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Infectious structures and response of maize plants to invasion by *Exserohilum turcicum* (Pass). in compatible and incompatible host pathogen systems

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ABSTRACT
Objective: To study the differential response of corn leaves to invasion by *Exserohilum turcicum* comparing compatible and incompatible host-pathogen systems.
Methodology and results: Maize leaves excised from 14 day-old plants were inoculated with 13 different *E. turcicum* isolates following the detached leaf bioassay technique. The infection process was studied by fluorescent microscopy after clearing leaves using a mixture of ethanol:choloroform, 75:25 (v/v) containing 0.15% trichloroacetic acid, and subsequent staining with calcofluor. Fourty-six percent of the isolates had bipolar germination with direct penetration. Penetration occurred by the formation of a fine penetration peg beneath the appressorium. A chlorotic reaction was observed in areas beneath and adjacent to the appressoria and germ tubes. Hyphal growth was scanty in the resistant reaction and abundant in the susceptible reaction. However, there were no significant differences in spore germination and penetration between the resistant and susceptible reactions.
Conclusion and application of findings: Although the initial pathological histology of *E. turcicum* infection appears to be similar in both resistant and susceptible reactions, mycelial growth is later restricted in the xylem vessels of resistant reactions. Inhibition of mycelial growth results into reduced lesions, delayed wilting and tissue necrosis. Biochemical or other factors that restrict mycelial growth could be studied further for exploitation in breeding programs for maize varieties with resistance to *E. turcicum*.

Key words: infection, maize, penetration, resistance, xylem

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INTRODUCTION

Exserohilum turcicum (Pass.) is the causal agent of northern leaf blight, which threatens maize production in many areas of the world. Yield losses of up to 50% have been reported especially when the disease sets in early in the season (Yeshitila, 2003). The disease also reduces the total sugar

content of maize grains, lowers germinative capacity (Cuq *et al*., 1993) and predisposes heavily infected plants to stalk rot (Gowda *et al.,* 1992; Cardwell *et al*., 1997). *E. turcicum* has high pathogenic variability (Mwangi, 1998; Yeshitila, 2003) and the disease is controlled mainly through the use of resistant varieties derived from qualitative and quantitative genes acting together or separately (Juliana *et al*., 2005).

Germination of *E. turcicum* conidia is bipolar and occurs 3-6 hours after inoculation. Germ tubes are 20-150µ long and in general, grow at an angle rather than parallel to the veins of the leaf. They produce simple or forked terminal appressoria from which penetration pegs develop (Muiru, 2008). Germ tubes develop more from apical than from basal cells of the spores. Penetration is usually direct and occurs only rarely through stomata. Infection pegs grow into or between epidermal cells of either the dorsal or the ventral side of the leaf (Hilu & Hooker, 1964). Penetration has been observed even after 6 hours, though it occurs mostly 12-18 hours after inoculation (Jennings & Ullstrup, 1957; Lilian *et al*., 2002). Following penetration, the fungus produces a vesicle-like structure 10-30µ diameter in or between the epidermal cells. This structure gives rise to secondary hyphae that proceed intracellularly in the mesophyll tissue in various directions. Some hyphae may grow towards the xylem vessels (Hilu & Hooker, 1964).

The hyphae continue advancing inside the chlorenchyma tissue resulting in lesion formation. The number of necrotic cells increases as the lesion develops and some cells die without any direct contact with hyphae. Cellular host response is characterized initially by light staining of chloroplasts which later lose their identity and finally disintegrate (Jennings & Ullstrup, 1957; Muiru, 2008). Nuclei enlarge slightly, and as cells become plasmolyzed, the nuclei disintegrate. Cells eventually die in the vicinity of a lesion. These cells later appear devoid of all cytoplasm, separate and become disorganized (Tagne *et al*., 2002). The bundle sheaths of large veins may serve as structural barriers to the lateral spread of the fungus. Hyphae, however advance into new interveinal areas through chlorenchyma tissue located between the bundle sheath and the epidermal layer (Hilu & Hooker, 1964, Lilian *et al*., 2002).

The hyphae in the lesions vary from 6-15µ wide and enlargement of the hyphae is limited by the size of the xylem vessels. The hyphae continue to grow from the xylem into the surrounding healthy tissues resulting in the enlargement of the lesions. The hyphae penetrate the normal bundle sheath and grow rapidly in adjacent mesophyll cells. The cells become plasmolyzed and their protoplasm appears granular and the whole cell dies very rapidly (Jennings & Ullstrup, 1957; Muiru, 2008). Except for the epidermal layer, all tissues including the xylem and phloem collapse. Mycelial strands aggregate into pseudoparenchymatous masses in sub-stomatal chambers. Conidiophores produced from these dense masses emerge through the stomata and produce conidia abundantly (Hilu & Hooker, 1964, Lilian *et al*., 2002).

