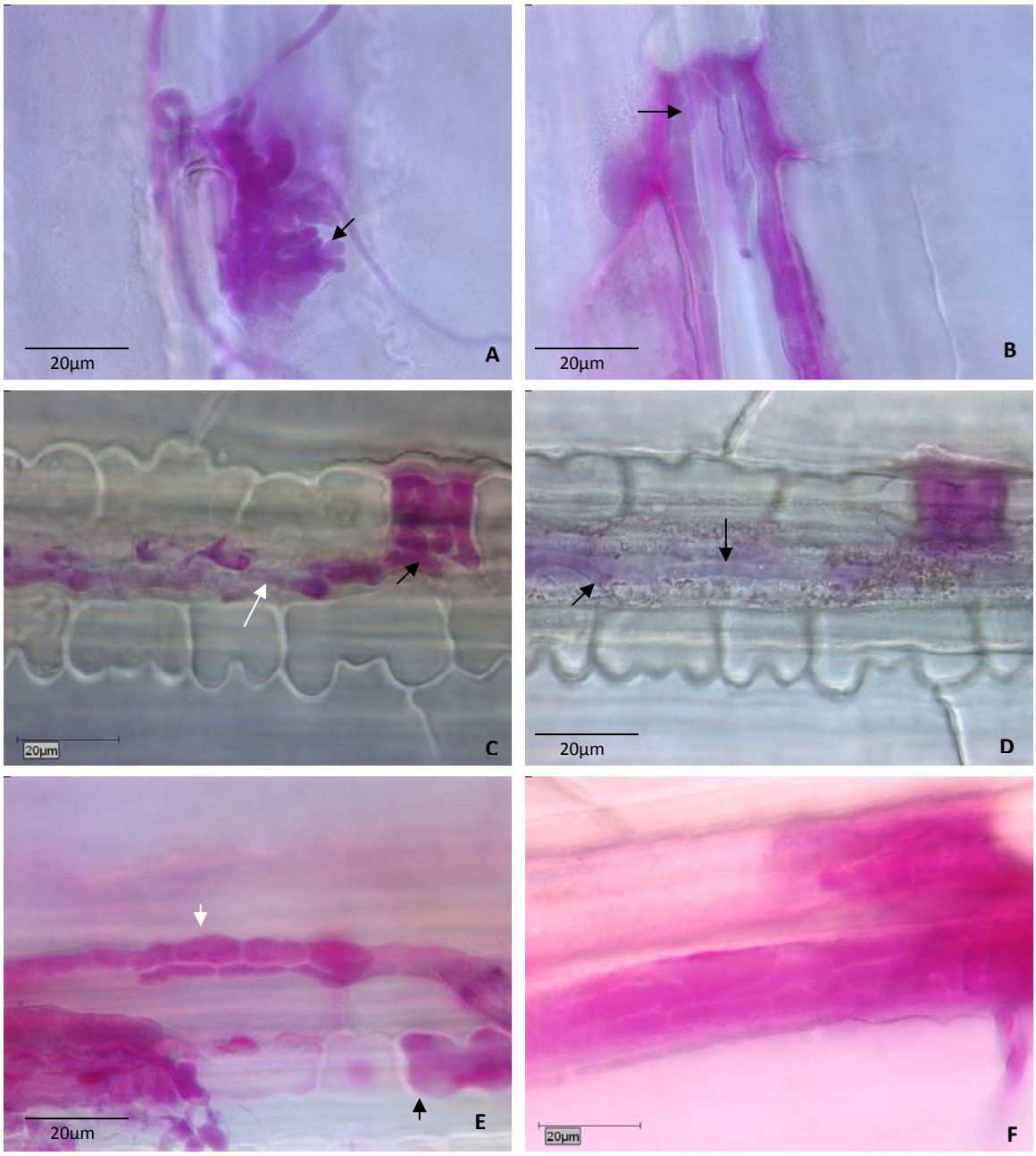


Figure 3.6. Subcuticular hyphal infection by *Fusarium graminearum* in maize leaves. A= hyphae growing as a hand-shaped subcuticular structure on bulliform cell. B= subcuticular hyphae growing along cell wall of bulliform cell. C= hyphae invading subcuticle of short epidermal cell (black arrow) and spreading into adjacent cell (white arrow). D= subcuticular hyphae in (C) penetrating into bulliform cell (arrow). E= hyphae invading subcuticle of short epidermal cell (black arrow) and apart of bulliform cell (white arrow). F= Hyphae growing parallel and dense in subcuticle of bulliform cell. Light microscope photographs.

Penetration and colonization of trichomes

The surface of the leaves of the 6th leaf had specialized epidermal trichomes (hairs). *F. graminearum* penetrated all three kinds of trichomes and the penetration occurred frequently with bi-cellular trichomes (Fig. 3.7 and 3.8). At first, the hyphae were observed to come in contact with the trichomes by adhering and growing along the trichomes or fastening around the trichomes (Fig. 3.7 B, C). After that, the hyphae penetrated into the trichomes either at the side or top of the cap cells (Fig. 3.7 D, E). After penetration, the hyphae grew inside the cap cells and attempted to penetrate through the wall between the cap and base cells (Fig. 3.7 F) and successfully colonized the base cell of the trichomes (Fig. 3.8 B). Hyphae that failed to penetrate the base cell appeared as hyphal outgrowths (Fig. 3.8 A). Penetration of the bi-cellular trichomes was observed to start as early as 48 hai.

Prickle trichomes were penetrated and colonized by *F. graminearum* 72 hai. The hyphae wrapped around prickle trichomes (Fig. 3.8 C) and formed a mass of hyphae around the top of prickle trichomes (Fig. 3.8.D). Another strategy that *F. graminearum* used to successfully penetrate prickle trichomes was that the germ tube or hyphae swelled at the contact point with prickle trichome tip (Data not show).

F. graminearum successfully penetrated and colonized macro trichomes and the hyphae wrapped around the base, middle or top of the trichomes (data not show). In some cases, the penetration point has not recognizable but the hyphae seemed to penetrate from the base of macro trichome (Fig. 3.8 E and F).

Following penetration, full growth and colonization of the trichomes, the fungus spread to other cells including adjacent epidermal cells. At this point, the hyphae enlarged considerably (Fig. 3.9).

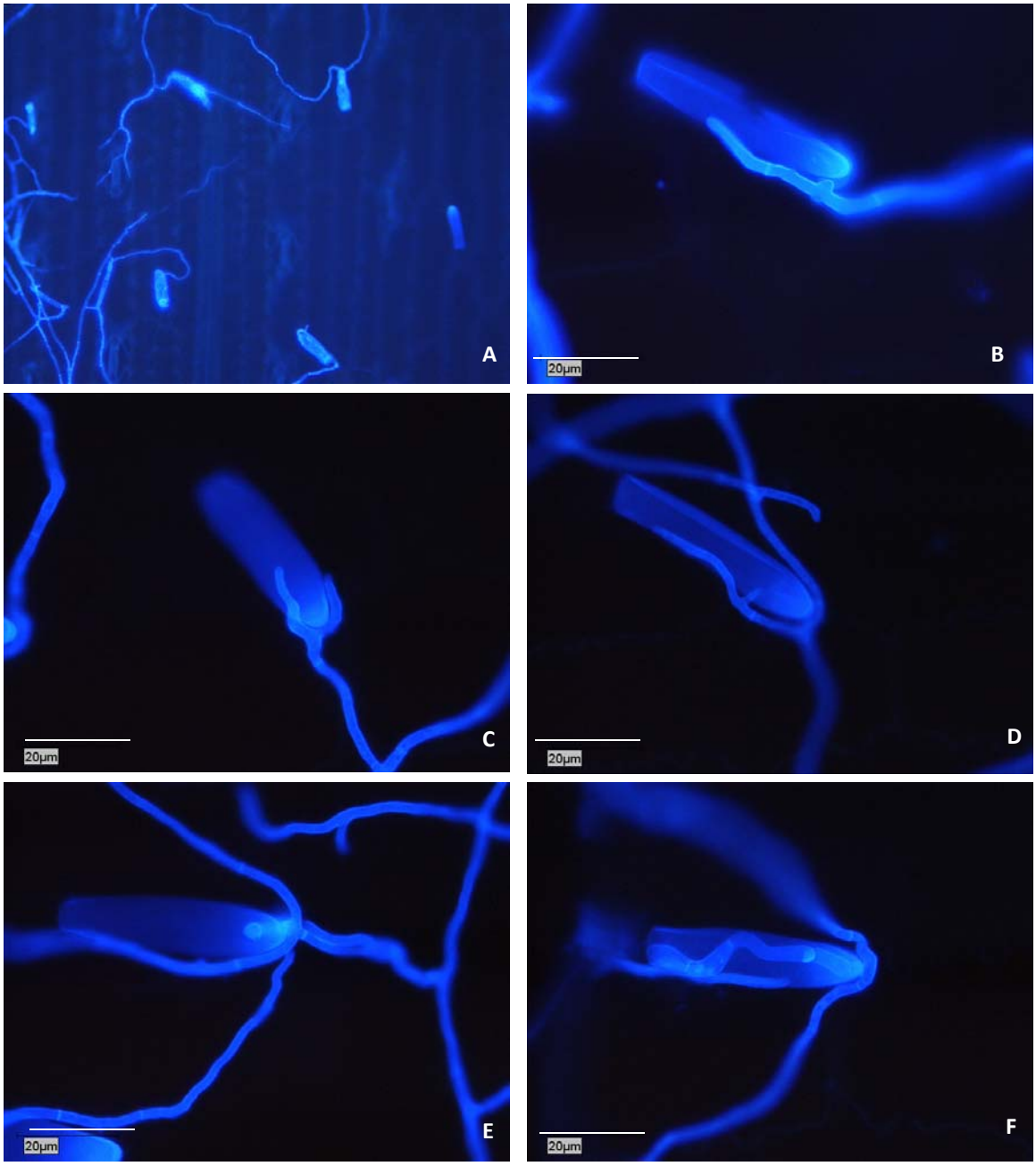


Figure 3.7. *Fusarium graminearum* infection into bi-cellular trichome on maize leaves, 48 hai. A= general view of infected trichomes. B= hypha in contact with bi-cellular trichome and forming infection hypha. C= hyphae clamping bi-cellular trichome. D= hyphae clamping bi-cellular trichome and infection hypha penetrating into cell at a site of trichome. E= hyphae clamping bi-cellular trichome and infection hypha penetrating into cell at the top of trichome. F= hypha penetrating successfully into bi-cellular trichome. Light microscope photographs.

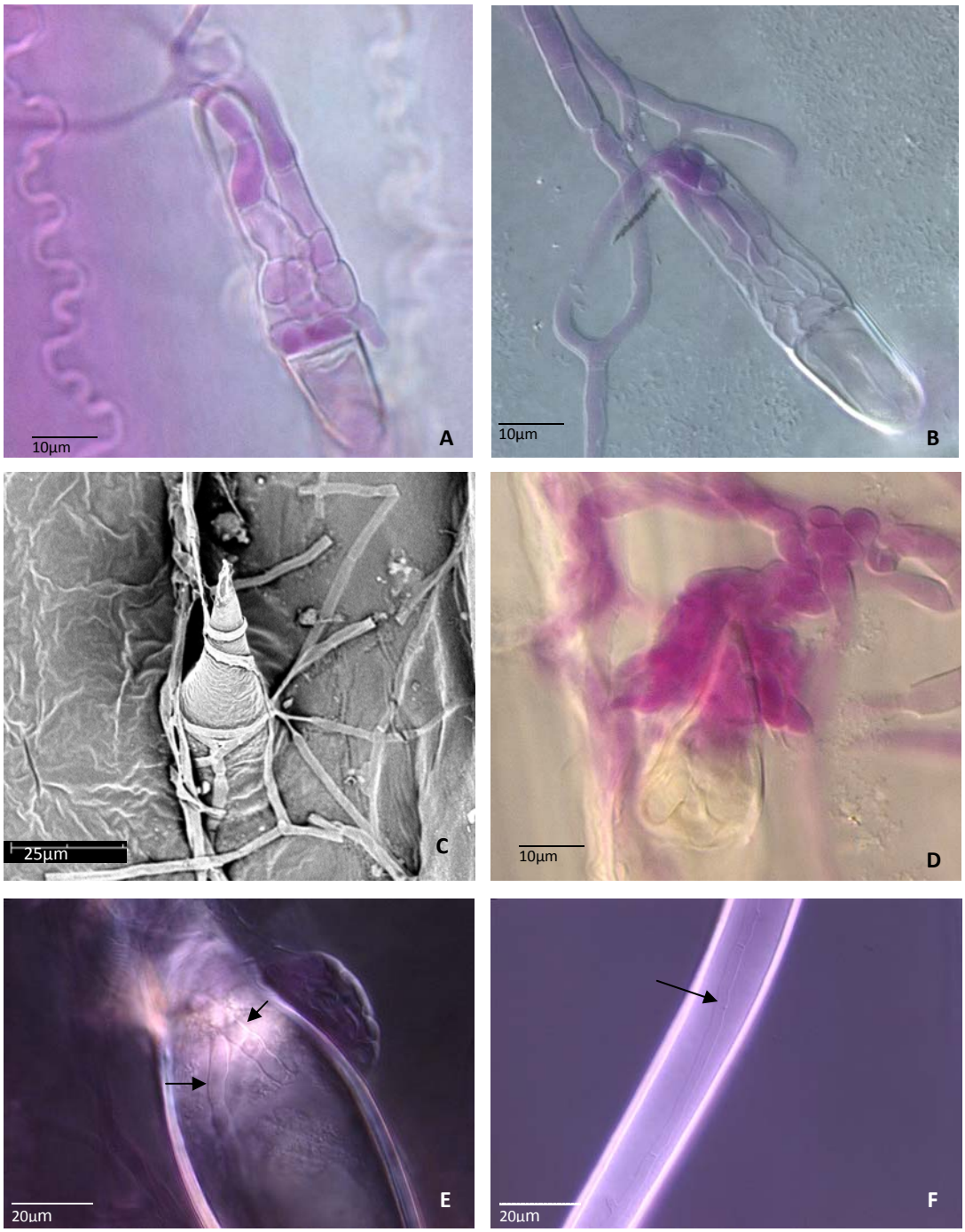


Figure 3.8. *Fusarium graminearum* infection of different types of trichomes of maize leaves. A= hyphae growing in bi-cellular trichome and swelling at cross cell wall between cap cell and base cell. B= hyphae growing in bi-cellular trichome and swelling at cross cell wall between cap cell and base cell and penetrating successfully the base cell, 72 hai. C= hyphae wrapping prickle trichome, 72 hai. D= many hyphae wrapping and penetrating prickle trichome, 7 dai. E= hyphae invading a base part of macro trichome. F= hyphae growing in top part of macro trichome, 7 dai. A, B, D, E, E: Light microscope photographs, C: Scanning electron microscope photograph.

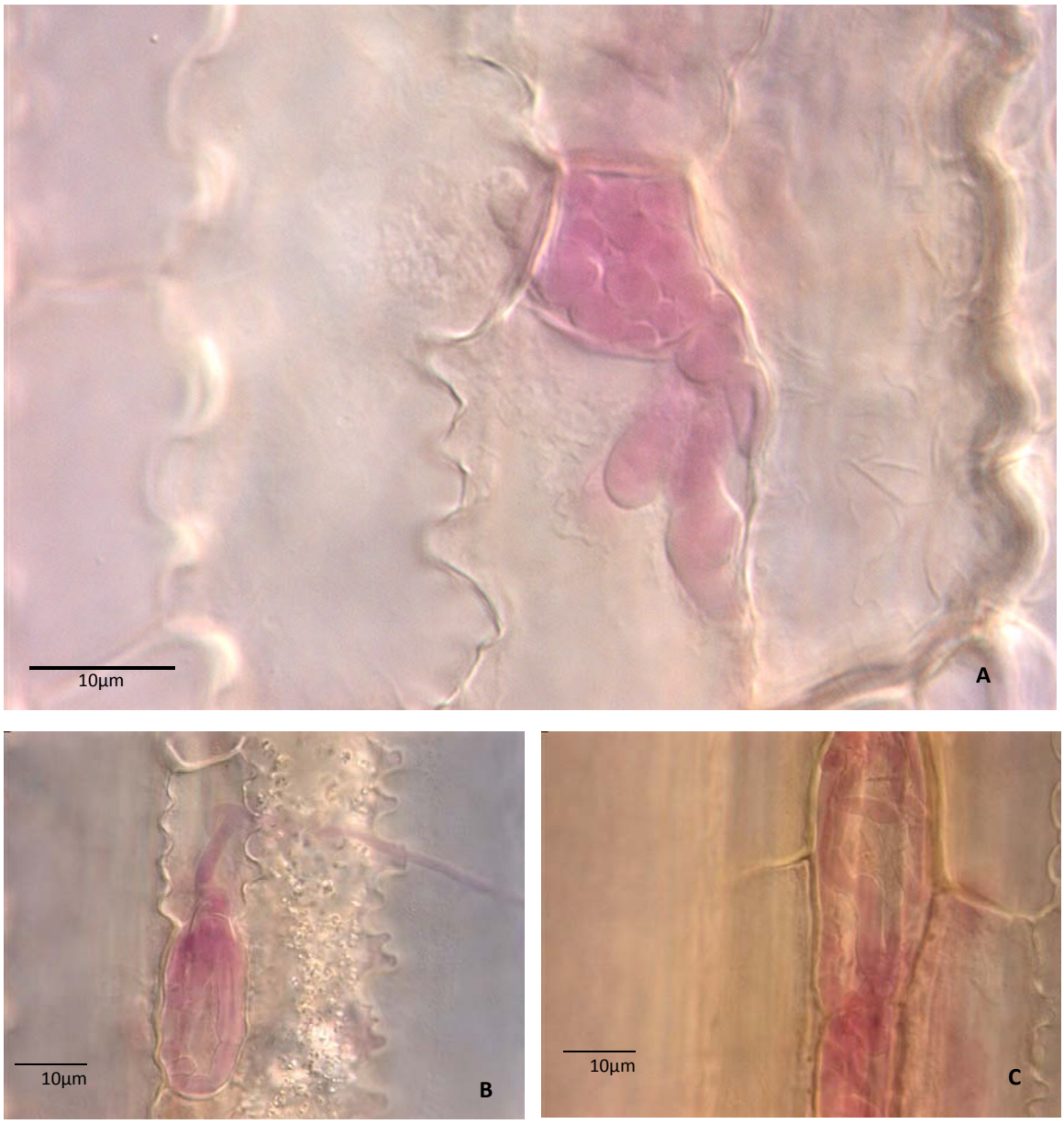


Figure.3.9. Hyphae of *Fusarium graminearum* spreading out. A= hyphae in base cell of infected bi-cellular trichome spreading out adjacent epidermal cell. B= hyphae growing in prickle trichome. D= hyphae in prickle trichome spreading out to adjacent cell. Light microscope photographs.

Infection through stomata and colonization of maize leaves

Different modes were employed for successful penetration into maize leaves via the stomata. The modes were: i) formation of appressorium like structures that originated from a single hypha upon coming in contact with the stomatal aperture (Fig. 3.10 A); ii) adherence of fungal hypha over the stomatal aperture (Fig. 3.10 C); and iii) formation of

a hyphal cushion on the stomata surface (Fig. 3.10 E). The process of hyphae adhering and penetrating stomata was observed 72 hai on the maize leaves. For all penetration techniques, an imprint circle underneath the appressorium was observed (Fig. 3.10 B, D). After penetration through the stomatal aperture, the hyphae colonized the substomatal cavity, i.e. the biggest airspace in maize leaf tissue (Fig. 3.10 F).

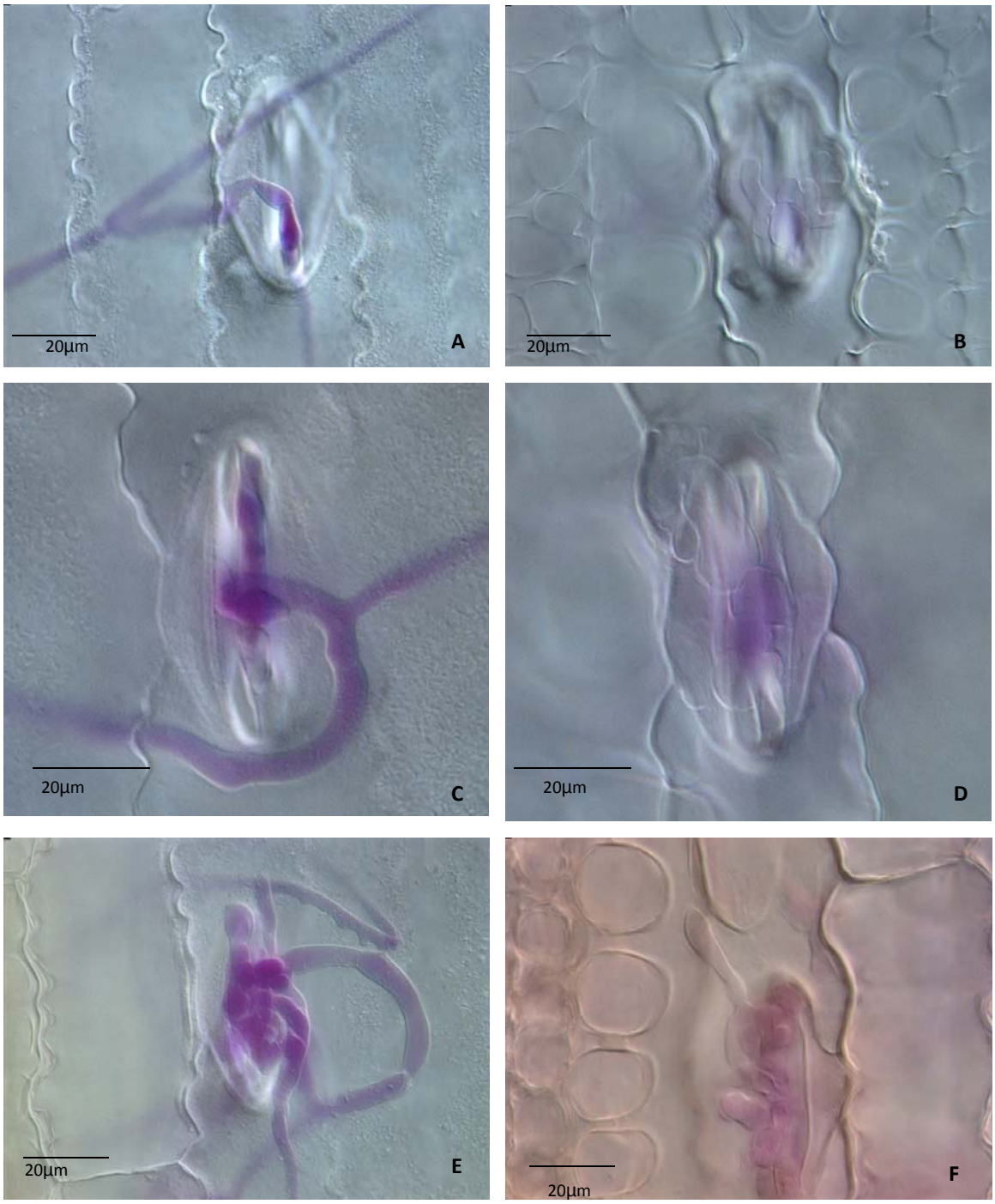


Figure 3.10. Infection of *Fusarium graminearum* through stomata of immature maize leaves, 3 dai. A= swelling at a hyphal tip occurring at stomatal aperture. B= imprint circle below swelling hyphal tip shown in A. C= swelling hypha adhering stomatal aperture. D= imprint of hyphae below adhering hypha shown in C. E= some hyphae attacking one stoma. F= successful infection hyphae in sub-stomata cavity and spreading out. Light microscope photographs.