In the resistant cultivars, hyphal growth in the leaves is less than that in the xylem of susceptible leaves. The hyphae in the xylem of resistant plants are also smaller than those in susceptible plants (Tagne *et al*., 2002). In resistant reactions, rapid death of plant cells due to infection by *E. turcicum* hyphae is not observed. The hyphae also fail to aggregate in the sub-stomatal chamber to initiate sexual reproduction of the fungus (Jennings & Ullstrup, 1957; Hilu & Hooker, 1964). In contrast to the susceptible lesion type, older lesions of monogenic resistant leaves are devoid of large air spaces and cells adjacent to a lesion have enlarged nuclei which may persist longer in the cells than in compatible reactions (Hilu & Hooker, 1964, Lilian *et al*., 2002). Though considerable work has been done on elucidation of host response to invasion by *E. turcicum*, not much has been done on fluorescent microscopy which is necessary in characterization of the resistance mechanism. The objective of this study was to

elucidate the host-pathogen interaction in both resistant and susceptible cultivars.

MATERIALS AND METHODS

The experiment was carried out in the Department of Crop Sciences, Division of Plant Pathology and Plant Protection, University of Göttingen, Germany. Thirteen isolates of *E.. turcicum* were isolated from diseased maize leaves collected from Kenya and Germany. The isolates were selected based on their morphological and cultural differences. Isolation was done by cutting leaf discs (1mm^2) along advancing margins of disease lesions and surface sterilizing in 2.5% sodium hypochlorite for 3 min. The tissues were rinsed in 3 changes of sterile distilled water and then plated on Potato Dextrose Agar (PDA) medium (potato extract 4 g, glucose 20 g, agar 15 g and water 1 litre) in 9-mm diameter Petri dishes. Cultures were incubated at room temperature (22±2°C) for 7 days and pure cultures obtained by sub-culturing onto fresh PDA medium. Monosporic cultures were obtained by preparing a dilute conidial suspension (1000 conidia per ml) and seeding this on water agar (agar 20 g, water 1 litre). After 12 hours of incubation at room temperature, individual conidia were located using a compound microscope at ×40 and single conidia individually transferred onto clean PDA medium using sterile mounted needles.

Histopathological studies: A modification of the method described by Wolf and Fredrich (1980) was adopted for general leaf clearing and staining. Conidial suspensions of monosporic cultures of *E. turcicum* (1.0 x 10^5 conidia ml^{-1}) were used to inoculate maize leaves excised at the 4-5 leaf growth stage and placed in moist chambers with both ends between folds of cotton wool. Inoculation was accomplished by placing drops (0.05ml) of conidial suspension on the maize leaves and marking with an indelible pen to note the exact location of inoculation. The inoculated leaves were incubated at 25 °C and fixing done after 24, 48, 72 and 96 hours. Leaf discs cut from the area where the drop of inoculum had been put were placed on filter papers inside glass Petri dishes. A mixture of ethanol: choloroform, 75:25 (v/v) containing 0.15% trichloroacetic acid was added to flood the leaf discs overnight at room temperature. The leaf discs were washed in deionized water and then stained in a dye consisting of one volume 15% trichloroacetic acid in water and one volume 0.6% Coomassie Brilliant Blue R-250 in 99% methanol. The dye solution was prepared a day before use. Leaves were stained for 3-5 minutes and washed again in deionized water. The stained preparations were preserved in a solution of glacial acetic acid-glycerol-water, 5:20:5 (v/v), either preserved in falcon tubes or on slides with the cover slips sealed with nail varnish. Observations were then made under microscope (×600) to study the conidia germination and the whole infection process noting histological changes of the pathogen structures and the plant cell.

Fluorescent staining of leaves with Calcofluor was done using a modification of the method described by Rohringer *et al*. (1977). Leaf discs cleared as explained above were incubated for 30 minutes in 0.1M Tris-HCl (pH=8.5). The leaf discs were then washed 4 times after every 10 minute with double distilled water followed by dipping in 0.1% cacofluor solution for 5 minutes. The leaf discs were washed 4 times as described above and then washed again in 25% glycerine for 30 minutes. The stained leaf discs were stored in 25% glycerin and examined using fluorescent microscopy techniques.