3.3.4.1.4. Infection of detached leaves

F. graminearum penetrated the leaf cells of detached leaves through trichomes, stomata and epidermal cells and the penetration process was similar for both attached and detached leaves. Hyphae were observed to grow on the leaf surface (Fig. 3.11 A), penetrate through the epidermis (Fig. 3.11 C) and invade the epidermal cells (Fig. 3.11 B). The hyphae then formed foot-like structures and penetrated through the cell walls (Fig. 3.11 E, F). Thereafter the hyphae colonized inter- and intra-cellular (Fig. 3.12 A) mesophyll cells, invaded sclerenchyma cells (Fig. 3.12 C), xylem and the phloem vessels (Fig. 3.12 B, D). The penetration of leaf cells through stomata was not different between attached and detached leaves, but the infection frequency was different. It was observed that the stomatal infection of the detached 4^{th} leaf occurred more frequent and earlier (3 dai) than those on the attached 4^{th} leaf (7 dai).

Re-emergence of hyphae was seen on the leaf surface as hyphal outgrowths through the stomata (Fig. 3.12 F). Additionally, hyphal re-emergence from short epidermal cells was detected (Fig. 3.12 E). This phenomenon was also seen in attached leaves but they were observed later.

3.3.4.1.5. Sporulation

Sporulation occurred from both superficial hyphae, i.e. mycelia growing from applied inoculum and from re-emerging hyphae, i.e. secondary infection hyphae.

Superficial hyphae were observed to grow and branch profusely on the maize leaf surface. Whereas sporulation was detected 48 hai from superficial hyphae, spores produced from re-emerging hyphae were observed 15 dai. Conidiophores were either single or fascicled and bore single macroconidia (Fig. 3.13 A-C). Interestingly, newly formed spore from spores was observed. Macroconidia were either lacking or only occasionally observed from very short single conidiophore (Fig. 3.13 D, E). All the conidia produced from secondary infection and superficial hyphae were similar in morphology (Fig. 3.13 F).

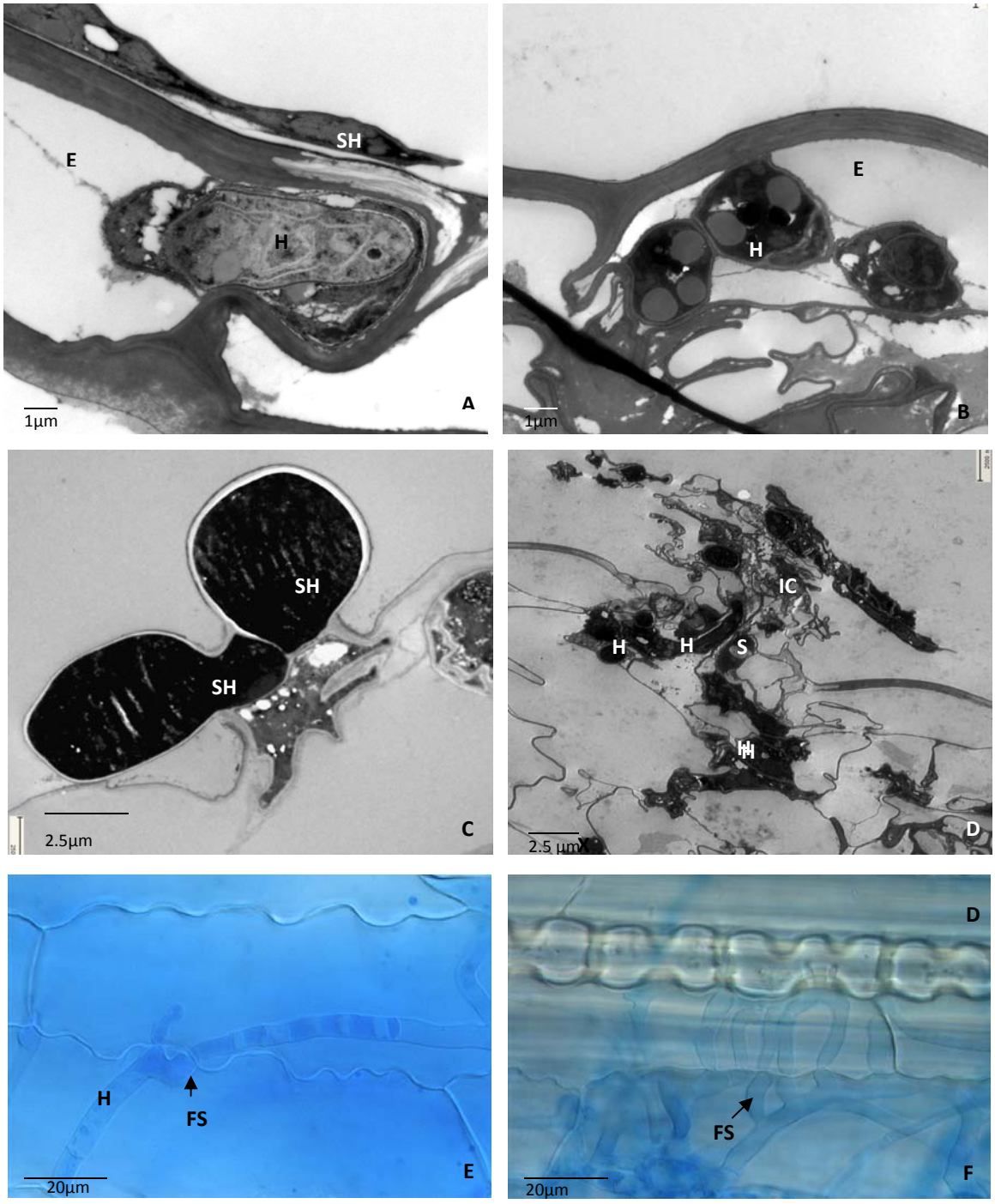


Figure 3.11. Infection of detached maize leaves cv. Tassilo by *Fusarium graminearum.* A= hyphae colonizing epidermal cell and hyphae on the leaf surface. B= hyphae growing densely in epidermal cell. C=hyphae degrading host cell wall (arrow). D= infected cushion forming over stomatal aperture and penetrating via stoma and colonizing substomata cavity. D, E= hyphae forming food – structure and passing through cell wall and colonizing cells. Abbreviation: **H** Hypha, **SH** superficial hyphae, **IC** Infection cushion, **FS** foot structure, **E** epidermic, **S** stoma. A-D: TEM photographs, E, F: Light microscope photographs.

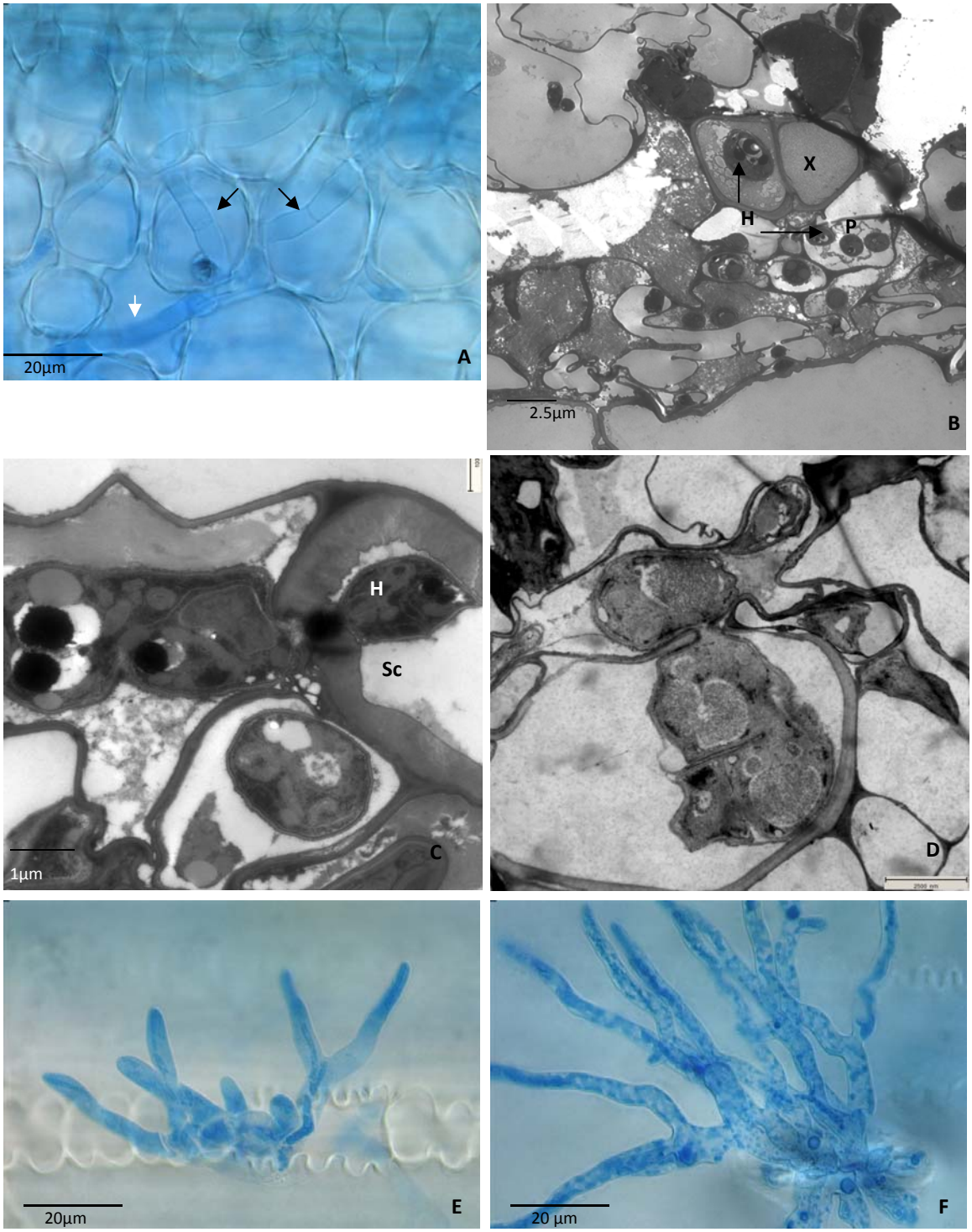


Figure 3.12. Infection of detached maize leaves by *Fusarium graminearum*. A= intercellular (white arrow) and intracellular hyphae in mesophyll (black arrow). B= hyphae colonizing xylem and phloem. C= hyphae invading sclerenchyma cell. D= hyphae penetrating into xylem. E= hyphae re-emerging through short cell. F= hyphae re-emerging via stoma. Abbreviation; **H** hyphae, **X** xylem, **P** phloem, **Sc** sclerenchyma cell. B, C, D: TEM photographs, A, E, F: Light microscope photographs.

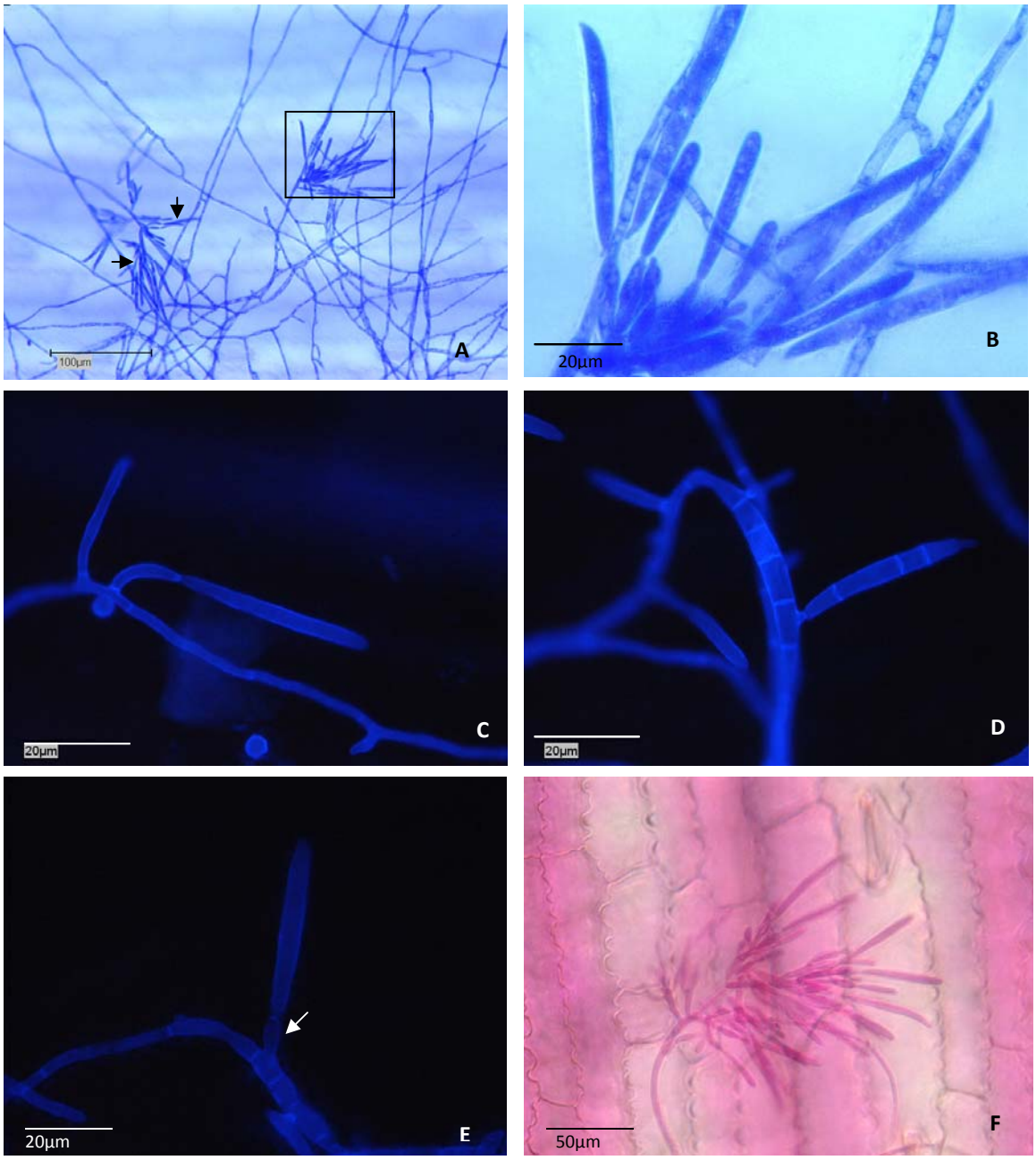


Figure 3.13. Sporulation of *Fusarium graminearum* on the maize leaf surface. A= macroconidiophores producing from hyphae on the leaf surface, 48 hai. B= Close - up box area in A, macroconidiophores. C= macroconidiaphore (monophialides) forming from hyphae on the leaf surface, 48 hai. D= spore sporulation from maccroconidia, 48 hai. E= conidiophore forming from macroconidia, 48 hai. F= macroconidiophores producing from re- emergence hyphae, 15 dai. Light microscope photographs.

3.3.4.2. Infection of maize leaves by *Fusarium proliferatum* and fungal sporulation

3.3.4.2.1. Germination of microconidia and mycelia growth

Microconidia of *F. proliferatum*, were usually single-celled, club shaped with a flattened base, and did not germinated 8 hai (Fig. 3.14 A). At 12 hai, some conidia formed initial germ tubes and 24 hai 35% of the conidia germinated with mostly one germ tube. A dense mycelial network was formed (figure 3.14 B).

3.3.4.2.2. Infection of asymptomatic mature leaves

Subcuticular infection was found, but penetration points were not clearly identifiable. Subcuticular hyphae were observed to grow in a coral shape starting at the corner of cell (Fig. 3.14 D, E) that spread to mesophyll cells (Fig. 3.14 F). *F. proliferatum* infection of the 4th leaf caused no disease symptoms, but infected cells were detected due to brown cell walls discoloration 7 dai (Fig. 3.14 C).

3.3.4.2.3. Infection of immature leaves with symptoms

Penetration and colonization of trichomes

Infection of leaf trichomes by *F. proliferatum* was observed. The most infected type was the bi-cellular trichome and the process of infection also occurred early 48 hai (Fig. 3.15). Germ tube or hyphae penetrated trichomes at the base or the tip of the cap cells of bi-cellular trichomes (Fig. 3.15 A, B). After infection, *F. proliferatum* spread from the cap cell to the base cell was limited since the hyphae failed to penetrate through the cell wall (Fig. 3.15 D). Penetration into prickle trichomes by *F. proliferatum* was also detected (Fig. 3.15 E) and occurred 7 dai through the top of the trichomes. Whereas *F. proliferatum* hyphae were observed to wrap around the base, middle or top the macro trichomes (Fig. 3.15 F), no successful penetration was observed.

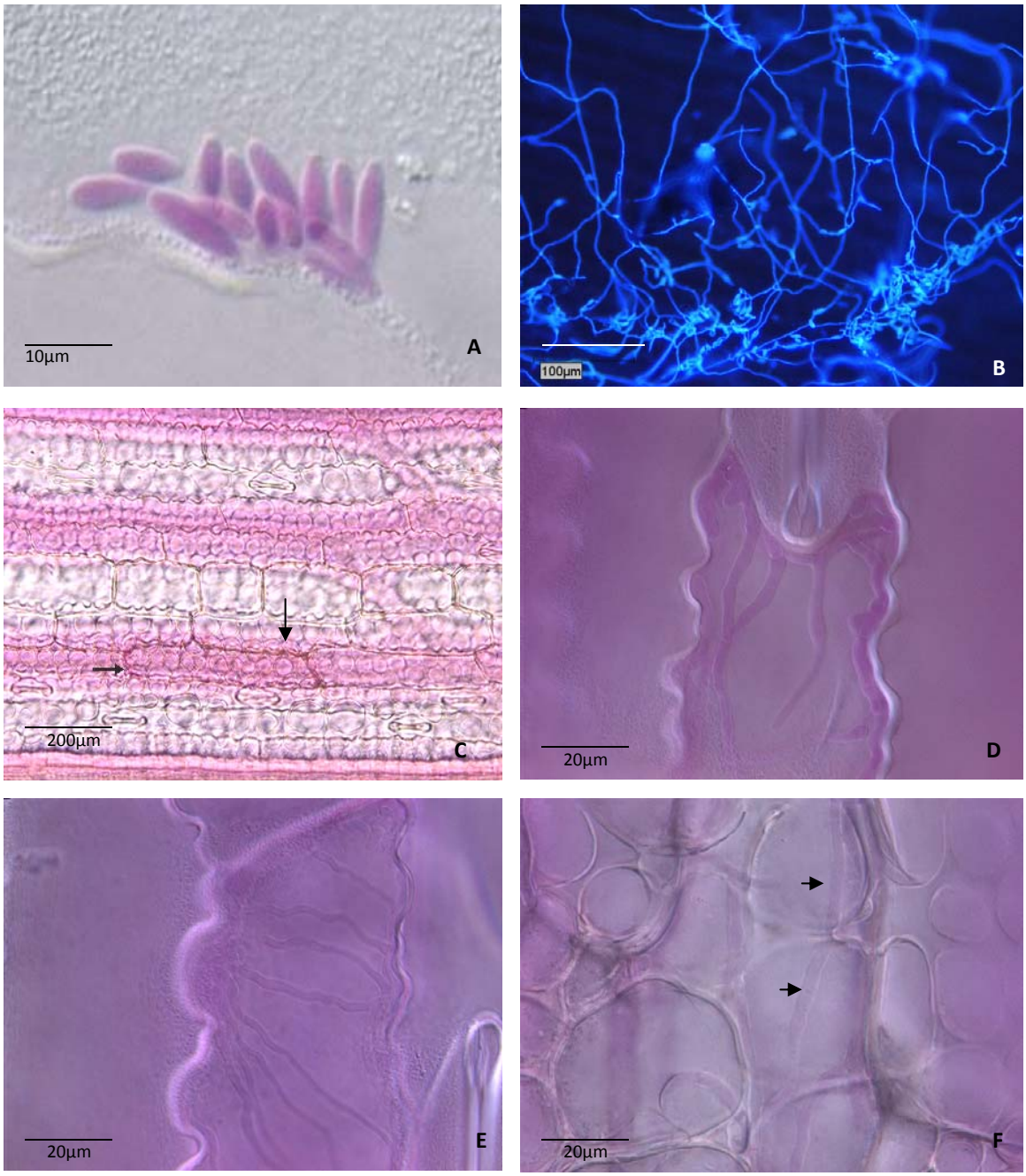


Figure 3.14. Infection of *Fusarium proliferatum* in asymptomatic maize leaves. A= microconidia, 8 hai. B= mycelia growing on leaf surface, 72 hai. C= infected epidermal cell (arrow). D, E= hyphae growing as a finger-shaped subcuticular structure, 7dai. F= hyphae invading mesophyll from subcuticular infection hyphae shown in D, 7dai. Light microscope photographs.

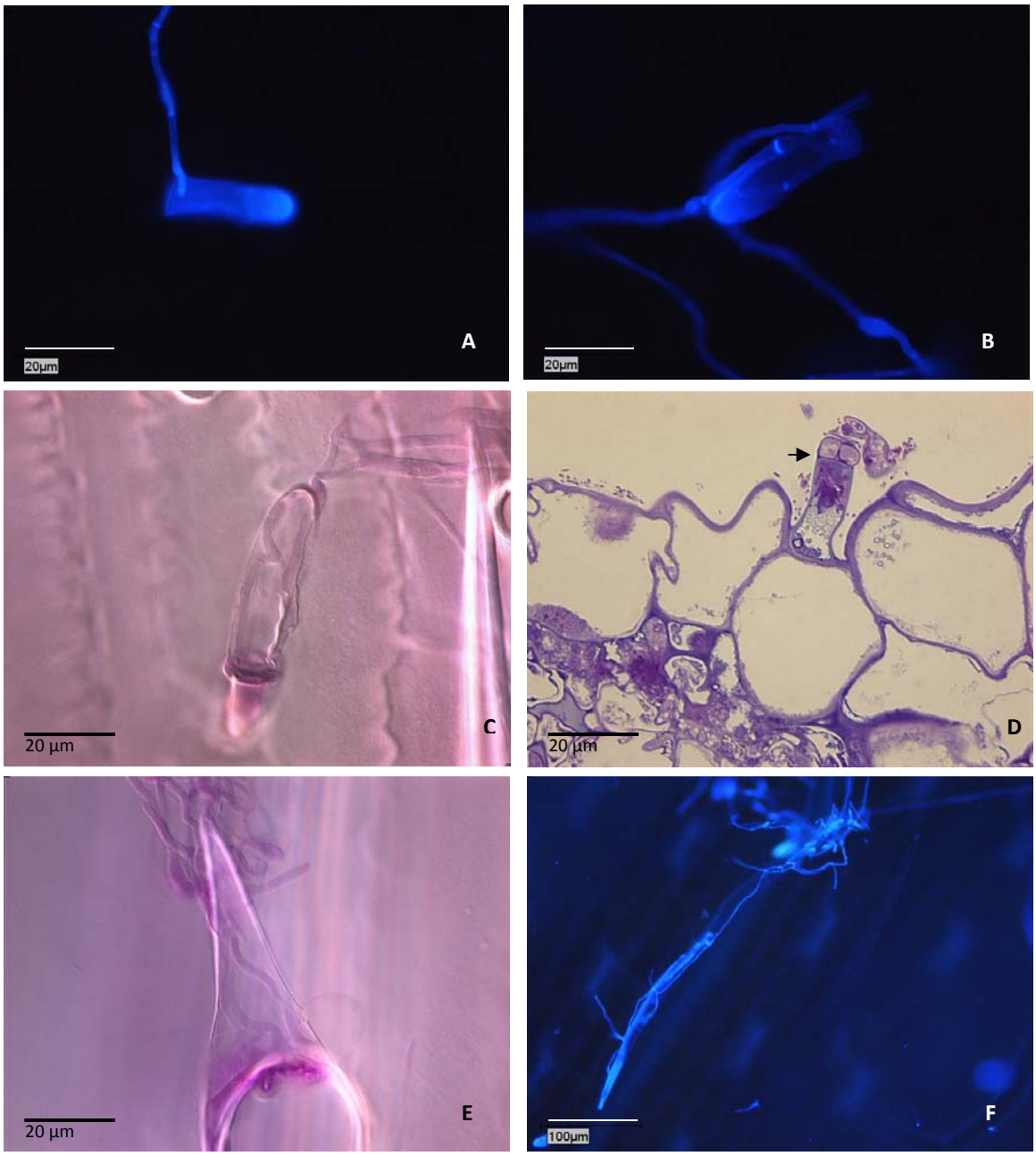


Figure 3.15. Infection of trichomes of maize leaves by *Fusarium proliferatum.* A= initial penetration into bi-cellular trichome, 48 hai. B, C= hyphae growing in cap cell of bi-cellular trichome. D= cross section of infected bi-cellular trichome (arrow), 7 dai. E= hyphae forming in prickle trichome. F= hyphae wrapping macro trichome. Light microscope photographs.

Infection through the stomata, colonization and re-emergence

Infection of *F. proliferatum* via the stomata was observed beginning 4th dai. The fungal hyphae penetrated either directly through or formed mats around the stomatal aperture (Fig. 3.16 A, B). Direct penetration, however, was less frequent compared to the formation of a hyphal mat as an infection cushion (Fig. 3.17 A, C). Later the hypha elongated and penetrated through the stomatal aperture into the substomatal cavity (Fig. 3.17 D). After reaching the cavity, septate hyphae were noted to enlarge, branch and colonize the substomatal cavity (Fig. 3.17 A, E black arrow). Dense hyphae were seen inside the cavity 25 to 30 dai, (Fig 3.16 C, D). After colonizing the cavity, the hyphae invaded intercellular spaces between parenchyma cells (Fig. 3.17 A, white arrow). From the 9 dai, fungal hyphae were observed to re-emerge from stomata near by or from necrotic tissue. Re-emergence of hyphae at the non- necrotic tissue also was observed later, 25dai (Fig. 3.16 E).

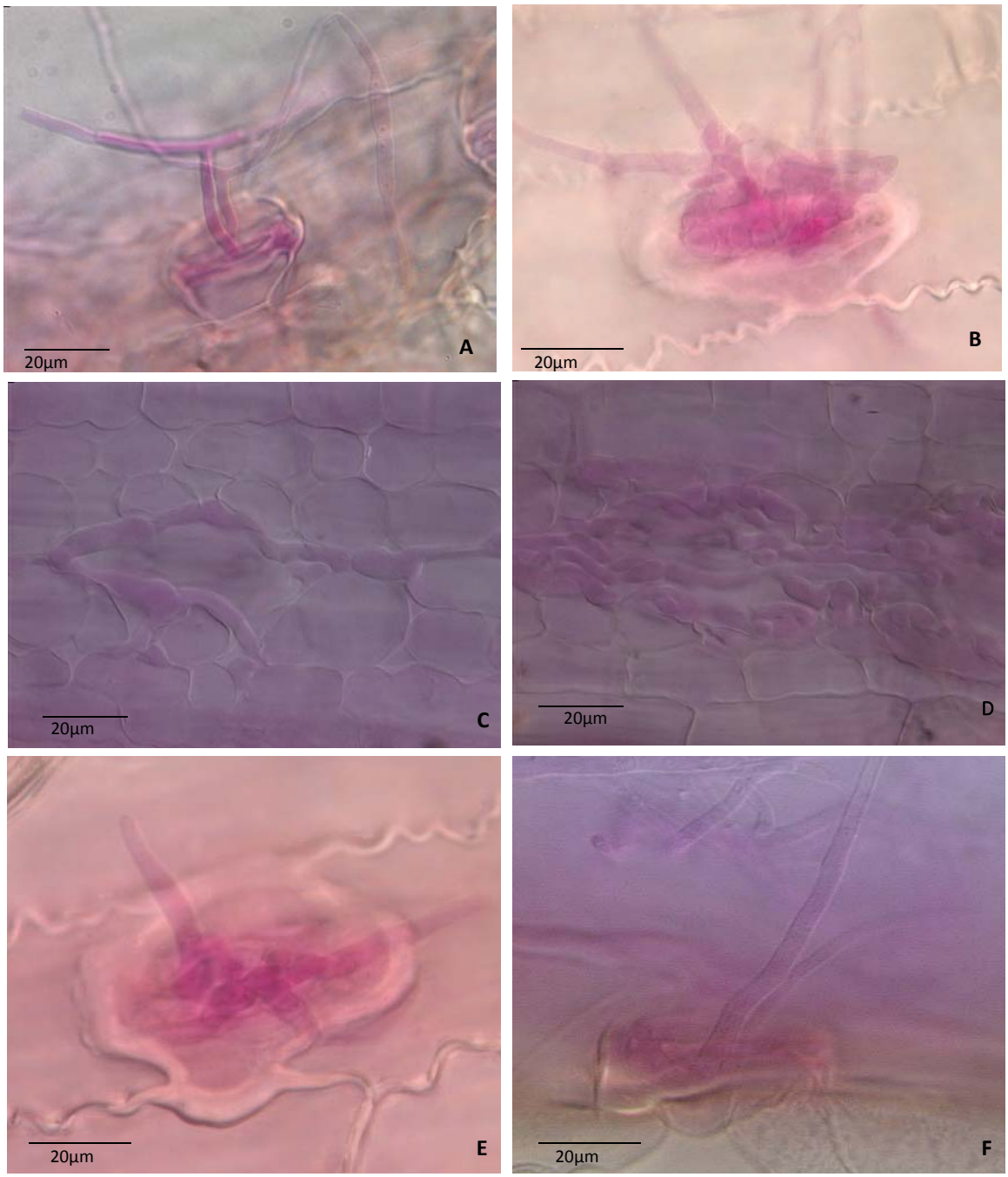


Figure 3.16. Infection via stomata of maize leaves by *Fusarium proliferatum*. A= hyphae penetrating directly through stomatal aperture. B= infection cushion on surface of stoma. C= hyphae growing in substomatal cavity. D= hyphae growing densely in substomatal cavity. E= hyphae re-emergence from stoma, 25 dai. F= hyphae re-emergence from stoma at edge lesion, 9 dai. Light microscope photographs.

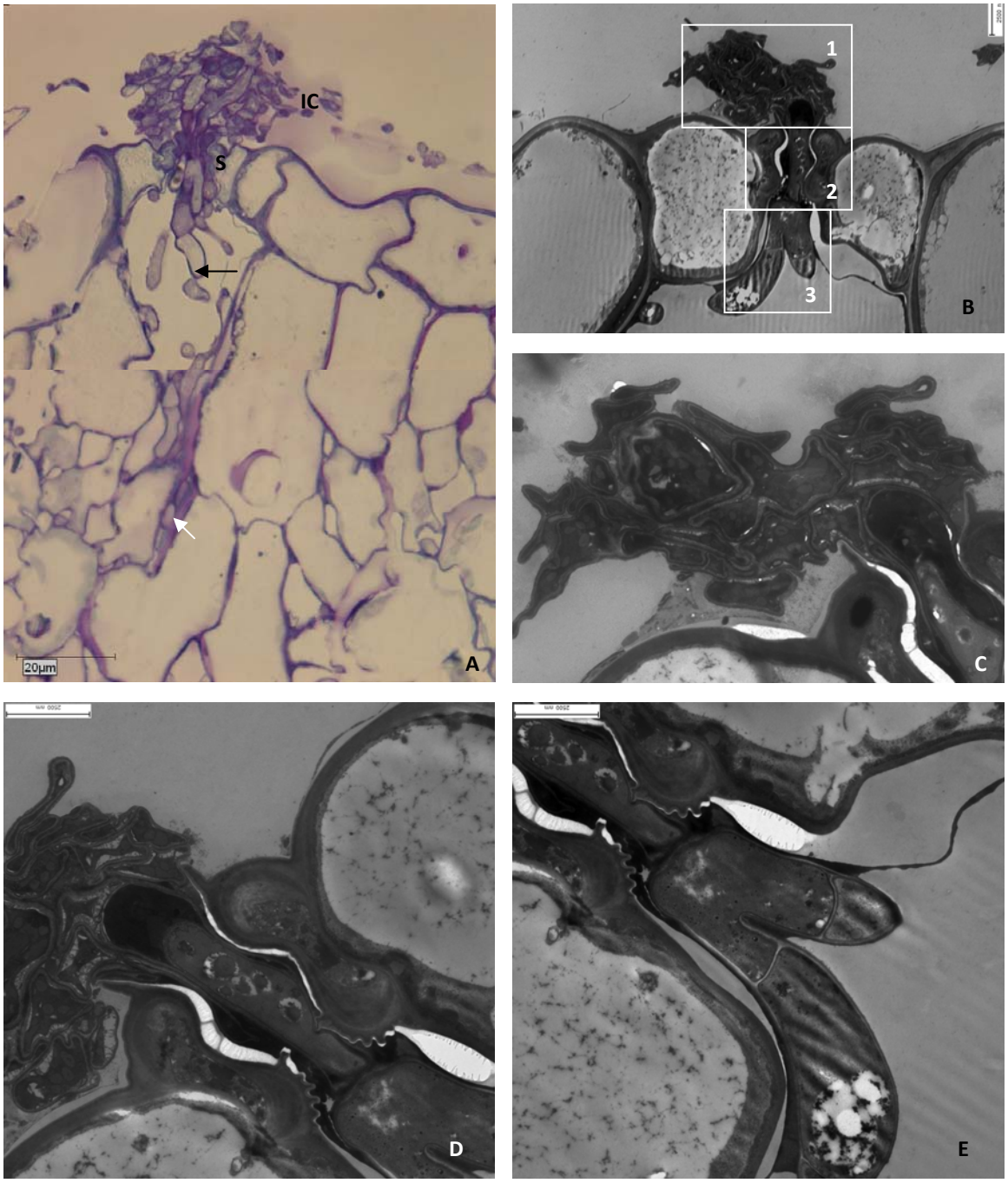


Figure 3.17. Infection via stomata of maize leaves by *Fusarium proliferatum*. A= vertical section of leaf having heavy symptom. Infection cushion (IC) forming on stomatal surface, penetrating through stomatal aperture (ST) and growing in substomatal cavity (black arrow) and intercellular space (white arrow). B= close –up infected stomata in A. C= box area 1 in B, close - up infected cushion. D= close - up box area 2 in B, hypha penetrating through stomatal aperture. E= close - up box area 3 in B, hypha enlarging and branching in substomatal cavity. A: Light microscope photographs. B-E: Transmission electron microscope photographs.

Inter and intracellular colonization

Semi-thin sections of leaf tissue with heavy symptoms showed mycelia to be present not only on the leaf surface but also inside the deformed leaf tissues (Fig. 3.18 A). There were hyphae in leaf sections sampled starting 7 dai (Fig. 3.18 B). The hyphae were observed to grow either in big or small intercellular spaces (Fig. 3.18 C). Dense hyphae formation was also seen within the intercellular spaces (Fig. 3.18 D). The growth of intercellular hyphae appeared to be associated with chloroplasts and nucleus disintegration in the adjoining cells. Moreover, the intercellular hyphae formed thin cells at their tips (Fig. 3.18 E) or foot-like structure (Fig. 3.18 F) which were able to penetrate through the cell walls. A thin neck, the point at which the fungal hyphae gained entry through the cell wall, was drastically reduced in diameter (Fig. 3.19 D).

Intracellular infection of parenchyma cells was only observed in heavily infected tissues (Fig. 3.19 A-D). However, in some cases, although tissues were destroyed, no hyphae were found. Heavily infected intracellular cells led to distortion or collapse of adjacent cells (Fig. 3.19 C, D).

3.3.4.2.4. Sporulation

Formation and sporulation of *F. proliferatum* were very intense, with both the microconidia and macroconidia observed on the leaf surfaces as well as inside infected tissues. The production of mono- and polyphialides occurred at the same time with conidia production and was observed from the 48 hai (Fig. 3.20 A, B). Microconidia produced from secondary infection hyphae arising from infected trichomes (Fig. 3.20 C) and from necrotic lesions (Fig. 3.20 D) occurred 9 dai. 3-6 celled macroconidia were observed on the leaf surface (Fig. 3.20 E, F). Internal sporulation was noted 7 dai. Microconidia arose from hyphae colonizing dead necrotic internal tissues and from the stomatal cavity. The microconidia were released from the stomatal aperture and trichomes (Fig. 3.21 A-D).

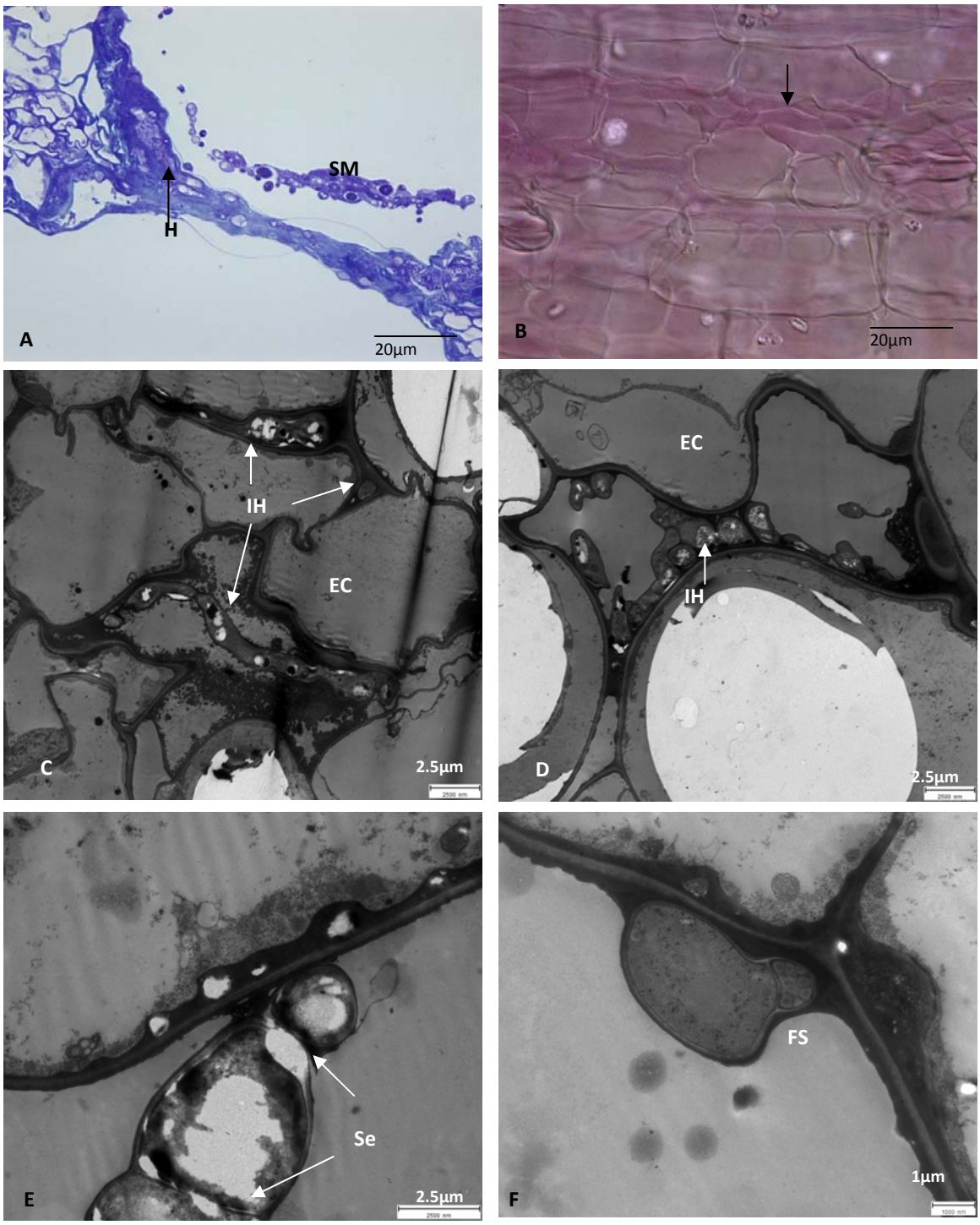


Figure 3.18. *Fusarium proliferatum* hyphae growing intercellular in a necrotic lesion of maize leaves. A= heavy colonization, mycelia growing abundantly on the leaf surface (SM) and in the tissue (H), 7 dai (vertical semi-thin section). B= hyphae growing intercellular spaces (whole leaf section). C= intercellular hyphae. D= dense intercellular hyphae (IH) and empty host cell (EC). E= septations of intercellular hypha (Se). F= Intracellular hypha forming foot – structure (FS). A, B: Light microscope photographs. C-F: Transmission electron microscope photographs.

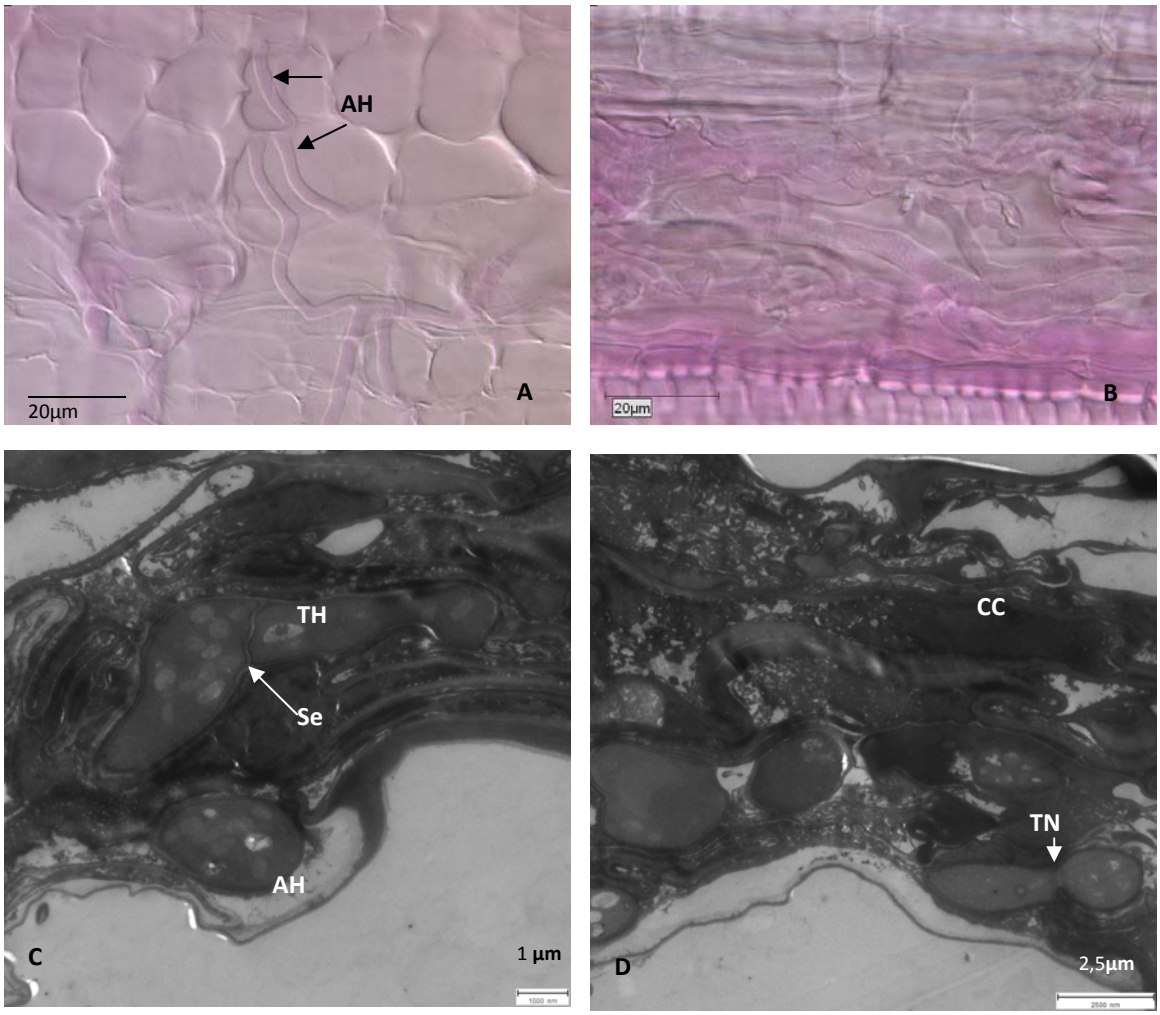


Figure 3.19. *Fusarium proliferatum* hyphae growing intracellular in dead maize leaves, 7dai. A= hyphae growing in parenchyma cells. B= hyphae growing abundantly in tissue at necrotic lesion. C= thick hyphae in mesophyll (TH) with septum (Se) and intracellular hyphae (AH). D= forming thin neck (TN) penetrating into mesophyll cell and cells collapsing (CC). A, B: Light microscope photographs, C, D: Transmission electron microscope photographs.