RESULTS

Conidia of six of the 13 isolates (46%) had bipolar germination with the germ tubes originating from the apical rather than from the basal cells of the spores (Table 1). Germination was observed to start occurring 8 hours after inoculation but most of the germination occurred 15-20 hours after inoculation. Formation of appresoria was observed 24 hours after inoculation and penetration into the leaf was mostly direct as opposed to penetration through the stomata (Plate 1). In the resistant reaction, hyphal growth in the xylem parenchyma was scanty compared to the susceptible reactions where the mycelia growth was dense. Fluorescent photomicrographs showed dense mycelial growth of pathogen on susceptible leaf tissues while resistant leaf tissues showed scanty mycelial growth (Plate 1 B & D). Formation of germ tubes from both ends of hyphal fragments were observed for some of the isolates, e.g. isolates G32, G9, G51 and G34 (Plate 1C) .

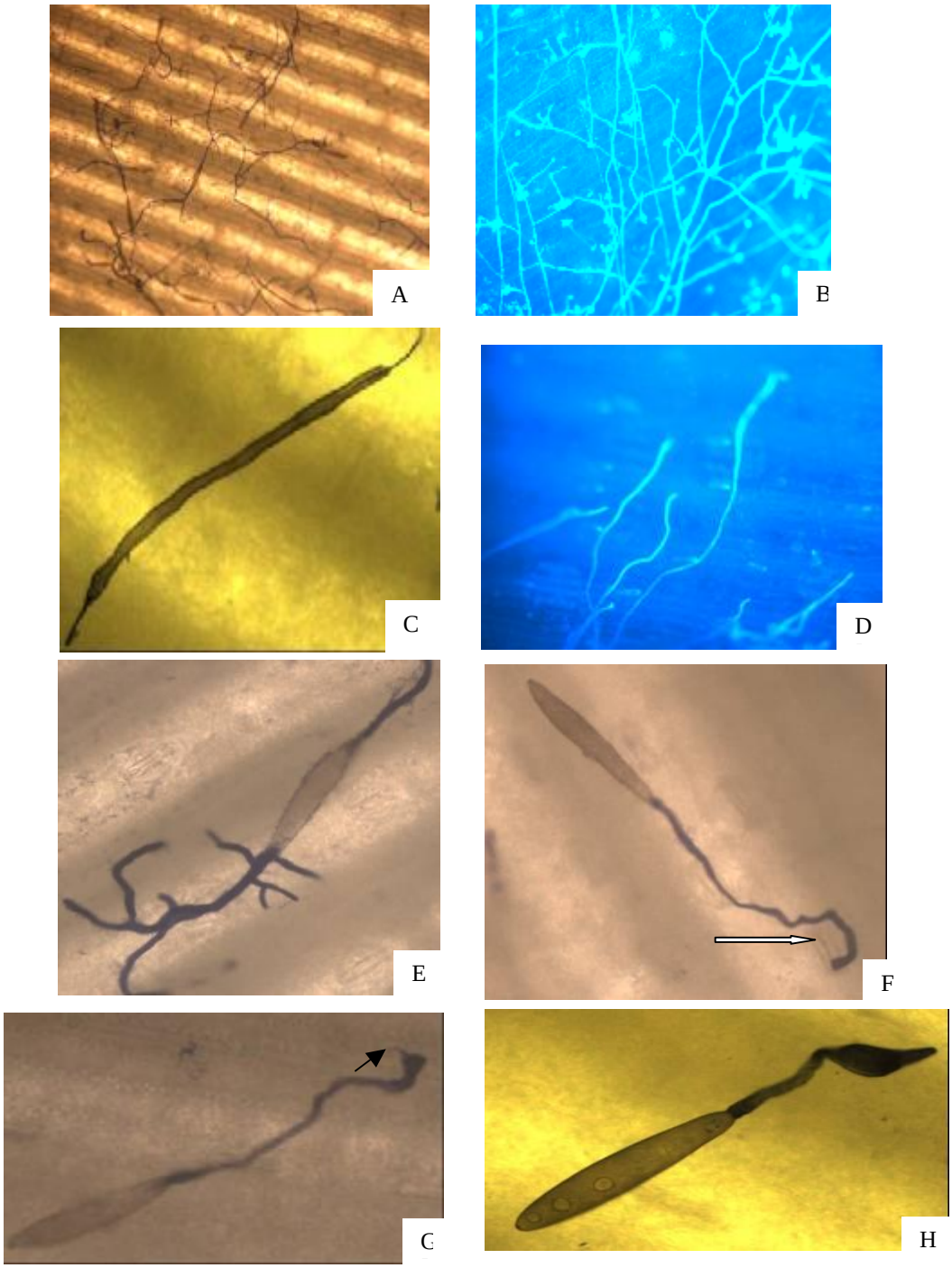

Plate 1: Photomicrophotographs of *Exserohilum turcicum* conidia and mycelia during the infection process. A: Hyphal formation 24 hours after inoculation with isolate ENG 1 (×400); B: Dense hyphal growth (24 hours after inoculation) from isolate G41 (×600); C: Hyphal fragment from isolate G32 (24 hours after inoculation) forming germ tubes (×600); D: Scanty hyphal growth (24 hours after inoculation) from isolate S55 (×600); E: Multiple branching of germ tube (18 hours after inoculation) from isolate G1(×400); F: Penetration through the stomata (12 hours after inoculation) of isolate S55 (×400) ; G: Formation of an infection peg (12 hours after inoculation) of isolate S64 (×400); H: Formation of an appresorium (12 hours after inoculation) from isolate ENG 2 (×600).