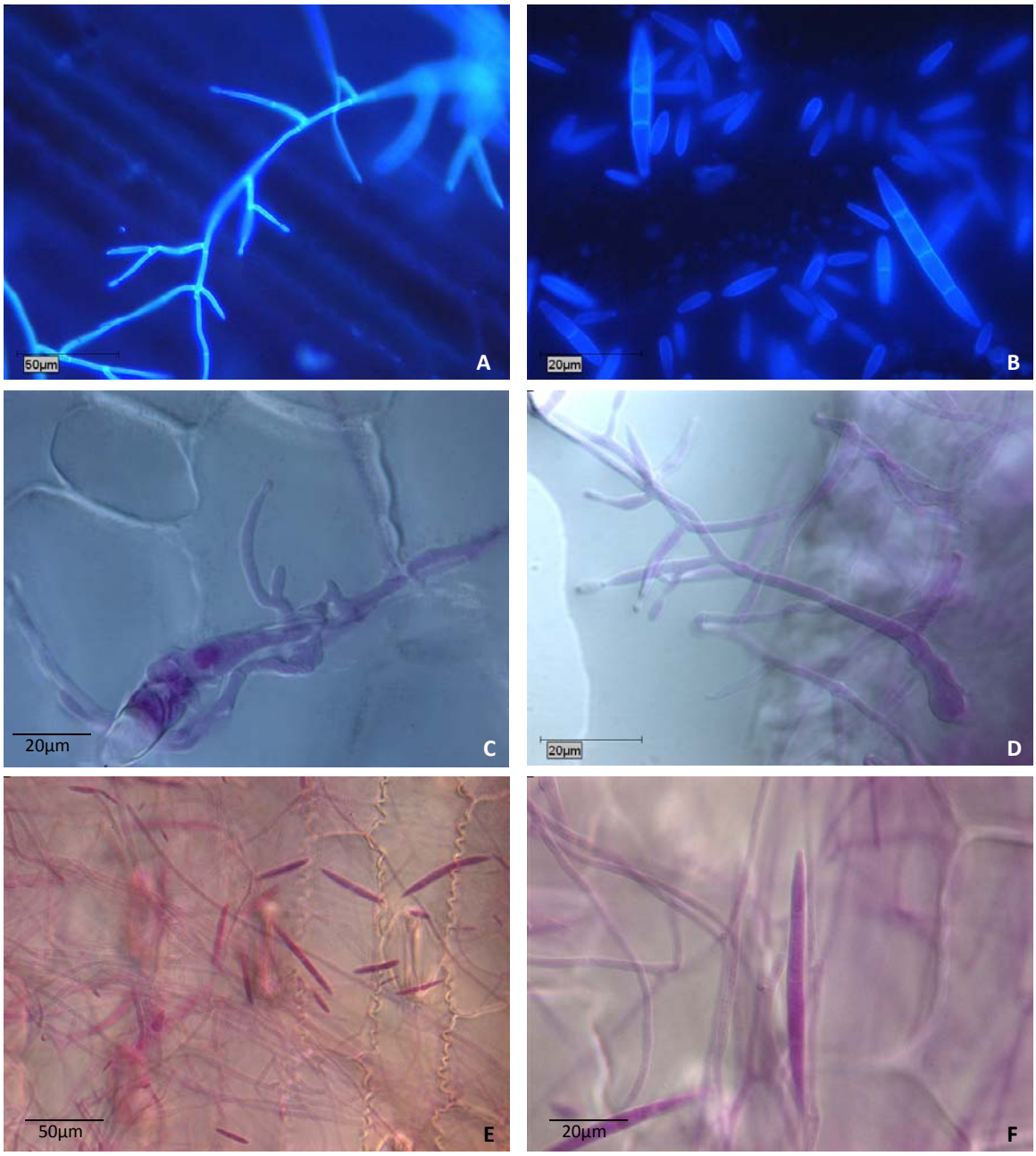


Figure 3.20. Sporulation of *F. proliferatum* on a maize leaf surface. A=microconidiophores (mono- and polyphialides) producing from hyphae on the leaf surface, 48 hai. B= microconidia and macroconidia on leaf surface. C= microconidiaphore forming from hyphae re- emerging from trichome, 9 dai. D= phialides forming from hyphae re- emerging from necrotic lesion, 9 dai. E= macroconidia forming at 15 dai. F= close- up macroconidia in E. Light microscope photographs.

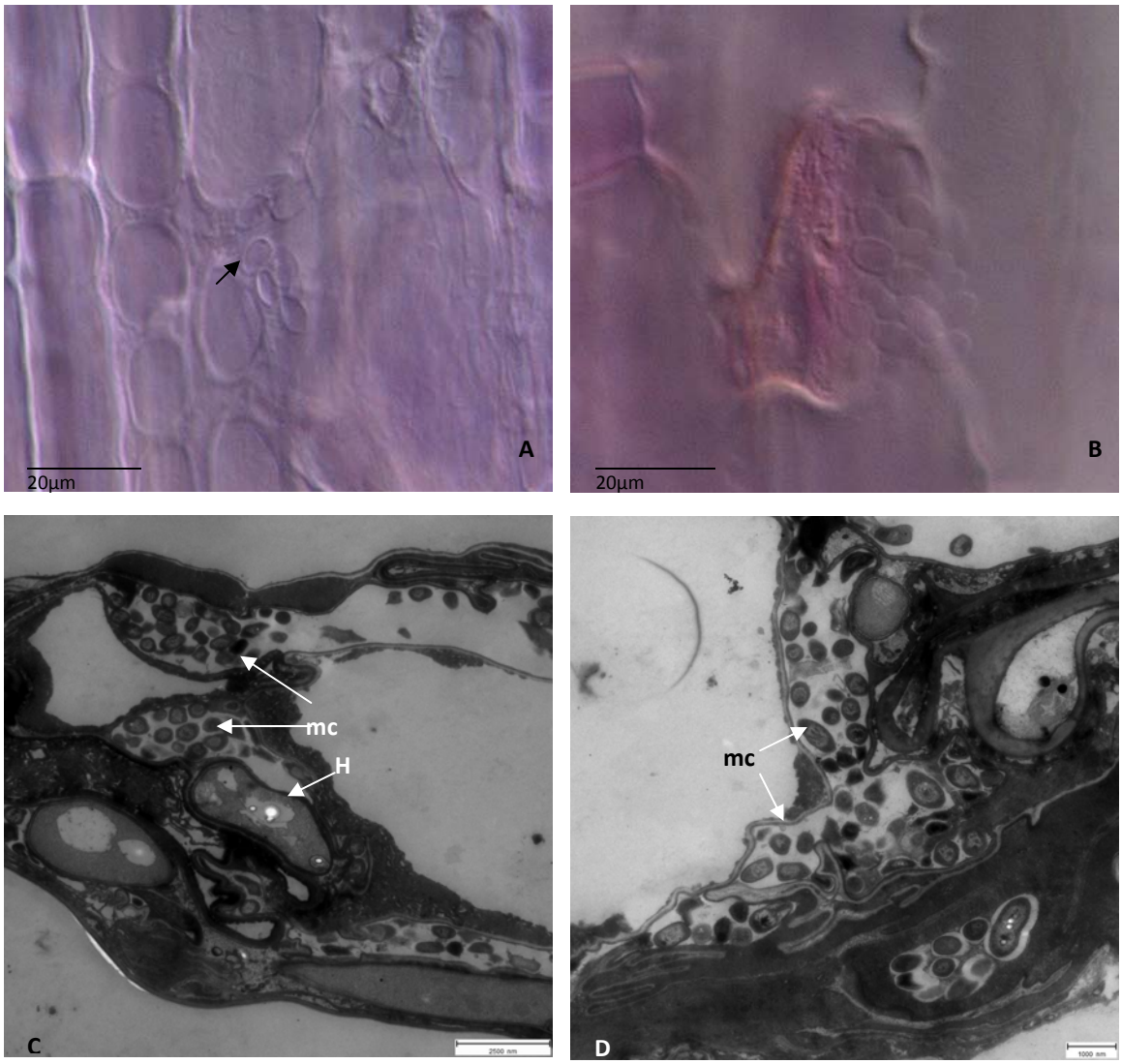


Figure 3.21. Microconidia formed inside maize leaf tissue by *Fusarium proliferatum*. A= microconidia forming in substomatal cavity (arrow), B= microconidia sporulating through stomata. C, D= hyphae (H) producing microconidia (mc) in destroyed leaf tissue, 7 dai. A, B: Light microscope photographs, C, D: Transmission electron microscope photographs.

3.3.4.3. Infection and sporulation of *F. verticillioides* on maize

3.3.4.3.1. Germination of microconidia and mycelia growth

The inoculum was for the most part composed of one-celled conidia (99 %) (Fig. 3.22 A). The one-celled conidia normally formed one germ tube (Fig. 3.22 B) 12 hai. Two germ tubes forming on one-celled conidia were also observed later. The germ tube was separated from the conidium by a septum (Fig. 3.22 B). *F. verticillioides* germ tubes elongated on the leaf surface and formed a less dense mycelial network 72 hai, (Fig. 3. 22 C) on the leaf surface compared to that formed by *F. graminearum* and *F. proliferatum*.

3.3.4.3.2. Infection of asymptomatic mature leaves

The infection process of *F. verticillioides* in asymptomatic tissue was difficult to follow. Hyphae in these tissues were sparce or were not stained with acid fuchsin. No signs of hyphae or germ tube penetration into the leaf tissue were detected*,* but hyphae were observed colonizing the mesophyll cells on a few occasions (Fig. 3.22 D).

3.3.4.3.3. Infection of immature leaves with symptoms

Penetration and colonization of trichomes

The penetration of bi-cellular trichomes by *F. verticillioides* was not as frequent as that of *F. graminearum* (Fig. 3.23). Nonetheless, after penetration and colonization of bi-cellular trichomes, *F. verticillioides* hyphae were observed to spread to adjacent cells. For example, hyphae spread into the subcuticle of the short cells (Fig. 3.23 E) and into the epidermal cells (Fig. 3.23 F). Prickle trichomes and macro trichomes were invaded by the wrapping of *F. verticillioides* hyphae around the trichomes (Fig. 3.23 A). However, they failed to colonize the interior of these trichomes.

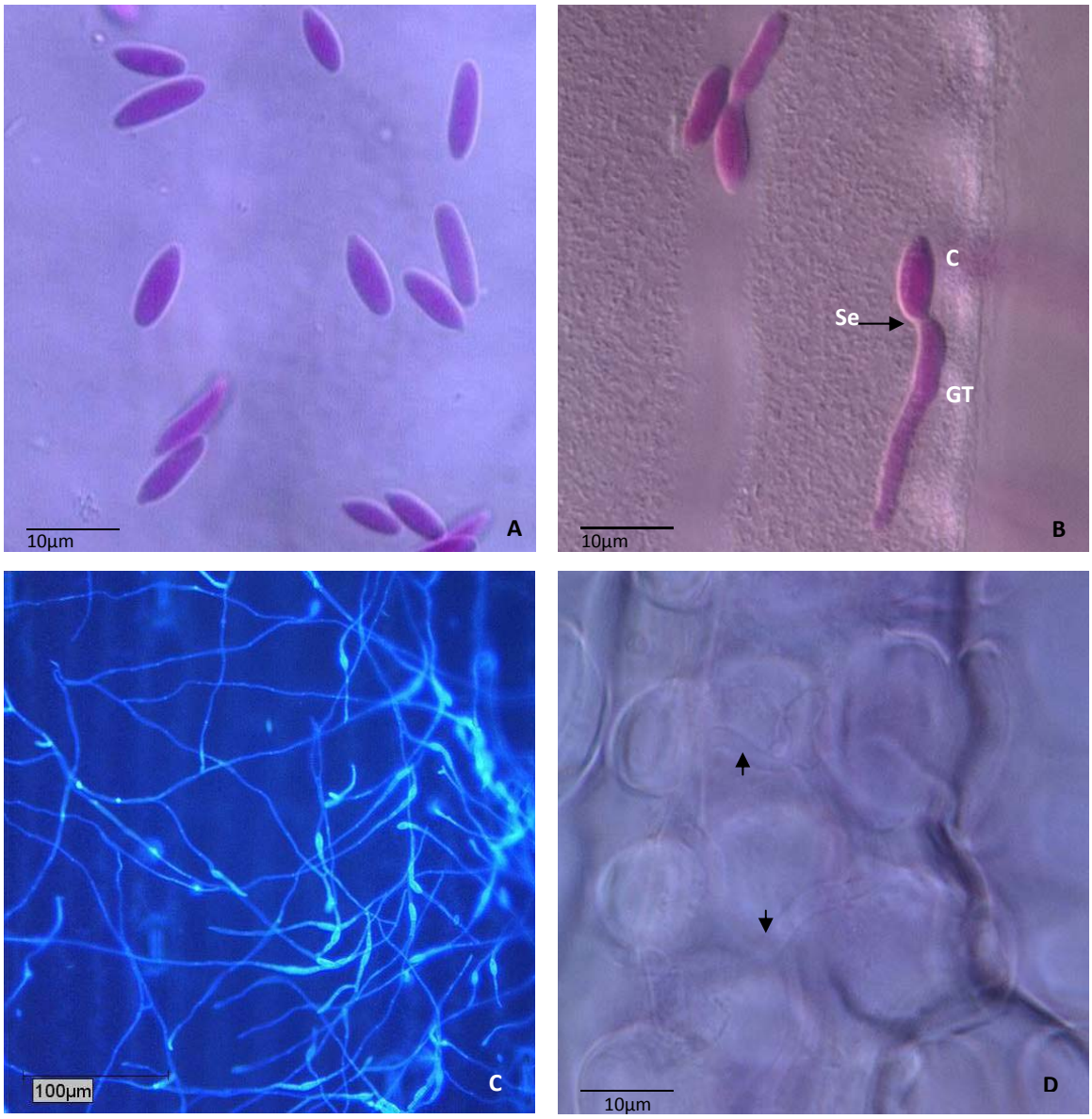


Figure 3.22. Asymptomatic infection of maize leaves by *Fusarium verticillioides*. A= microconidia, 8 hai. B= microconida (C) developing one germ tube (GT), a septum (Se), 24 hai. C= mycelia growing on leaf surface, 72 hai. D= inter – intra cellular hyphae (arrow) in leaf tissue, 7 dai. Light microscope photographs.

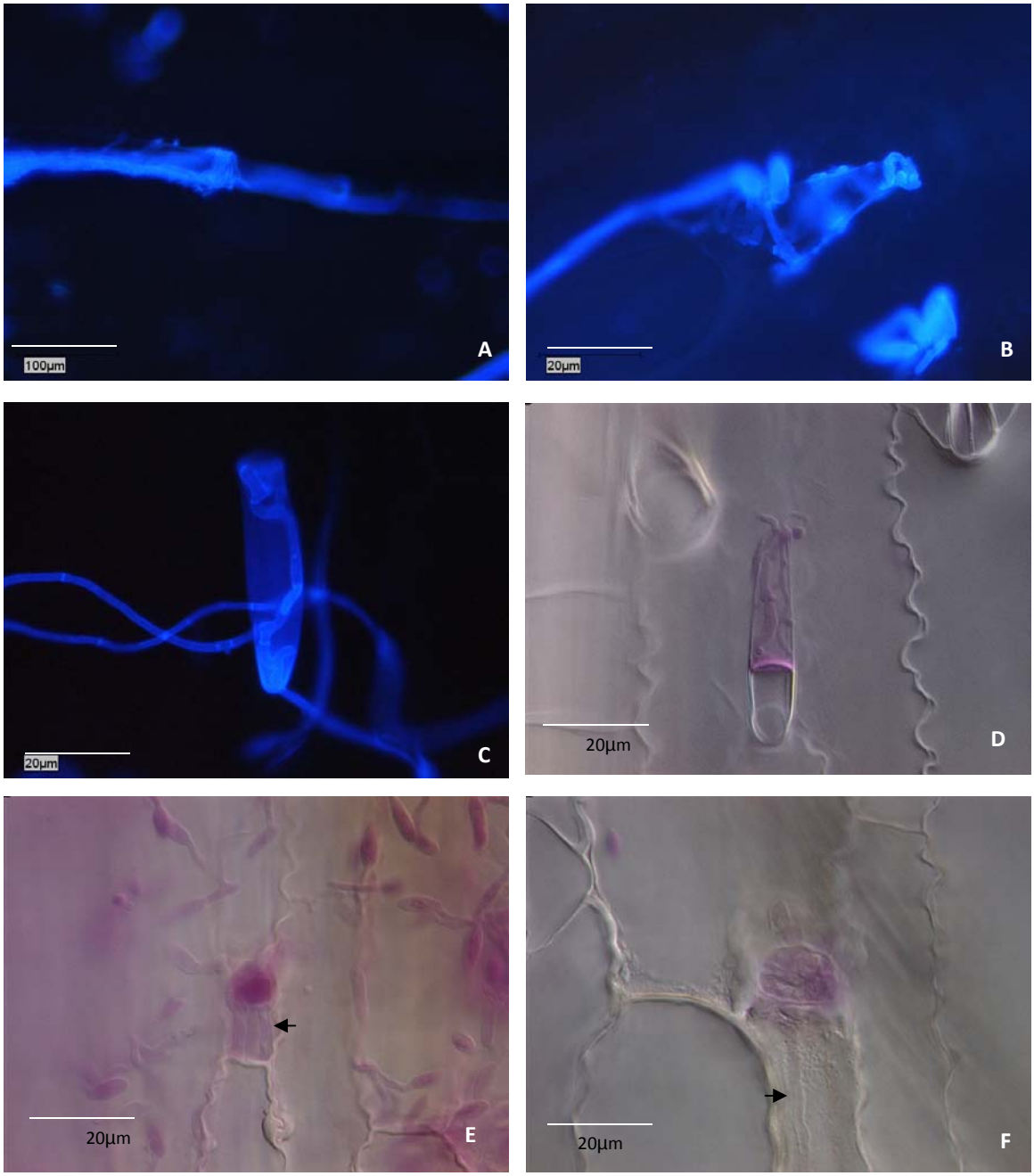


Figure 3.23. Infection of trichomes of maize leaves by *F. verticillioides*. A= hyphae wrapping macro trichome. B= hyphae wrapping from the tip to middle of prickle trichome. C, D= hyphae colonizing bi- cellular trichomes. E= hyphae spreading into subcuticular (arrow). F= hyphae spreading into epidermal cell (arrow). Light microscope photographs.

Infection through stomata and colonization

Fusarium verticillioides infected the stomata using either a single or multiple germ tube(s) (Fig. 3.24 A, B). Penetration by multiple germ tubes was observed more often than with a single germ tube. Both single and multiple germ tubes formed a round or flattened appressoria coming into contact with the stomatal surface. After penetration, the hyphae colonized the substomatal cavity (Fig. 3.25B), and grew downwards into the spaces among parenchyma cells (Fig. 3.24 D). Subcuticular infection was detected at long epidermal cells and subsidiary cells of stoma (Fig. 3.24 C).

Intercellular colonization

Vertical sections of leaves inoculated with *F. verticillioides* showed details of tissue infection, the clarity of which depended on the level of infection and disease symptoms. On leaves showing mild disease symptoms, hyphae colonizing intercellular spaces were clearly observed (Fig. 3.25 A). In the large spaces, hyphae grew quickly (Fig. 3.25 C). In the small or narrow spaces, the hyphae adhered to the cell walls or tried to penetrate cell walls which were observed to be thicker (Fig. 3.25 D).

3.3.4.3.4. Sporulation

Similarly, *F. verticillioides* produced conidia either from superficial hyphae or from secondary infection hyphae. Microconidia formed from superficial hyphae (Fig. 3.26 B) and even from germ tubes (Fig. 3.26 A). However, the sporulation of *F. verticillioides* from superficial hyphae was detected much later than for *F. graminearum* and *F. proliferatum,* i.e. 72 hai. Monophialides (Fig. 3.26 C) formed on the leaf surface and sporulated with a chain of conidia at 18 dai.

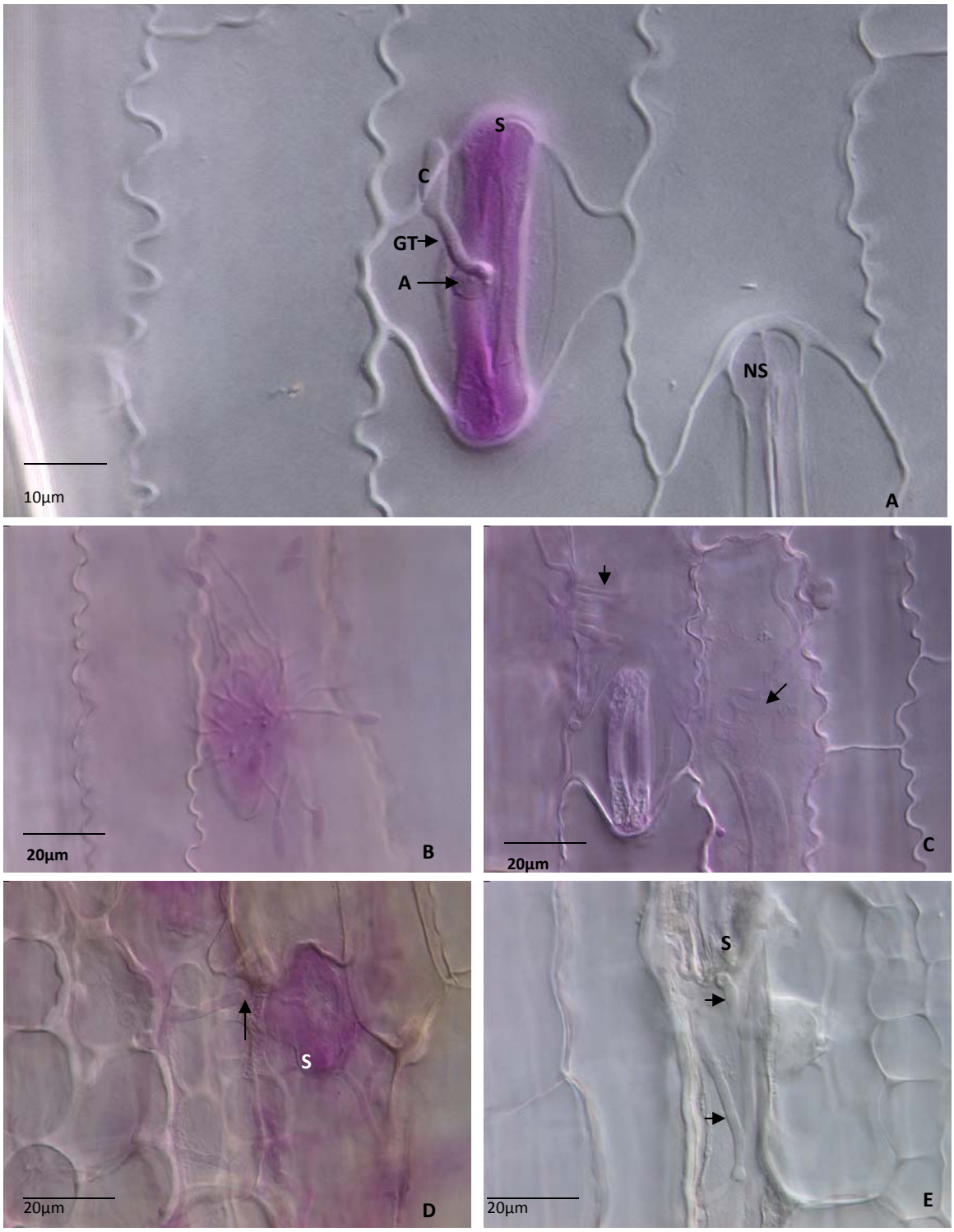


Figure 3.24. Infection through stomata of maize leaves by *Fusarium verticillioides.* A= conidium (C) germinating, germ tube (GT) forming appressorium (A), Infected stoma (S) staining pink with acid Fuchsin. Non-infected stoma (NS) is unstained. B= multi germ tubes and hyphae penetrating through stoma. C= hyphae infecting subcuticucle (arrow) of long cells which are near by stoma. D, E= hyphae spreading from infected stomata. Light microscope photographs.

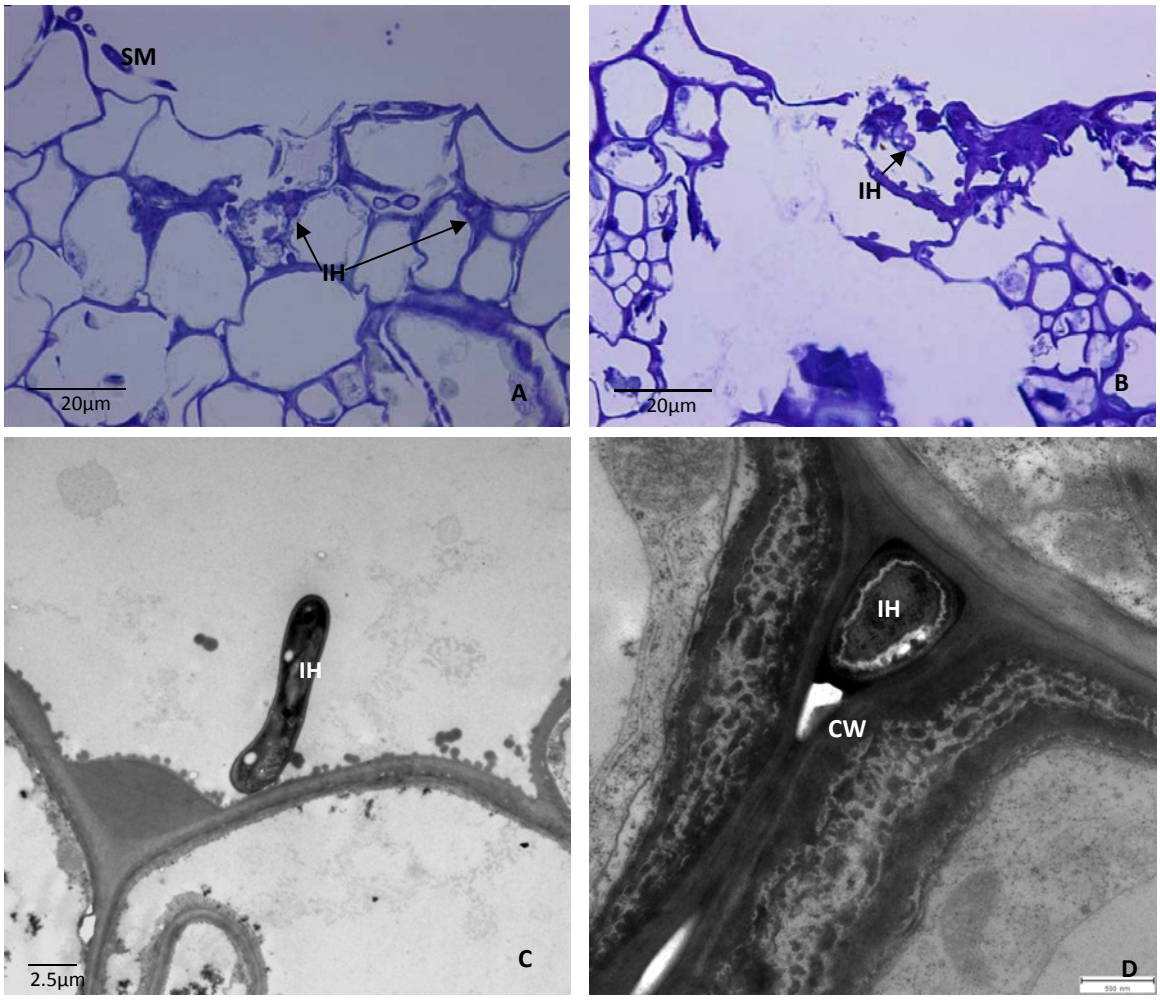


Figure 3.25. Colonization of maize leaf tissue by *Fusarium verticillioides*. A= superficial mycelia (SM) colonizing intercellular spaces (IH). B= colonizing substomatal cavity. C= invading intercellular spaces (IH). D= invading intercellular space (IH) and host cell walls (CW). A, B: Light microscope photographs. C, D: Transmission electron microscope photographs.

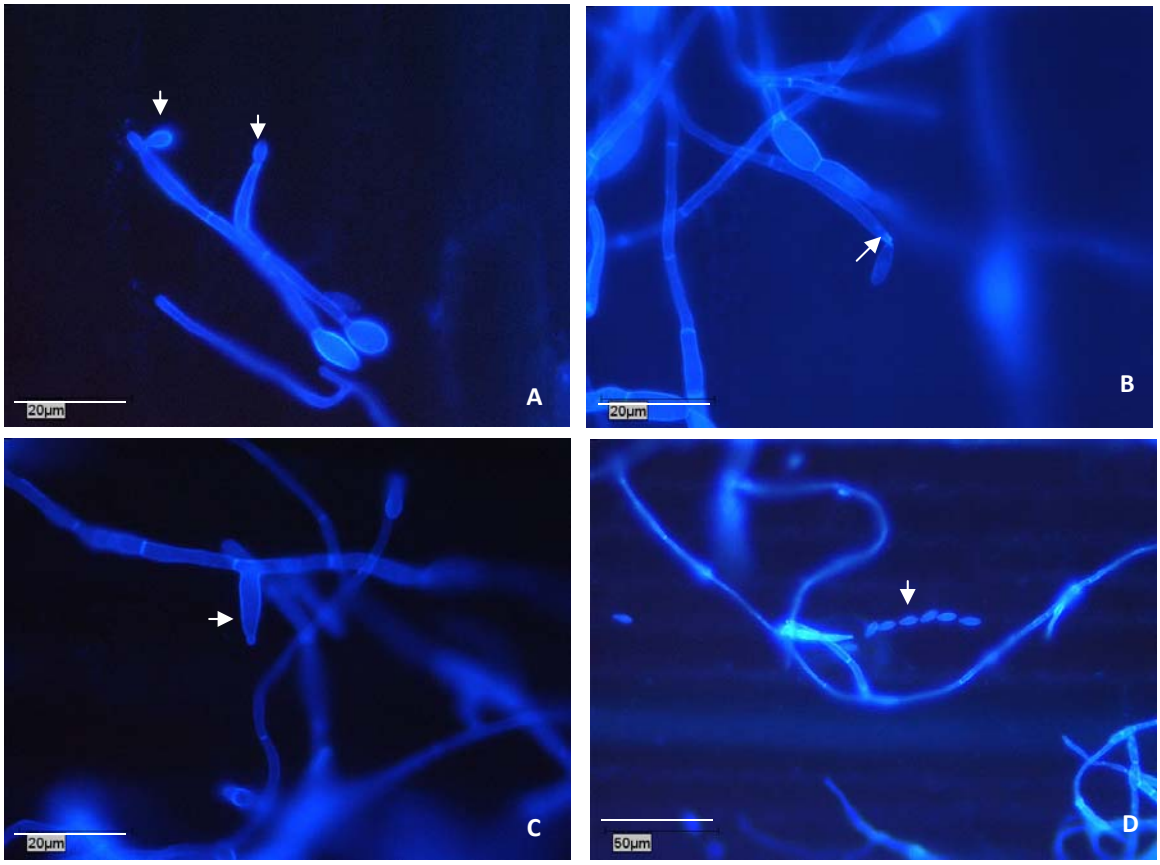


Figure 3.26. Sporulation of *Fusarium verticillioides* on the leaf surface of maize. A= microconidia producing from germ tube and sporulating, 72 hai. B= microconidia producing from aerial hypha, 72 hai. C= monophialides forming from hyphae, 18 dai. D= microconidial chain forming from monophialides, 18dai. Light microscope photographs.

3.3.5. Comparison of hyphal growth and modes of infection

3.3.5.1. Hyphal growth

Fusarium hyphae varied in size depending on the species and environment in which it was found. For instance, superficial hyphae were noted to be thinner than hyphae colonizing inside the leaf tissue. On the leaf surface, *F. graminearum* hyphae were significantly thicker than *F. proliferatum* and *F. verticillioides* (Table 3.4). However, after penetration, the hyphae enlarged and significantly increased in size by 40 -55 % in comparison to hyphae on the leaf surface. Whereas the diameter of hyphae ranged from 1.0 to 5.6 µm on the leaf surface, the hypha*l* diameter of those found inside the leaf tissue ranged from 1.3 to 11.4 µm.

Table 3.4. Diameter of hyphae (µm) of *Fusarium* spp. on the maize leaf surface and in leaf tissue.

F. species	On leaf surface			In leaf tissue			Sig. [2]
	Mean	max	min	Mean	max	min	
Fg	3.28 ± 0.11[1]	6.5	1.6	5.08 ± 0.14	11.4	2.8	**
Fp	2.33 ± 0.05	3.8	1.4	3.27 ± 0.08	5.3	1.8	**
Fv	1.69 ± 0.05	2.9	1.0	2.47 ± 0.09	4.6	1.3	**

[1] Standard error of mean with n=94 for *Fusarium graminearum* (Fg), n= 84 for *F. proliferatum* (Fp) and n=76 for *F. verticillioides* (Fv) .

[2] **: Significant difference (z-Test: Two sample for means) between inside the tissue and on the surface

3.3.5.2. Infection of trichomes

Although all the three fungal species, i.e. *F. graminearum*, *F. proliferatum* and *F. verticillioides* had the ability to infect the trichomes of the maize leaves, the penetration levels were significantly different among the three species. The penetration for *F. graminearum* into trichomes was significantly higher than for *F. proliferatum* and *F.*

verticillioides 7 dai (P = 0.004). However, 15 dai there was no significant difference in trichome infection of the three species (P = 0.182) (Fig. 3.27).

3.3.5.3. Infection via stomata

The infection of the stomata by *F. proliferatum* was highest and significantly different from *F. graminearum* and *F. verticillioides* 7 (P = 0.004) and 15 dai (P = 0.001, Fig. 3.28).

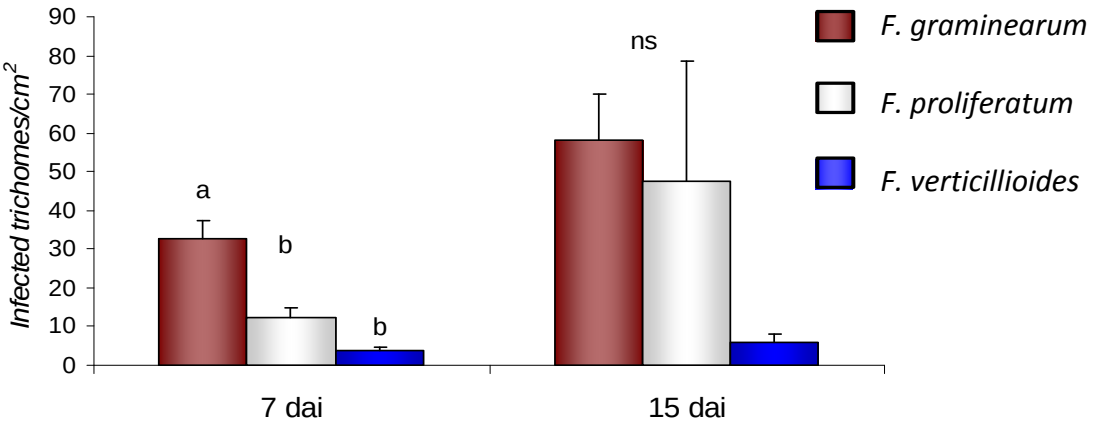


Figure 3.27. Infection of trichomes by *Fusarium* species on maize leaves at 7 and 15 dai. *F. graminearum* isolate AG 23d, *F. proliferatum* isolate AG31g and *F. verticillioides* isolate AG11i. For each sampling time, mean values followed by the same letters are not significantly different P≤ 0.05 (Duncan's test). Error bars represent the standard error of the mean.

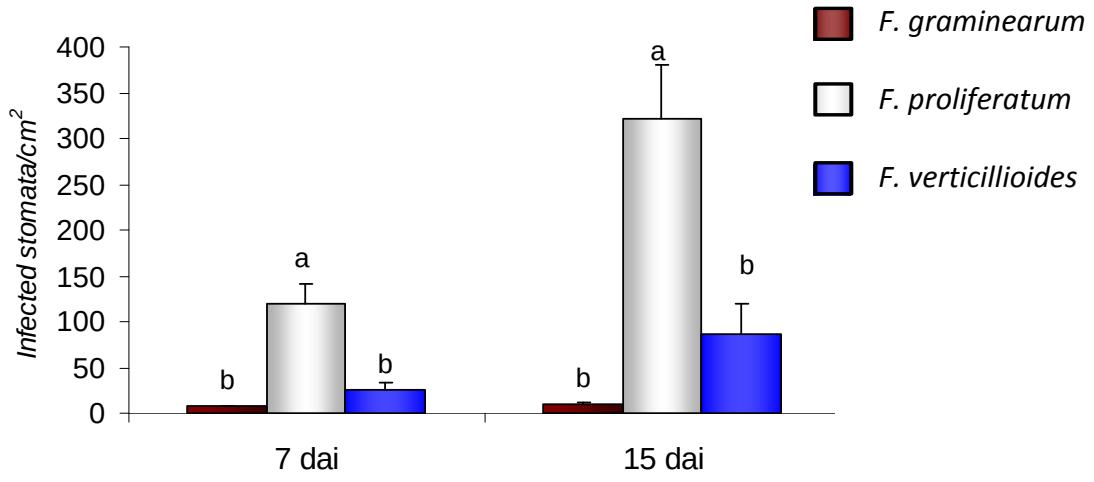


Figure 3.28. Infection via stomata by *Fusarium* species on the maize leaves at 7 and 15 dai. *F. graminearum* isolate AG 23d, *F. proliferatum* isolate AG31g and *F. verticillioides* isolate AG11i. For each sampling time, mean values followed by the same letters are not significantly different P≤ 0.05 (Duncan's test). Error bars represent the standard error of the mean.

3.4. Discussions

Many *Fusarium* species have been known to have an endophytic phase during colonization of the host (Bacon and Hinton, 1996; Yates et al., 1997; Yates et al., 1999; Vieira, 2000; Oiah et al., 2006; Larran et al., 2007) by which the fungi penetrated and colonized host cells without damages of host tissue. In the present investigation, hyphae of all three *Fusarium* species were found in symptomless leaves. Direct penetration and appressorium formation were observed by *F. graminearum* while *F. proliferatum* and *F. verticillioides* penetrated directly. These findings are in line with previous research findings in which *F. moniliforme* penetrated maize roots directly (Lawrence, 1981) and infected the maize plant symptomless (Bacon and Hinton, 1996). They reported that hyphae colonized intercellular spaces of secondary and, primary roots and internodes from systemic infection. The results of the present study revealed that *F. graminearum*, *F. proliferatum* and *F. verticillioides* infected the leaves locally and invaded either inter- or intracellularly. *F. graminearum* invaded epidermal cells whereas *F. proliferatum* and *F. verticillioides* occupied mesophyll cells. However, at an infected site only one to three cells were colonized, adjacent cells were not infected by all three fungi. These results are similar to those of Schulz (1999) who described that *Fusarium* sp. colonized barley inter- and intracellularly.

The penetration sites of asymptomatic infections by *Fusarium* were not conspicuous (Wagacha et al., 2012). In this study, hyphae grew randomly or along grooved /anticlinal cell walls on the surface of the leaf host tissue, formed mycelia networks and then penetrated the maize leaf. The penetration point for *F. proliferatum* and *F. verticillioides* were not clearly observed, but the penetration site of *F. graminearum* was usually observed at the corner of cell walls following the formation of an appressorium. These characteristics could be explained by morphological and biological properties of the leaf. For example, hyphae may adhere to the grooves/anticlines easier than smooth surfaces or hyphae may have been capable of perceiving signals of water, free spaces and nutrients from those sites. Intercellular endophytic colonization requires nutrients from the apolast for growth (Schulz and Boyle, 2005). Nutrients within apolast host cells were

diverse and plentiful for fungi growth and reproduction (Canny, 1995; Bacon and White, 2000; Tejera et al., 2006). Knight (2011) also found hyphae of *F. pseudograminearum* growing along grooves of wheat sheath surface.

The symptomless state persists beyond the seedling stage (Bacon and Hinton, 1996) in a balance between fungi and host (Schulz et al., 1999). It depends on plant genetics (Ochor et al., 1987; Bai and Shaner, 1996) and environmental conditions (Kuldau and Yates, 2000) during or later in the infection process. In this study, mature leaves showed the asymptomatic stage, did not change over time to symptoms of disease.

However, the *Fusarium* species are also known to form symptom after infection (Ding et al., 2011). *Fusarium* spp. caused symptoms on the kernels, crowns, leaf sheaths, and stems of maize plants (Gilbertson et al., 1985; Gilbertson, 1986; Hampton et al., 1997; Munkvold and Desjardins, 1997; Reid, 2002; Pastirčák, 2004; Santiago et al., 2007; Dutton, 2009); The results in section 2.3.5 showed that maize leaves with symptoms differed depending on the *Fusarium* species, leaf morphology and plant physiology. The symptoms only appeared when the immature leaves (folded leaves) were inoculated. On the infected leaves with disease symptoms, there were typical infection patterns of the species tested: *F. graminearum*, *F. proliferatum* and *F. verticillioides.* (1) Infection into trichomes of maize leaves by *Fusarium* was the first described in this study. Infection of trichomes was found very early after inoculation. *F. graminearum* hyphae adhered and clapped bi-cellular trichome from the tip and penetrated the trichomes (Fig. 3.7). The infection of trichomes by these species may be explained by the structure of the trichomes which had no wax on the surface and thin cell walls when compared to other epidermal cells (Fig. 3.1). These findings were in line with previous research findings in which hypocotyl trichomes of *Phaseolus vulganis* were wrapped and colonized by *F. solani* f. sp. *phaseoli* (Mulligan et al., 1990); in another study, Knight (2011) found that *F. pseudograminearum* wrapped around trichomes and invaded the base of trichomes of wheat leaf sheaths*,* but the penetration via trichomes was not observed. Stephen (2008) only reported the interaction between the germ tube of *F.*

graminearum and trichomes of the wheat leaf sheath. Trichomes were epidermal cells extruding out of the leaf surface with scattered distribution. Therefore, after infection via trichomes disease symptoms appeared but they were not heavy. The symptoms of disease were detected as small light yellow spots or no symptoms could be recognized.

(2) Infection of stomata was a strategy the fungi used to infect the host in the present studies. Knight (2011) also reported that *F. pseudograminearum* frequently infected stomata of wheat leaf sheath tissue that resulted in lesion formation. In the present study, the three species of *Fusarium* infected maize leaf via stomata. This infection pathway occurred frequently on inoculated immature leaves but was rarely observed on the inoculated mature leaves. This may be explained by the fact that the stomata of immature leaves may be more often open because of the high humidity conditions found in the whorl. Evidence of hyphal penetration via the opened stomata was observed on the detached 4th leaf in humidity chambers with *F. graminearum*. Although the three *Fusarium* species in this study had the capacity to infect via stomata, they showed varying behavioural patterns. *F. graminearum* formed appressoria or infection cushions or even penetrated directly. Direct penetration or forming appressoria also was observed by *F. verticilioides*. The formation of an infection cushion or direct penetration also was seen by *F. proliferatum*. These findings are in line with research of Boenisch and Schäfer (2011) in which *F. graminearum* formed lobate appressoria and infection cushions to penetrate caryopses, paleas, lemmas, and glumes of wheat plants. On the other hand, *F. moniliforme* was observed to directly penetrate epidermal cells of seedling maize (Lawrence, 1981; Murillo et al., 1999). Kang and Buchenauer (2000a) found that *F. culmorum* occasionally penetrated via stomata on the inner surface of the lemma of wheat spikes.

Another mechanism by which the three species of *Fusarium* gained entry into the leaf tissues was through the cuticles. Upon penetration, the fungus was observed to grow and colonize the subcuticular spaces of short epidermal cells. In other investigations *F. graminearum* and *F. culmorum* hyphae also were observed to grow inside the subcuticles of glume, palea, and lemma of wheat spikes (Kang and Buchenauer, 2000a;

Pritsch et al., 2000; Jansen et al., 2005). These species may penetrate the cuticle with short infection hypha (Mary Wanjiru et al., 2002) or secrete enzyme that degrade cuticle (Kang and Buchenauer, 2000b). In the present study hyphae of *Fusarium* were observed under the cuticle of epidermal cells, particularly in short epidermal cells. Moreover, the cuticle of detached leaves which were degraded by the presence of *F. graminearum* was occasional found.

Colonization by species of *Fusarium* of the xylem and phloem were reported by others. *F. pseudograminearum* colonized vascular bundles in the leaf sheath and culm of wheat plant (Knight, 2011). Lawrence (1981) also found xylem vessel occluded by *F. moniliforme* in the leaf and shank of sweet corn. *F. graminearum* occluded vessels and sieve tubes and destroyed the phloem of susceptible wheat lemma (Ribichich et al., 2000) and in the xylem vessels in the rachis of wheat (Mary Wanjiru et al., 2002). In the current study the colonization of both the phloem and xylem were only found by *F. graminearum* in the detached leaves under high humidity conditions. This suggests that the colonization of the vascular bundle may occur in conditions such as wounding, during senescence of the leaves or in high humidity regimes. Colonization of the vascular bundles may lead to quicker spread through the plant and advance spread in the plant population after sporulation.