DISCUSSION

Spore germination and leaf penetration by the various *E. turcicum* isolates evaluated in this study were similar in resistant and susceptible reactions, which confirms the findings of Jennings & Ullstrup (1957) and Lilian *et al*., (2002). Hilu & Hooker (1964) reported that about 90% of all penetrations are direct and typically occur over the juncture of vertical epidermal walls whereas 10% of penetrations are stomatal. Our study had similar findings with about 85% of all the isolates penetrating directly. The pathogen requires an appressorium to be able to penetrate the host (Jennings & Ullstrup, 1957), which probably explains why all the isolates were observed to form appressoria. Other pathogens, e.g. *Leptosphaeria maculans* have been reported to have similar penetration mechanisms (Naseri *et al*., 2008).

Penetration elicited localized host responses resulting in macroscopically visible flecks as previously reported by Lilian *et al*. (2002). At least one penetration peg is associated with each fleck which includes 80-100 killed cells (Hilu & Hooker, 1964). Growth in xylem vessels results in vascular plugging and complete breakdown of chlorenchyma cells between parasitized bundles, which is associated with the onset of wilting. These wilting symptoms are typically observed especially with young seedlings in greenhouses. At the structural level the pathological histology of *E. turcicum* appears to be similar between the resistant and susceptible systems and the difference is only clearly manifested when the pathogen invades the xylem vessels. There is thus no discernible difference between the resistant and susceptible reactions during the early stages of the infection process.

Table 1: Conidial germination and mode of penetration of various *E. turcicum* isolates.

Isolate	Type of germination	Mode of penetration	Production of germ tube at end of hyphal fragments
G34	Bipolar	Direct	+
B5	Bipolar	Direct and stomatal	_
G51	Unipolar	Direct	+
ENG 1	Bipolar	Direct	_
G19	Unipolar and bipolar	Direct	_
G9	Unipolar	Direct and stomatal	+
SORTE 5	Unipolar	Direct	_
ENG 2	Unipolar	Direct	_
G41	Bipolar	Direct	_
G32	Bipolar	Direct and stomatal	+
G1	Bipolar	Direct	_
S55	Unipolar	Direct and stomatal	_
S64	Unipolar	Direct and stomatal	_

Key: + Present; _ Absent

Resistance to *E. turcicum* is partly expressed in the xylem tissue and the pathogen is able to establish early in susceptible reactions with mycelial growth progressively filling vessels and tracheids. Hilu & Hooker (1964) and Lilian *et al*., (2002) demonstrated that cells of resistant varieties are characterized by enlargement of vessels near the infection site and these are likely to limit a faster establishment of the pathogen resulting in less sporulation and reduced hyphal growth in the intercellular spaces. Similar occurrences, though not observed in our study, could probably explain the occurrence of sparse mycelial growth observed in the resistant host pathogen interaction compared to the dense mycelia growth observed in the compatible system. Jennings and Ullstrup (1957) also demonstrated that longitudinal and lateral growth of hyphae gets retarded in resistant varieties and the hyphae are smaller and their branching is limited. Resistance in *E. turcicum* is associated with inhibition of mycelial growth so that wilting and necrosis is delayed resulting in fewer and smaller lesions than those occurring in susceptible tissue.

This study had focused on maize as a host in the histopathological studies though studies using other hosts such as Sudan grass (*Sorghum vulgare var sudanense*) and broomcorn (*Sorghum vulgare var technium*) have reported similar pathological histology (Hilu & Hooker 1964; Lilian *et al*., 2002, Naseri *et al*., 2008). The pathological histology of *E. turcicum* is unique compared to those of other leaf diseases caused by *Helminthosporium* in that growth of the fungus is most profuse in xylem tissue, while growth of others is almost confined to the chlorenchyma. With *E. turcicum* pectic enzymes are not involved in lesion formation. Rather, wilting occurs due to extensive plugging by hyphae thus physically obstructing water flow (Jennings & Ullstrup, 1957; Tagne *et al*., 2002). The production of germ tubes from the tips of the broken hyphal strands could be a contributor to aggressiveness and isolates producing germ tubes from the broken hyphal fragments were found to be comparatively more aggressive than the isolates without this attribute.

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