Symptom of infection on the maize leaves led to deformation of leaf blades and cells. In this study, tissues heavily infected by *F. proliferatum* had cells that were distorted. The chloroplasts and nucleus of surrounding cells were also disintegrated. However, in *F. verticillioides* lightly infected tissues, cell walls deposited callose (Fig 3.25 D).

The importance of mycotoxins in the infection process probably relates to damage to plant cells and callose generation to hinder fungal growth into cells. Bushnell et al. (2010) found that plasmalemmas were damaged and chloroplasts were lost when leaves were treated with DON. Kang and Buchenauer (2000a) reported that the colonization of the wheat spike by *F. culmorum* degenerated host cytoplasm and organelles collapse of parenchyma cells. Callose were deposited in the cells surrounding initially infected

vessels after infection by *F. oxysporum* (Mueller et al., 1994; Rodriguez-Galvez and Mendgen, 1995).

Fungal sporulation allows for dispersal, preservation and genetic diversity. Pritsch et al. (2000) observed *F. graminearum* sporulating within 48 to 76 hai on inoculated glumes. Early spore formation was also observed in this investigation. The three *Fusarium* species sporulated readily on the maize leaf surface. *F. graminearum* sporulated very early after inoculation (48 hai), *F. proliferatum* and *F. verticillioides* formed spores later (72 hai). The macroconidia of *F. graminearum* were active in the production of secondary macroconidia (Fig. 3.11 D, E). This is the first time that *Fusarium* was reported to produce secondary spores. Sporulation on the leaf surface was detected later, 9-18 dai. The late spore formation observed may have been due to fungal hyphae that re-emerged from the infected tissues. Interestingly, the spores were observed inside the leaf tissues (Fig. 3.21) and these spores were observed to be released through stomata and trichomes at 7 dai. However, this phenomenon was only observed in *F. proliferatum* inoculated maize leaves. The sporulation of other *Fusarium* spp inside the leaves of other plants has been reported before. For instance, Wagacha (2012) reported that *F. tricinctum* produced spores inside the leaves of wheat plants. On the other hand, *F. verticillioides* spores were detected inside maize seedlings, 21 dai (Oren et al., 2003). Contrary to findings of Oren et al., (2003), *F. verticillioides* was not detected sporulating inside maize leaf tissues in the present study. The contrast between these results and those of Oren et al. (2003) may be explained by the type of infected cells (mesocotyl cells) and inoculation method used. The phenomenon of secondary spore production that was witnessed in the present study constitutes a potential avenue for the preservation of genetic diversity during unexpected environmental conditions. The sporulation of *Fusarium* species during early stages of growth to later periods of infection process implied that the infected maize leaves may widely and efficiently disseminate spores to upper leaves and to silks.

4. Assessment of infection by *Fusarium graminearum, F. proliferatum* and *F. verticillioides* on maize leaves using quantitative PCR and microbiological assays

4.1. Introduction

Maize (*Zea mays*) the third biggest crop growing worldwide, is frequently infected by *Fusarium* species. *Fusarium* colonizes all parts of maize including the root, leaf sheath, stalk, leaves and cob (Jardine and Leslie, 1999; Yates, 2007; Murillo-Williams and Munkvold, 2008; Polisenska et al., 2008). *Fusarium* species also are known to parasite wheat, barley and sorghum and survive over winter in crop residues (Leslie et al., 1990; Parry et al., 1995; Bhat et al., 1997; Cotten and Munkvold, 1998; Dill-Macky and Jones, 2000; Goswami and Kistler, 2005; Naef and Defago, 2006; Maiorano et al., 2008; Svitlica et al., 2008; Keller et al., 2010; Keller et al., 2011). *F. moniliforme*, *F. proliferatum*, and *F. subglutinans* can survive in maize residues on the soil surface in the field or in the soil for more than a year (Cotten and Munkvold, 1998).

Assessment of *Fusarium* infection in host tissue is complicated because *Fusaium* spp. Infection can lead to the development of symptoms but also symptomless infections (Bacon and Hinton, 1996; Yates et al., 1997; Vieira, 2000; Yates et al., 2005). Researchers have evaluated colonization of *Fusarium* in host tissue using microbiological, histopathological and molecular methods and assays. The assessment of the level of infection of a pathogen by using a scale of visible disease has been used for a long time. The method does not allow assessment of symptomless infections. Assessment, however, fungal isolation or the use of real time polymerase chain reaction (PCR) can be applied effectively to detect the pathogen in symptomless tissue. With the help of a selective medium, Czapek-Dox agar containing iprodione and dicloran (CZID) (Abildgren et al., 1987), *Fusarium* species were effectively isolated. CZID induced the growth of *Fusarium* colonies but limited the growth of other fungi.

There have been many applications of real time quantitative PCR in the: identification of pathogens, host-pathogen interactions, selecting resistant cultivars and studying the infection process of pathogens (Schnerr et al., 2001; Schaad and Frederick, 2002;

Vandemark and Barker, 2003; Vandemark and Ariss, 2007; Görtz et al., 2010). Real time quantitative PCR was also applied in detection and quantification of *Fusarium* species (Möller et al., 1999; Mulè et al., 2004; Strausbaugh et al., 2005; Sarlin et al., 2006; Stephens et al., 2008; Yli-Mattila et al., 2008; Nicolaisen et al., 2009; Nutz et al., 2011; Obanor et al., 2012).

Knight et al. (2012) and Hogg et al. (2007) applied real time quantitative PCR and a visual disease scale to assess Fusarium crown rot of wheat**.** However, Knight et al. (2012) reported a strong correlation between visual discoloration of the leaf sheath and fungal biomass in wheat tissues. Hogg et al. (2007) also obtained the same results in 2004 but not in 2005. Moradi et al. (2010) compared microbiological and real-time PCR assay on infection levels of wheat kernels. Percy et al (2012) and Malligan (2008) compared the development of visible symptoms of crown rot of wheat and frequency of re-isolation of *F. pseudograminearum* from plant tissues.

Results from histopathological assessment in section 3 showed that *F. graminearum, F. proliferatum* and *F. verticillioides* infected maize leaves either without symptom development or with symptoms. The overall objective of the research conducted here was to examine whether there is a relationship between disease severity, infection and colonization and fungal biomass during the development of the three *Fusarium* species in maize leaves. The following specific objectives were investigated:

i. determine disease severity of infection by the three *Fusarium* species on maize leaves
ii. assess the development of the three species of *Fusarium* in maize leaves tissue by using microbiological assay.
iii. examine the development of these the species of *Fusarium* in maize leaves using real time PCR.
iv. evaluate the relationships between disease severity, colonization and fungal biomass during the development of these three *Fusarium* species in maize leaves.

4.2. Materials and methods

4.2.1. Fungal pathogen and inoculum preparation

Fusarium proliferatum (Matsushima) Nirenberg, isolate AG31g, *F. verticillioides* (Sacc.) Nirenberg, isolate AG11i and *F. graminearum* isolate AG 23d were used in the study. The isolates were originally obtained from maize kernels collected in Germany (Görtz, 2008). For the propagation of *Fusarium* conidia either full-strength or low-strength Potato Dextrose Agar (LSPDA) or Potato Dextrose Broth (PDB) were used. Ingredients of the culture media were described in section 2.2.2. Fungal inoculum was obtained from stock cultures prepared according to Moradi (2008) as outlined in section 2.2.2.

4.2.2. Cultivation of plant

Maize cv. Tassilo was used in the studies. The maize seeds were surface sterilized with hot water (Rahman et al., 2008) and grown as described in section 2.2.2.

4.2.3. Experimental design

Maize seedlings were inoculated as described in section 2.2.3.5. In brief, 15 day old maize seedlings were inoculated by: i) hand spraying on the 4^{th} leaf (mature leaf) and ii) adding a droplet (750µl) of fungal suspension into the whorl of the 6^{th} emerging leaf (immature leaves). Control plants were treated with water in a similar manner. Inoculated plants were incubated in a growth chamber under high humidity for 48 h and then maintained at day and night temperatures ranging from 18-20 ^{o}C or 22-24 ^{o}C, and a relative humidity of 45-55% and 75-83%, respectively. A photoperiod of 15 hours and light intensity of 180000-20000 lux was used.

A second experiment was carried out under greenhouse condition under high and low humidity conditions. Maize plant cultivation and inoculation were carried out as described above. After inoculation, plants were kept in high humidity condition for 48 hai and then divided into two humidity regimes: (1) 50-60% relative humidity (RH, low humidity condition) and (2) 80-90 % RH (high humidity condition) under a 16 hr photoperiod. Light intensity was 4000-5000 lux for both humidity conditions. Temperature and humidity were recorded by data logger (appendices 4.1).

4.2.4. Plant growth

Plant growth was recorded at 5, 10, 20 and 40 days after inoculation (dai). At each sampling, eight plants were randomly selected and plant height, crop biomass and leaf weight determined. Maize height was measured from the soil surface to the top of the longest leaf. Plant biomass was measured cutting off the stem at the soil surface and determining fresh stem weight using a balance. For leaf weight, the 4^{th} leaf was cut at the collar and its fresh weight recorded.

4.2.5. Disease incidence and disease severity

Disease incidence was measured as that proportion of plants that were diseased and disease severity was estimated as percentage of the leaf area showing symptoms out of total leaf area. Disease incidence and disease severity were scored at 10, 20 and 40 days after inoculation. Eight plants were evaluated for each sampling time.

4.2.6. Re-isolation

Re-isolation of the fungi from the plant leaves was performed after surface sterilization of the 4^{th} leaf, 6^{th} and 7^{th} leaves 10, 20, and 40 dai. Eight plants (replications) per treatment were evaluated for each sampling time.

Maize leaves were cut randomly into small pieces (0.5 cm^2) and placed into tea paper bags and then immersed in NaOCl (1.3%) solution for two minutes. The leaves in the bags were then rinsed twice in sterile distilled water for two minutes each and then dried on sterile tissue paper inside a laminar airflow cabinet. After that, seven pieces of leaf tissues per treatment were plated onto CZID-agar plates. To assess the effectiveness of the surface sterilization procedure, tissue imprints were made on CZID-agar plates prior to plating (Schulz and Boyle, 2005). All plates were incubated at room temperature (22 ± 3 ^oC) for 5-7 days before colonization assessment was carried out. The frequency of colonization (re-isolation frequency) was calculated as number of pieces exhibiting fungal outgrowth of *Fusarium* per total number of tissue pieces examined multiplied by 100.

4.2.7. Microscopy

4.2.7.1. Stereo microscopy

A Leica MZ16 F stereo microscope (Leica Microsystems, Wetzlar, Germany) was used for assessment of microscopic characteristics of symptom development on the maize leaves. Fresh specimens were directly observed and photographed with a fitted camera and observed on a connected screen.

4.2.7.2. Light microscopy

For the observation of fungal structures on the surface of the inoculated leaves, fresh specimens were cut (1 cm^2), mounted on a microscopic slide in diethanol (0.01%) and covered with a cover slip. The specimens were observed with the BP 340-380/FT 400/LP 430 filter combination using the Leitz microscope in a fluorescence mode (Leitz DMR photomicroscope from Leica). Images were photographed with a digital camera and saved using the program "Discus" (Technisches Büro Hilgers, Koenigswinter, Germany).

4.2.8. Fungal biomass analysis

4.2.8.1. DNA extraction from fungal culture

Three *Fusarium* species *F. graminearum, F. verticillioides* and *F. proliferatum* were grown in potato dextrose broth (24 g/L, Merck, Darmstadt, Germany) in 100 mL Erlenmeyer flasks on a shaker in an incubator at 22 ^{o}C and 120 rpm. The mycelia were harvested after 5 days by filtration and stored in 15-mL tubes then freeze-dried. A sterile steel stick was put in each tube and whirled by vortex until mycelia became a powder form. 18-20 mg of ground mycelia were used for DNA extraction. A Qiagen DNeasy (QIAGEN N.V., Netherlands) plant mini kit was used to perform DNA extractions. DNA were eluted into 100 µL of elution buffer and stored at -20°C until required.

4.2.8.2. Fungal DNA extraction from leaf samples

Fungal biomass was analyzed in inoculated $4^{th}$and 6^{th} , 7^{th} leaves at 0, 2, 5, 10, 20 and 40 dai. Four replications per treatment and each replication consisting of 2 plants were evaluated for each sampling time. Leaf samples were stored in -20 and then freeze-dried. Lyophilised leaves were ground to a fine powder using an unltracentrifugal mill MM200 (Retsch, Germany). 18-20 mg of ground maize leaf tissue was used for DNA

extraction. A Qiagen DNeasy plant mini kit was used to perform DNA extractions. The extraction process followed instructions of the producer (appendices 4.2). DNA were eluted into 100µL of elution buffer and stored at -20°C until required.

4.2.8.3. Polymerase chain reaction (PCR)

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

Primers and Probes

Primers and probes used for quantification of genomic DNA of *F. graminearum* were designed by Waalwijk et al. (2004) and for the Fumonisins producing species *F. proliferatum* and *F. verticillioides* designed by Waalwijk et al. (2008).

Fusarium spp.	Primers/ probes	Sequences (5'-3')
F. graminearum	*F. graminearum* MGB-F	GGCGCTTCTCGTGAACACA
	F. graminearum MGB-R	TGGCTAAACAGCACGAATGC
	F. graminearum MGB-probe	AGATATGTCTCTTCAAGTCT
Fumonisins producing species	Taqfum-2F	ATGCAAGAGGCGAGGCAA
	Vpgen-3R	GGCTCTCA/GGAGCTTGGCAT
	FUM-probe1	CAATGCCATCTTCTTG

F. graminearum

The reaction mixture for *F. graminearum* (30 µl) 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 2 min at 50 ^{o}C and 10 min at 95 ^{o}C, followed by 40 cycles of 95 ^{o}C for 15 s and 60 ^{o}C for 1 min.

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 mixture for *F. proliferatum or F. verticillioides* 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.

The amplification consisted of a single cycle of 20 s at 95 ^{o}C, followed by 40 cycles of 95 ^{o}C for 01 s and 60 ^{o}C for 20 s.

4.2.8.4. Quantification of genomic DNA

DNA concentrations of *Fusarium* species in the standards were determined spectro-photometrically by measuring optical density at 260nm using a nanophotometer (Implen, Munich, Germany).

Pure DNA of the different *Fusarium* species were diluted into four 10-fold serial dilutions (9, 90, 900, 9000 pg/µl). Quantification of fungal DNA was performed using the standard curve method. The standard curve included at least 5 points of dilution, each of them in duplicate. Plotting these points on a standard curve determined the linearity, the efficiency, the sensitivity and the reproducibility of assay by the Step One software version 2.2.2. The correlation coefficient (R^2-value) of standard curves was at least 0.99.

4.2.9. Data analysis

All data were tested for normality and homogeneity of variance using the Kolmogorov or Shapiro-Wilk tests prior to subjecting data to analysis of variance (ANOVA). IRRISTAT statistical package (version 5.0, International Rice Research Institute) was used to analyze the data. Data on disease incidence were arcsine square root transformed before carrying out ANOVA. When significant differences occurred across treatments, the mean comparisons were performed by using Duncan's new multiple range test or LSD at 5% significant level. Pearsons correlations coefficients were used to calculate the relationship between the variables such as re-isolation frequency, disease severity, fungal DNA content.

4.3. Results

4.3.1. Relationship between fungal biomass and symptom manifestation of infected maize plants by *F. graminearum, F. proliferatum* and *F. verticillioides* under controlled conditions

4.3.1.1. Disease severity

Symptoms appeared only after inoculation of immature leaves (the 6th, and 7th leaves). The symptoms of each *Fusarium* species were described in section 2.3.5. Disease severity in this experiment varied from 5.38 to 11.67 % and there were no significant differences between the *Fusarium* species tested and sampling times (Table 4.1).

4.3.1.2. Fungal biomass

Ten days after inoculation, the highest DNA content was on the treatments inoculated on the 6th, 7th leaves with *F. proliferatum* (814 *pg/ mg DW)* and significantly different with *F. proliferatum* inoculated on the 4th leaf as well as for *F. graminearum* and *F. verticillioides*. However, at 20 dai, fungal biomass was reduced and no significant effects either for the *Fusarium* species or the 4th and 6th, 7th leaves were detected (Table 4.2).

Table 4.1. Disease severity (%) on maize leaves cv. Tassilo inoculated with *Fusarium* species at 10 and 20 days after inoculation under controlled conditions.

F. species	10 dai	20 dai	Mean
F. graminearum	7.00 ± 1.29 (1)	10.17 ± 2.66	8.58
F. proliferatum	6.42 ± 1.31	11.67 ± 4.25	9.04
F. verticillioides	5.38 ± 2.41	10.88 ± 4.88	8.13

(1): Standard error of mean (n=4)

Table 4.2. Content of fungal DNA *(pg/ mg DW)* in maize leaves cv. Tassilo following inoculation of the 4th, 6thand 7th leaves with species of *Fusaium.*

Leaves	*F.* species	10 dai		20 dai	
4th leaf	*F. graminearum*	82.4 ± 11.6 (1)	b	40.9 ± 17.2	
	F. proliferatum	277.2 ± 81.0	b	111.6 ± 25.3	
	F. verticillioides	275.1 ± 39.0	b	179.7 ± 30.6	
6th,7th leaves	*F. graminearum*	251.2 ± 77.3	b	119.1 ± 36.2	
	F. proliferatum	814.3 ± 171.8	a	554.0 ± 244.2	
	F. verticillioides	224.2 ± 42.3	b	120.2 ± 39.8	

(1): Standard error of mean (n=4).
In column, means followed by the same letters or without letter are not significantly different at P≤0.05 (Duncan's test).

4.3.1.3. Correlations between disease severity and fungal biomass

The relationship between disease severity and fungal biomass was positive, but the correlation was not significant at the 0.05 level (Table 4.3).

Table 4.3. Correlation coefficients (r) for disease severity and fungal biomass of *Fusarium* species at 10 and 20 days after inoculation of the 6th and 7th leaves.

Sampling times	Severity and fungal biomass
10 dai	0.296 (1) ns (P=0.350, n=12)
20 dai	0.534 ns (P=0.074, n=12)

(1) Pearson's =r, n=12. Ns: Correlation is not significant at the 0.05 level (2-tailed).

4.3.2. Relationships between fungal biomass, symptom manifestation and infection of maize plant by *F. graminearum, F. proliferatum* and *F. verticillioides* under low and high humidity conditions

4.3.2.1. Effect of *Fusarium* infection on maize plant growth

The height of the stems of the maize plants was neither affected by fungi infection nor humidity condition at 5, 10 and 20 dai. However, at 40 dai, plant growth was increased under high RH compared to low RH (P= 0.000). The inoculation with the *Fusarium* species had no effect on the development of plant height (Table 4.4).

Similarly, the weight of the maize plants was not significantly different when plants were inoculated with the different species of *Fusarium* (Table 4.5). Humidity conditions, however, influenced plant weight at 40 dai (P= 000) but did not significantly affect early growth stages at 5, 10 and 20 dai.

The weight of the 4th leaf was 1 -1.1 gram/leaf. The Infection of *Fusarium* did not have a significant effect on the weight of the 4th leaf over all sampling times in the different humidity conditions. However, at 40 dai, the weight of the 4th leaf was reduced 2-10 times in comparison with leaf weight at 5 to 20 dai (Table 4.6).

4.3.2.2. Effect of *Fusarium* species on disease incidence, disease severity and symptom development

Symptom development occurred 5 dai for *F. graminearum* and 7 dai by *F. proliferatum* and *F. verticillioides*. The incidence of disease varied from 50 to 87.5%, but they were not significantly different between the *Fusarium* species, humidity conditions, and time course (Table 4.7).

The 6 and 7th leaves showed holes, chlorotic spots and streaks with damage from 2.0 – 6.7 %. Disease severity was not significantly affected by the species of *Fusarium* nor humidity condition (P>0.05). However, the development of lesions was obviously different. For example, the lesions caused by *F. graminearum* under low RH were grey in the centre, brown spots and had edges between healthy and unhealthy tissue (Fig. 4.1 A) while lesions under high RH appeared water-soaked in the centre with a yellow area

and no edges between healthy and unhealthy region (Fig. 4.1 B). Mycelia of *F. graminearum* grew denser under high RH (Fig. 4.1 D) than in low RH (Fig. 4.1 C). Similarly, yellow necrotic lesions with brown edges (Fig. 4.1 E) were observed by *F. verticillioides* in low RH while in high RH the lesions were transparent streaks without limited edges (Fig. 4.1 F). The symptoms caused by *F. proliferatum* were exhibited a dark brown boundary line between the holes and the green interior of the leaf under low RH (Fig. 4.2 A) but in high RH, the boundary line was not clear, only a yellowish line appeared between the holes and the green interior of the leaf (Fig. 4.2 B). In addition, under high RH, dense mats of mycelia formed but were rarely seen under low RH (Fig. 4.2 C, D).

Table 4.4. Effect of *Fusarium* infection on height (cm) of maize plants from 5 to 40 days after inoculation under low and high relative humidity.

Humidity	Treatments	5 dai	10 dai	20 dai	40 dai
Low relative humidity	*F. graminearum*	69.3	74.5	98.6	130.9 b(1)
	F. proliferatum	70.3	78.1	101.0	132.1 b
	F. verticillioides	67.9	74.5	96.6	137.8 b
	Treated water		78.4		140.9 b
High relative humidity	*F. graminearum*	70.5	75.6	100.8	146.0 a
	F. proliferatum	69.4	78.7	98.0	154.8 a
	F. verticillioides	70.0	77.3	98.5	149.8 a
	Treated water		79.4		150.3 a

(1) In column, means followed by the same letters or without letters are not significantly different at P≤0.05 (Duncan's test).

Table 4.5. Effect of *Fusarium* infection on weight (gram) of maize plants from 5 to 40 days after inoculation under low and high relative humidity.

Humidity	Treatments	5 dai	10 dai	20 dai	40 dai
Low relative humidity	*F. graminearum*	9.0	12.3	29.3	54.1 b[(1)]
	F. proliferatum	8.5	12.1	25.1	56.1 b
	F. verticillioides	9.1	13	27.3	61.5 b
	Treated water		14.2		50.5 b
High relative humidity	*F. graminearum*	8.6	11.0	27.5	65.2 a
	F. proliferatum	8.4	13.1	27.3	65.7 a
	F. verticillioides	8.4	11.1	24.9	66.2 a
	Treated water		14.1		60.5 a

[(1)]In column, means followed by the same letters or without letters are not significantly different at P≤0.05 (Duncan's test).

Table 4.6. Effect of *Fusarium* infection on weight (gram) of the 4th leaf of maize plants from 5 to 40 days after inoculation under low and high relative humidity.

Humidity [(1)]	Treatments	5 dai	10 dai	20 dai	40 dai
Low relative humidity	*F. graminearum*	1.1	1.1	1.2	0.1
	F. proliferatum	0.9	1.1	1.1	0.3
	F. verticillioides	1.1	1.2	1.1	0.2
	Treated water		1.1		0.2
High relative humidity	*F. graminearum*	1.2	1.1	1.1	0.1
	F. proliferatum	1.0	1.1	1.1	0.3
	F. verticillioides	1.4	1.2	1.0	0.5
	Treated water		1.1		0.2

Table 4.7. Disease incidence (%) on maize plants inoculated with species of *Fusarium* from 10 to 40 days after inoculation under low and high humidity condition.

Humidity	Fungi	10 dai	20 dai	40 dai
Low relative humidity	*F. graminearum*	62.5	75.0	50.0
	F. proliferatum	75.0	75.0	62.5
	F. verticillioides	75.0	75.0	50.0
High relative humidity	*F. graminearum*	75.0	62.5	87.5
	F. proliferatum	50.0	87.5	50.0
	F. verticillioides	75.0	87.5	75.0

Table 4.8. Disease severity (%) on maize leaves inoculated with species of *Fusarium* from 10 to 40 days after inoculation under low and high humidity condition.

Humidity	Fungi	10 dai	20 dai	40 dai
Low relative humidity	*F. graminearum*	2.39	2.25	2.50
	F. proliferatum	2.44	3.06	4.69
	F. verticillioides	2.00	2.50	2.19
High relative humidity	*F. graminearum*	5.69	6.44	5.94
	F. proliferatum	2.63	2.75	3.19
	F. verticillioides	6.69	4.13	4.00

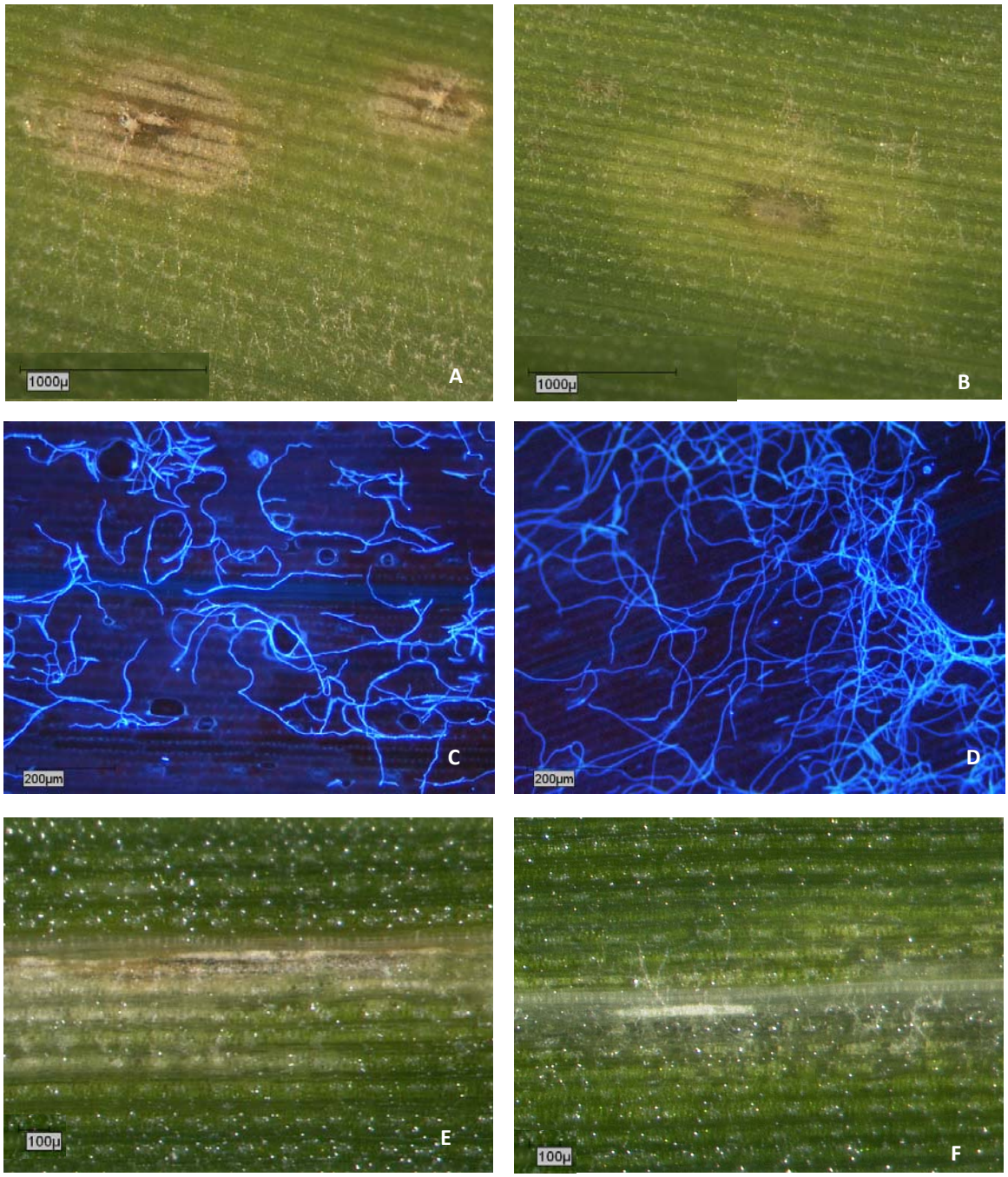


Figure 4.1. Symptoms and mycelium growth on maize leaves inoculated with *Fusarium graminearum* under low humidity conditions (A, C), and high humidity conditions (B, D). Symptoms on maize leaves inoculated with *F. verticillioides* under low humidity conditions (E) and high humidity conditions (F), 20 dai. A, B, E, F: stereo microscope photographs, C, D: light microscope photographs.

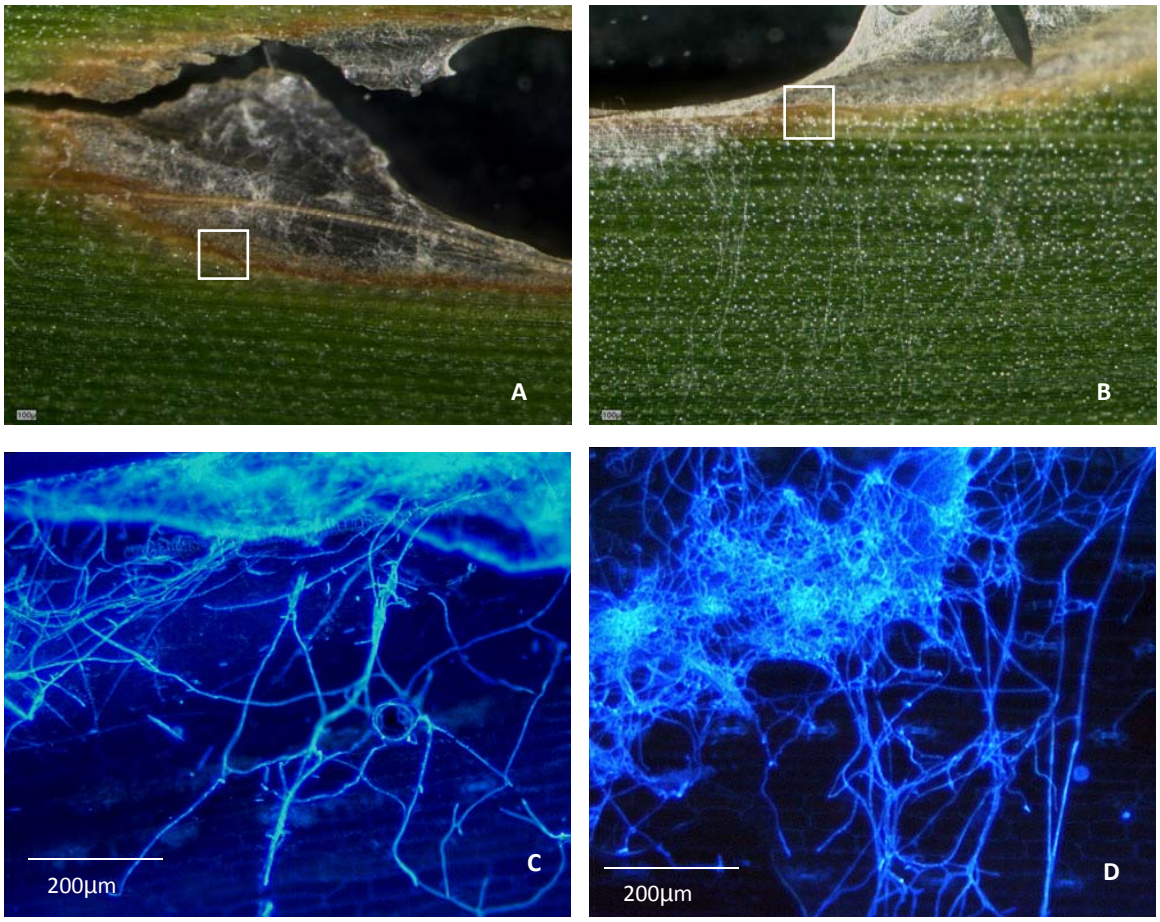


Figure 4.2. Symptoms and presence of mycelia on maize leaves inoculated with *Fusarium proliferatum* under low humidity conditions (A) and high humidity conditions (B). C= box area in A, close-up of mycelia growth, D= box area in B, close-up of mycelia growth, 20 dai. A, B: stereo microscope photographs, C, D: light microscope photographs.

4.3.2.3. Re-isolation frequency

The colonization of *Fusarium* species was affected by humidity condition. Colonization by *Fusarium* spp. under high RH conditions was significantly higher than under low RH conditions (P=000). Leaf age ($4^{th,}$ 6^{th} and 7^{th} leaf) did not conspicuously influence fungal colonization at 10 dai (P= 0.53). However, at 20 and 40 dai, fungal colonization was significant (P= 0.007 and 0.028 respectively). For example, the frequency of re-isolation from the 4^{th} leaf was lower than that of the 6^{th} and 7^{th} leaves. Colonization of *F. proliferatum* and *F. verticillioides* were higher than *F. graminearum* at 10 dai (P= 0.002, Fig. 4.3). A statistical interaction among the three factors (fungal species, humidity and

inoculation site) were found at 20 and 40 dai with P= 0.014 and 0.001 respectively (Fig. 4.3). At 20 dai, the frequency of re-isolation of *F. verticillioides* was significantly higher (46%) under high RH conditions than that under low humidity conditions (26%). At 40 dai, the frequency of colonization by *F. graminearum* (86%) and *F. proliferatum* (69%) increased under high RH whereas colonization by *F. graminearum* (19%) decreased under low RH (Fig. 4.3). The frequency of colonization did not increase from 10 to 20 dai but it increased greatly at 40 dai.

4.3.2.4. Biomass of *Fusarium* species in maize leaves

After inoculation, maize plants were incubated in the same chamber for a total of 48 hours. Therefore, samples and data were collected only once.

Although the same concentration of conidia of each *Fusarium* species was used for inoculation, *F. graminearum* DNA content (6155 pg/mg DW) was significantly higher than *F. proliferatum* (2944 pg/mg DW) and *F. verticillioides* (2281 pg/mg DW) (P= 0.000) at 0 dai. However, fungal DNA content was not significantly different between the 4th and the 6th and 7th leaves 0 dai (P = 0.207, Fig. 4.4).

Two dai, fungal DNA extracted from the 4th leaf was highest and followed the 6th and 7th leaves for *F. graminearum* and its DNA content was highest followed by *F. proliferatum* and *F. verticillioides*. However, there was no interaction between the 4th or 6th and 7th leaves and *Fusarium* species at this early phase after inoculation 2 dai (P= 0.093) (Fig. 4.4).

Fungal DNA concentration in plants cultivated under high RH was significantly higher than that in low RH at 5, 10 and 20 dai (Fig. 4.4). Fungal DNA on the 4th leaf was higher than that on the 6th and 7th leaves at 10, 20 and 40 dai (P=0.006, 0.001 and 0.000, respectively). *F. graminearum* DNA content was significantly higher than *F. proliferatum* and *F. verticillioides* 5 dai. The interactions among humidity conditions, age of leaves and *Fusarium* species were found. *F. graminearum* and *F. proliferatum* DNA content in the 4th leaf in high RH conditions were very high, 210680 pg/mg DW for *F. graminearum* and 94024 pg/mg DW for *F. proliferatum* (Fig. 4.4, 40 dai). Fungal DNA varied over time,

i.e. fungal DNA of all species increased from 0 dai to 5 dai (3796 to 4674 pg/mg DW). Then the content decreased from 5 dai to 20 dai (4674 to 1602 pg/mg DW) and significantly increase again afterward (1602 to 28983 pg/mg DW) (multifactor analysis, LSD 5%= 3541; P=000; n=120).

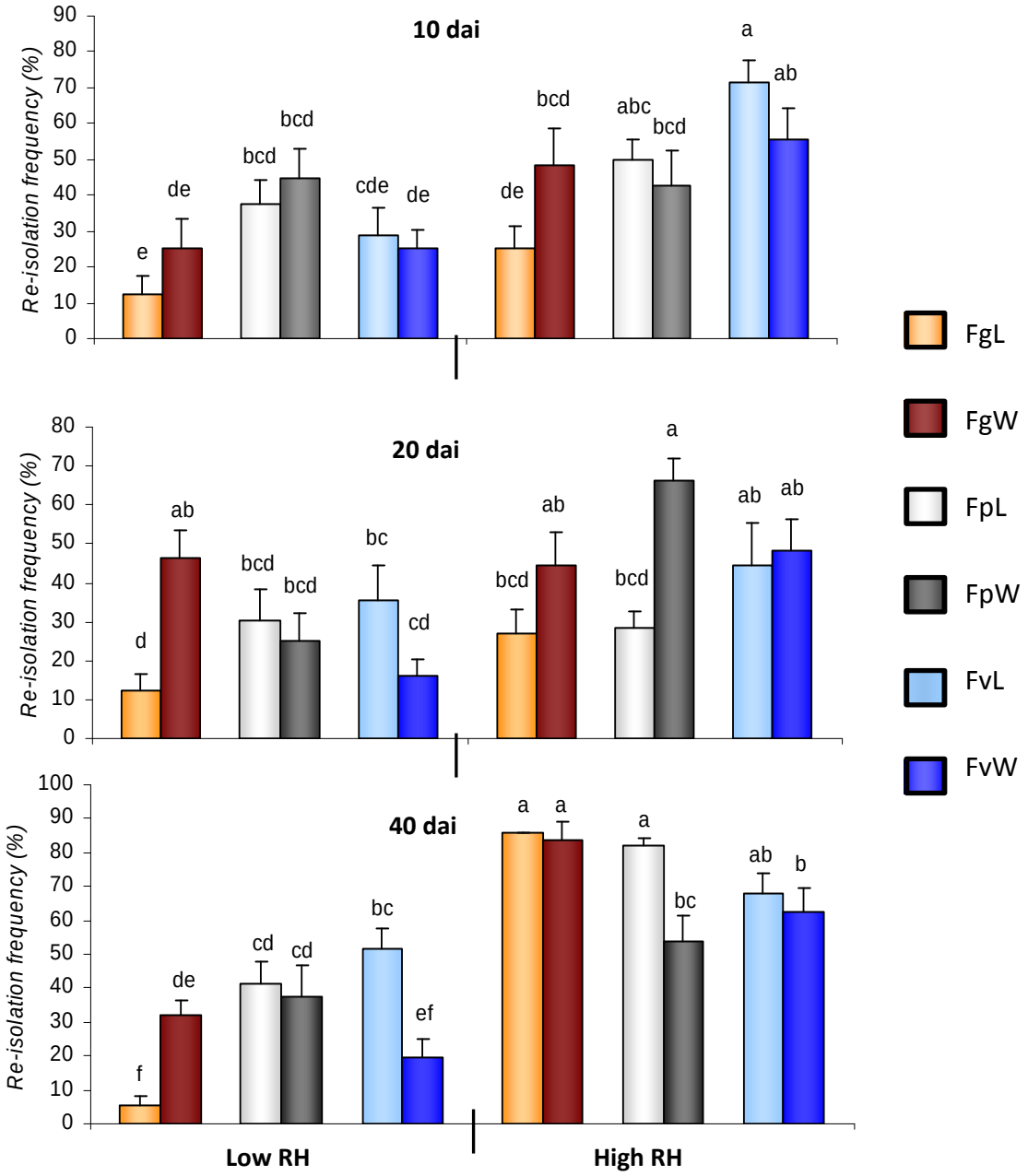
4.3.2.5. Correlations: Colonization, fungal biomass, disease severity

Analysis of correlations between colonization, severity of symptoms and fungal biomass were determined for the leaves exhibiting symptoms of disease on the 6^{th} and 7^{th} leaves. Correlation analysis was also determined for colonization and fungal DNA content on the symptomless 4^{th} leaf.

For the leaves without symptoms, a significant correlation between colonization and fungal biomass occurred at 40 dai (r=0.623, P=0.001, n=24, Table 4.9) but was not correlated at 10 and 20 dai.

For leaves with symptoms, the colonization was not strongly correlated with disease severity at 10 and 20 dai, but it was statistically positively correlated at 40 dai.

Fungal biomass correlated with disease severity at the beginning of disease development 10 dai and this correlation became weaker over time (table 4.10). The colonization did not correlate with fungal biomass.



Fugure 4.3. Frequency of *Fusarium* infection of maize leaves under low and high relative humidity (RH). L, the 4th leaf and W, the 6th, 7th leaves were inoculate by *F. graminearum* (Fg), *F. proliferatum* (Fp) and *F. verticillioides* (Fv). Bars follow by the same letters are not significantly different at P≤ 0.05 (Duncan's test). Error bars represent the standard error of the mean.

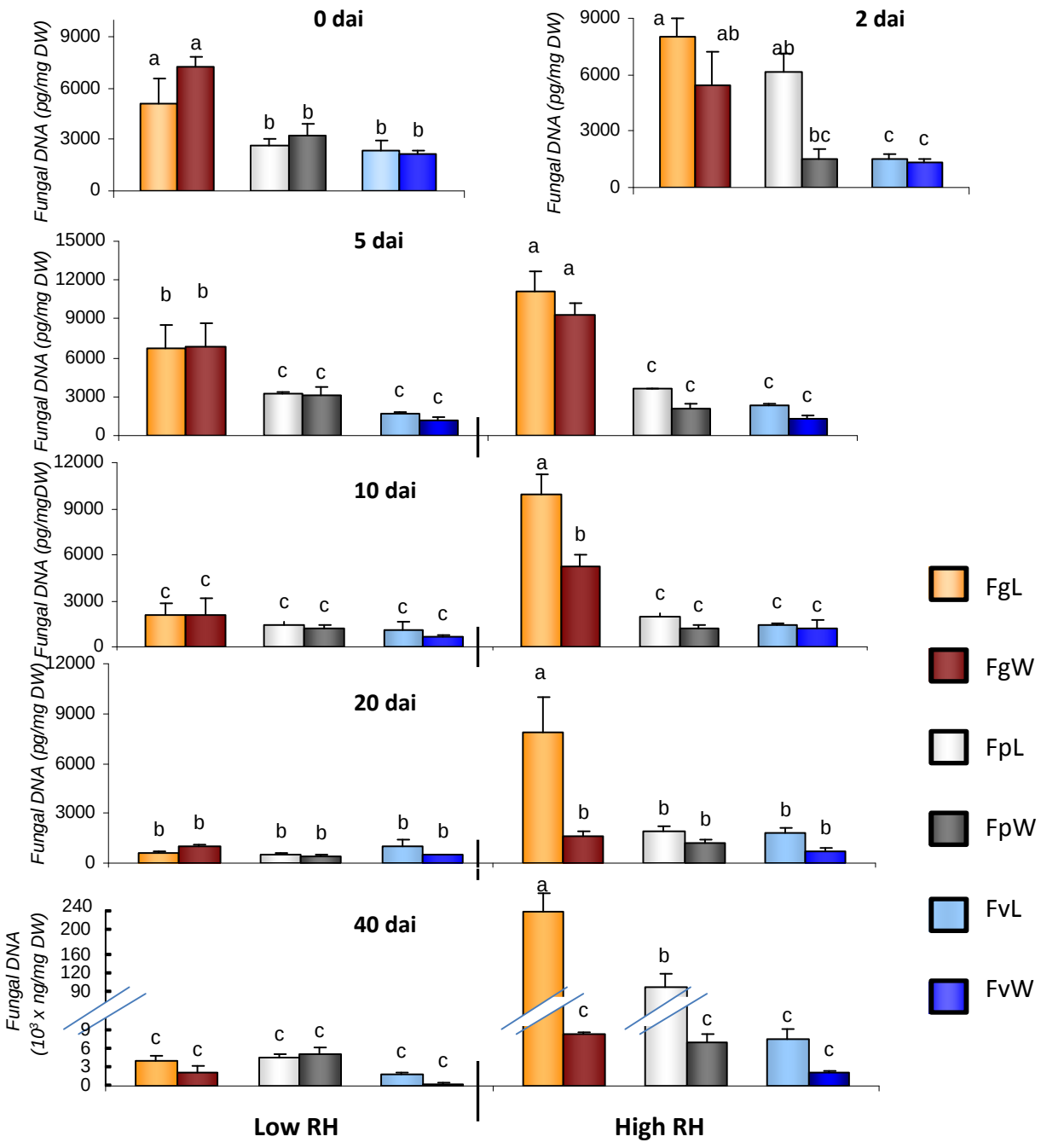


Figure 4.4. Fungal biomass in inoculated maize leaves under low and high relative humidity (RH). L, the 4th leaf and W, the 6th, 7th leaves were inoculate by *F. graminearum* (Fg), *F. proliferatum* (Fp) and *F. verticillioides* (Fv). Bars follow by same letters are not significantly different at P≤ 0.05 (Duncan's test). Error bars represent the standard error of the mean.

Table 4.9. Correlation coefficients (r) for colonization and fungal biomass of *Fusarium* species from 10 to 40 days after inoculation of the 4th leaf.

Sampling times	Colonization and fungal biomass
10 dai	-0.066 (1) ns (P=0.426)
20 dai	0.182 ns (P=0.396)
40 dai	0.623 ** (P=0.001)

(1) Pearson's =r, n=24.
Ns: Correlation is not significant at the 0.05 level, * *: Correlation is significant at the 0.01 level (2-tailed).

Table 4.10. Correlation coefficients (r) for severity, colonization, and fungal biomass of *Fusarium* species from 10 to 40 days after inoculation of the 6th and 7th leaves.

Sampling times	Severity and colonization	Severity and fungal biomass	Colonization and fungal biomass
10 dai	0.344 (1) ns (P=0.099)	0.412 * (P= 0.046)	0.235 ns (P= 0.269)
20 dai	0.228 ns (P=0.284)	0.306 ns (P= 0.143)	0.247 ns (P=0.244)
40 dai	0.436 * (P=0.033)	-0.098 ns (P= 0.648)	0.374 ns (P= 0.072)

(1) Pearson's =r, n=24.
Ns: Correlation is not significant at the 0.05 level, *: Correlation is significant at the 0.05 level (2-tailed).

4.4. Discussions

Quantification of the amount of fungal DNA in host plants using polymerase chain reaction (PCR) is common (Mulè et al., 2004; Waalwijk et al., 2004; Strausbaugh et al., 2005; Waalwijk et al., 2008; Nicolaisen et al., 2009; Faria et al., 2011; Atoui et al., 2012). Besides this new technique, microbiological bio-assay, isolation frequency and visual disease ratings have been used. In this study, both microbiological and real time PCR assay were used to evaluate the infection process of three species of *Fusarium* on maize

leaves. The comparison between disease severity with colonization, disease severity with quantification of fungal biomass, colonization with quantification of fungal biomass may yield much more information on the host pathogen interrelationships.

F. graminearum biomass was higher than that of *F. proliferatum* and *F. verticillioides* at 0 and 2 dai when incubated in high humidity conditions although the same amount of conidia was applied. This may be explained by the fact that the conidia of *F. graminearum* are macroconidia (5-7 cells) while *F. proliferatum* and *F. verticillioides* conidia are microconidia with one to 2 cells only. Macroconidia formed 2 to 6 germ tubes while microconidia formed 1-2 germ tubes and mycelia of *F. graminearum* grew denser than that of other species (section 3).

The infection process of *Fusarium* includes spore germination, penetration and colonization, and further spread in the leaf (section 3). Similar findings were reported by Kang and Buchenauer (2000a) and Boenisch and Schäfer (2011). Stephens et al. (2008) used both histological and real-time quantitative polymerase chain reaction analyses to assess *F. graminearum* infection processes. Phase 1 was spore germination with the increase of fungal biomass during 0-2 dai. Phase 2 was colonization with the decrease of fungal biomass and phase three was extensive colonization which increased fungal biomass from 14-35 dai. In this study, the infection process also occurred in 3 phases: fungal DNA content increased from 0 to 5 dai, then decreased to 20 dai and increased again afterward.

F. graminearum biomass production under high humidity conditions was always 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. Moradi (2010) reported that using microbiological and real-time PCR assays gave different results for difference *Fusarium* species except for *F. graminearum* infected wheat kernels. The results obtained in this study demonstrated that *F. graminearum* not only grew endophytically in the leaf tissue but also grew densely over the leaf surface while *F.*

proliferatum and *F. verticillioides* infected the tissue but with a lower amount of superficial infection on the leaf surface than *F. graminearum*. Rapid growth and producing large amounts of dense mycelia were characteristic for *F. graminearum* (Nelson et al., 1983) and *F. graminearum* grew well in high humidity (Bottalico, 1998; Miller, 2001). Those characteristics may explain for the differences in the results of the two experiments in growth chamber and in the greenhouse maintained at high relative humidity. *F. graminearum* DNA biomass was highest following *F. proliferatum* and *F. verticillioides* under high humidity conditions in the greenhouse while *F. graminearum* DNA biomass was lowest followed by *F. proliferatum* and *F. verticillioides* under low RH in the growth chamber.

Biomass of *F. graminearum* and *F. proliferatum* in the 4th leaf under high relative humidity at 40 dai (Fig. 4.4) rapidly increased. This implied that (1) the fungus grew continuously and sporulated, and (2) the fungus grew both parasitically and saprophytically (Parry et al., 1995; Munkvold and Desjardins, 1997; Leonard and Bushnell, 2003; Trail, 2009). At 40 days after inoculation, most of the 4th leaf became senescent, brown and dry. Leaf senescence under a high humidity environment is favorable conditions for fungal growth. In the previous (section 3) findings, all three *Fusarium* species were able to produce spores on the infected leaves. Other results were reported by Wagacha et. al. (2012) who showed that all five *Fusarium* species tested formed spores when the senescing infected leaves were incubated at 100 % relative humidity for 48 hr. In the present in their study, sporulation was observed and mycelia grew densely under high humidity conditions than under low humidity. The parasitic and saprophytic capacity of *Fusarium* on maize leaves increased fungal biomass 20-30 fold and may have led to the distribution of inoculum potential for infection of the upper leaves, cobs and even into the next seasonal crops. Dill-Macky and Jones (2000) found that incidence and severity of Fusarium head blight were highest if wheat was grown following a maize crop. The growth of *Fusarium* in low relative humidity was lower than that in high relative humidity. This implied that *Fusarium* spore formation

and continuous growth either as a pathogen parasite or saprophyte were influenced by humidity.

The incidence and severity of *F. graminearum*, *F. proliferatum* and *F. verticillioides* were similar under high and low relative humidity, but colonization was higher under high humidity than under low humidity. These findings may suggest that the effect of humidity on disease of maize leaves was on the development of *Fusarium* in the host tissue rather than on the appearance of symptoms due to surface parasitism. These results were similar to that reported by Beddis and Burgess (1992) who reported that the effect of water stress on the incidence of *F. graminearum* in wheat seedling was not different under unstressed and stressed water conditions but the colonization was higher under water stressed conditions in seedlings. Papendick and Cook (1974) also showed death of the host by fungi when water potential was low (-35 to -45 bars) at maturity.

The observations made in this study were that plant height, plant weight and infected leaf weight were not reduced when the plants were inoculated by *Fusarium.* In contrast, Knight (2012) reported an increase in leaf sheath weight when infected with *F. pseudograminearum* on wheat seedling. Miedaner (1988) however, found a negative effect on wheat root and shoot weight when inoculated by *F. culmorum.* Similarly, maize seedlings infected by *F. verticillioides* had reduced root and leaf development (Williams et al., 2007). Soil infested by *F. verticillioides* decreased maize seedling weight while seed inoculation had no effect (Oren et al., 2003). Williams also found symptoms of disease on the leaves such as stunting, necrotic lesions. In the present research, the symptoms of Fusarium disease occurred on immature leaves but the severity was low. These results may have been the reason that mild symptoms have not conspicuously affected development of the plants. Knight (2011) reported small lesions on the emerging leaf blade were isolated and failed to develop on fully photosynthetic leaves. However, relative humidity influenced leaf growth, photosynthesis and disease appearance. High turgor pressure and less transpiration under high RH predisposes leaf

enlargement. When RH is low, transpiration increases and there is a decrease in dry matter (Anonym, 2013). These findings may explain the results in this study that plant height and weight were lower under low humidity than under high humidity conditions at 40 dai.

Finding correlations between assessments by visual disease ratings and applying qPCR was done by Knight (2012). Knight found that there were correlations between *F. pseudograminearum* DNA and visual rating of crown rot of wheat leaf sheaths. These relations became weaker at increased sampling times but always were positively correlated. Hogg et al. (2007) reported positive correlations for crown, node 1 and node 2 of spring and durum wheat of disease severity and *Fusarium* biomass (*F. pseudograminearum*) and wheat yield in 2004 but no significant correlations for spring wheat in 2005. In the present study, the comparisons of the fungal biomass assessed by qPCR and disease severity on the $6^{th}$and 7^{th} leaves showed a positive and significant correlation at 10 dai. Then correlations became weaker at later time points. These correlations, however, were not significant in the experiments conducted in growth chambers. The differences in environmental conditions between the two experiments may have effect the results. For example, light intensity (18000-20000 lux in growth chamber and 4000-5000 lux in the greenhouse) and other unmeasured factors (the circle of air and microclimate) may have added effects on the outcome. In addition, symptom occurrence is not only affected by the infection of hyphae alone, but also by other factors such as: mycotoxin, hydrolytic enzyme production or physiological condition of the plant (Oren et al., 2003). Also the production of Fumonisins and the disruption of sphingolipid metabolism in maize roots (Williams et al., 2007) and Fumonisins production needed for foliar disease occurrence on maize seedlings by *F. verticillioides* (Glenn et al., 2008), or the trichothecenes that increase the spread of *F. graminearum* on maize (Harris et al., 1999) may have played a role here too.

In the present studies, the relationship between colonization and disease severity was positive and was significantly correlated at 40 dai. This implied that symptom

appearance was not only the result of fungal growth in the tissue but was also controlled by other factors. The physiological condition of leaves, such as immature, mature or senescence, may have affected the infection process of *the Fusarium* species. The correlation coefficient between re-isolation frequency and fungal DNA content was negative for the 4^{th} leaf (r = -0.066, P= 0.426) while those for the $6^{th}$and 7^{th} leaves was positive (r = 0.235, P=0.269) at 10 dai. However, at 40 dai, correlations for the 4^{th} leaf became stronger (r=0.623, P= 0.001) and for the $6^{th}$and 7^{th} leaves also increased (r= 0.374, P=0.072). These results of the present study demonstrated that that the infection of *Fusarium* on the maize leaves intensifies when the maize leaves became senescent.

In this study, colonization of *Fusarium* and fungal biomass depended on the *Fusarium* species and environmental condition present. Microbiological assay demonstrated the colonization of *Fusarium* in the maize tissue while real time PCR analyzed both superficial and in tissue infection. Under high humidity conditions, *F. graminearum* characteristically produced more superficial infection than *F. proliferatum* and *F. verticillioides* but the colonization of *F. graminearum* in maize leaf tissues was lower than that of *F. proliferatum* and *F. verticillioides.*

5. Summary

Studies on the infection and colonization of maize leaves by *Fusarium graminearum, F. proliferatum* and *F. verticillioides* provided insights into the interaction between *Fusarium* and the host plant maize. This study aimed to identify factors affecting the infection of the three *Fusarium* species into maize leaves, how they infect and colonize leaf tissue and the relation between colonization, disease severity and fungal biomass.

- Two growth stages, GS 15 and GS 35, of maize plants were inoculated with *F. proliferatum* and *F. verticillioides.* The rates of colonization of the leaves inoculated at the growth stage GS 15 was 34% higher than that detected performed at GS 35 (26%) at 13 dai. The influence of growth stage on infection was not different at 26 and 39 dai. The frequency re-isolation of *F. proliferatum* from surface sterilize leaf tissue was 38 % higher than that of *F. verticillioides,* 29 % at 26 dai.
- The level of colonization of the three species depended on spore concentration. Higher spore concentration (10^5, 10^6 and $2x10^6$ spore/mL) resulted in higher re-isolation frequency (18, 44, 51 %), respectively. There was no effect of spore concentration on the level of colonization of *F. proliferatum* and *F. verticillioides* in the maize cultivars, Ronaldinio and Tassilo tested. Disease incidence did not differ between the two cultivars nor was it influenced by spore concentration.
- Light regimes of 5800-6000 lux for 9h/day and 18000-20000 lux for 15h/day, did not affect infection and disease incidence of *F. proliferatum* and *F. verticillioides* on two maize cultivars. Fungal infection (71.9%) was higher on the lower leaves than on the upper leaves (42.1%).
- The comparative analysis of different inoculation sites (mature leaves, the 4^{th} leaf and immature leaves or emerging leaves, the 6^{th}, 7^{th} leaves) on the infection and symptom manifestation of maize plants by *F. graminearum*, *F. proliferatum* and *F. verticillioides* revealed that these *Fusarium* formed symptoms (86.4-90%) on the immature leaves only and asymptomatic infections on the mature leaves. Disease severity was 7.3, 10.0 and 7.1 % for *F. graminearum, F. proliferatum* and *F. verticillioides*, respectively. Colonization was lower on asymptomatic leaves than on

leaves showing typical symptoms of infection. The rates of colonization by *F. graminearum, F. proliferatum* and *F. verticillioides* were 26, 38, 57 % in the asymptomatic leaves and 68, 58 and 57 % in the leaves showing symptoms, respectively.

- Macroscopic symptoms were observed on leaves inoculated with *F. graminearum* starting 4-5 dai and with *F. proliferatum* and *F. verticillioides* from 7-8 dai. The typical symptoms of *F. graminearum* were small water-soaked lesions at the beginning. The lesions then turned into yellow spots with shades of brown or grey in the centre as infection progressed. Small lesions or spots were detected at the site where fungal inoculum was applied into maize whorl. The typical symptoms of *F. proliferatum* and *F. verticillioides* were necrotic lesions, holes and streaks. The holes caused by *F. proliferatum* were usually bigger than that of *F. verticillioides.* Symptoms of *F. proliferatum* and *F. verticillioides* appeared below the site where fungal inoculum was applied into maize whorl or on the leaf tips of the newly emerging leaves after inoculation. Heavily infected leaves showed symptoms of severe deformation or "deadhearts".
- Conidial germination of *Fusarium* species on glass occurred 8 hai whereas germination on the surface of maize leaves was first observed 12 hai. The rate of germination of conidia at 24 hai was 35-41% on the leaf surface and 45-67 % on the diagnostic microscope slides. The densest mycelia network on leaves was formed by *F. graminearum* followed by *F. proliferatum* and *F. verticillioides. F. graminearum* infection of host tissues on asymptomatic leaves, led to the formation of appressoria-like structures which were not found by *F. proliferatum* and *F. verticillioides.* Inter- and intracellular colonization was detected whereas the intracellular infection was limited in one to three leaf cells.
- On leaves showing disease symptom, the penetration of *Fusarium* included subcuticular growth, direct entry through epidermal cells, trichomes, and stomata. The detailed description of the infection process of *F. graminearum* via the trichomes was reported for the first time in this study.

- Infection of leaf tissues via the trichomes was more frequent for *F. graminearum* than *F. proliferatum* and *F. verticillioides.* Infection of leaf tissues via the stomata by forming appressoria was seen by *F. graminearum* and *F. verticillioides and the* formation of a cushion and direct penetration were seen for all three species.
- Frequency of infection via the stomata was highest for *F. proliferatum* followed by *F. verticillioides* and *F. graminearum.* Inter- and intracellular infection of epidermal and mesophyll cells were observed for all three species. On the detached leaves, however, *F. graminearum* colonized inter- and intracellular parenchyma cells as well as sclerenchyma cells, xylem and the phloem vessels.
- Sporulation of three species occurred from superficial hyphae and from re-emerging hyphae. Superficial hyphae sporulated early, starting 48 hai and re-emerging hyphae sporulated 9 dai for *F. proliferatum,* 15 dai for *F. graminearum* and 18 dai for *F. verticillioides*. Interestingly, *F. proliferatum* formed microconidia inside infected tissues and sporulated through stomata and trichomes. Superficial hyphae and hyphae re-emerging from stomata and epidermal cells of *F. proliferatum* also produced macroconidia.
- The height and weight of the maize plants were not affected by infection of any of the three *Fusarium*. However, high humidity did stimulate plant growth as measured in height and weight of maize plants at 40 dai. Disease incidence and disease severity were not significantly different regardless of *Fusarium* species inoculated nor due to humidity conditions, and time of exposure.
- The concentration of fungal DNA in the tissue increased from 0 to 5 dai, then decreased from 5 to 20 dai and increased again thereafter. Fungal DNA content on the 4^{th} leaf and on the 6^{th} and 7th leaf did not differ under low humidity condition from 0 to 40 dai. However there were differences in DNA under high humidity condition on plants inoculated with *F. graminearum* from 10 to 40 dai and with *F. proliferatum* at 40 dai. At 40 dai, fungal DNA content on the 4^{th} leaf under high humidity condition was high (210680, 94024, 7451pg/mg DW for *F. graminearum*, *F. proliferatum* and *F. verticillioides,* respectively).

- A significant positive correlation between fungal biomass (assessed by qPCR) and disease severity on leave showing symptoms of disease was measured 10 dai and became weaker 20 and 40 dai. These correlations, however, were not significant in other experiment. Significant positive correlations of the disease severity and colonization were detected 40 dai. There was a significant positive correlation between rate of colonization and fungal biomass at 40 dai on asymptomatic leaves compared to a non-significant correlation for the leaves showing disease symptoms.

The three *Fusarium* species infected and colonized maize leaves with and without causing symptoms and they sporulated early and over long periods of time after infection. This indicates the importance of leaf infections in the diseases cycle as a mean of extending *Fusarium* infection to silks, kernels and later over the field and into next cropping season. In general, there were no significant differences in the levels of aggressiveness between *F. proliferatum*, *F. graminearum*, and *F. verticillioides* in causing infection and disease on maize leaves.

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Appendix

4.2.1. Temperature and humidity recorded by data logger

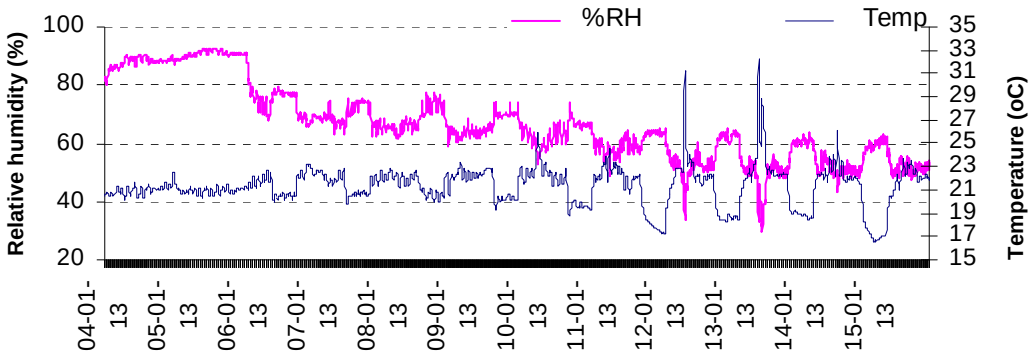

Figure 4.1. Humidity and temperature in normal chamber condition from 0 to 11 dai. Data were recorded by datalogger.

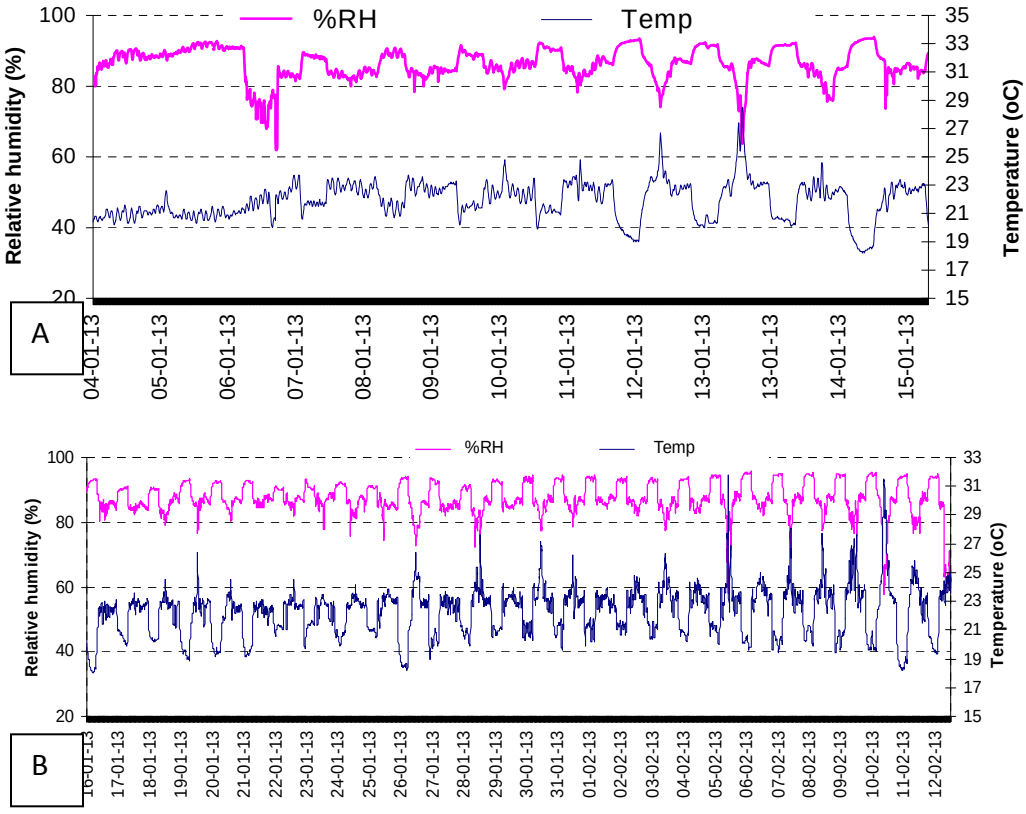

Figure 4.2. Humidity and temperature in high relative humidity. A: from 0 to 11 dai, B: 12 to 40 dai. Data were recorded by datalogger.

4.2. 2. Fungal DNA extraction from leaf samples

Lyophilised leaves were ground to a fine powder using an unltracentrifugal mill MM200 (Retsch, Germany). 18-20 mg of ground maize leaf tissue was used for DNA extraction. A Qiagen DNeasy plant mini kit was used to perform DNA extractions. The extraction process followed instructions of the producer (Qiagen, 2012) as below.

1. Add 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) to a maximum of 20 mg (dried) disrupted plant or fungal tissue and vortex vigorously.
2. Incubate the mixture for 10 min at 65°C. Mix 2 or 3 times during incubation by inverting tube.
3. Add 130 µl Buffer AP2 to the lysate, mix, and incubate for 5 min on ice.
4. Centrifuge the lysate for 5 min at 14,000 rpm.
5. Pipet the lysate into the QIAshredder Mini spin column, placed in a 2 ml collection tube, and centrifuge for 2 min at 14,000 rpm.
6. Transfer the flow-through fraction from step 5 into a new tube (not supplied) without disturbing the cell-debris pellet.
7. Add 1.5 volumes of Buffer AP3/E to the cleared lysate, and mix by pipetting.
8. Pipet 650 µl of the mixture from step 7, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at 8000 rpm and discard the flow-through.
9. Repeat step 8 with remaining sample. Discard flow-through and collection tube.
10. Place the DNeasy Mini spin column into a new 2 ml collection tube (supplied), add 500 µl Buffer AW, and centrifuge for 1 min at 8000 rpm. Discard the flow-through and reuse the collection tube in step 11.
11. Add 500 µl Buffer AW to the DNeasy Mini spin column, and centrifuge for 2 min at 20,000 x g (14,000 rpm) to dry the membrane.
12. Transfer the DNeasy Mini spin column to a 1.5 ml or 2 ml microcentrifuge tube and pipet 100 µl Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature and then centrifuge for 1 min at 8000 rpm to elute.
13. Repeat step 12 once.

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