Infection Process in Resistant and Susceptible Maize (Zea mays L.) Genotypes to Cercospora zeae-maydis (Type II)

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Abstract


The infection process of Cercospora zeae-maydis type II (syn. Cercospora zeina Meisel and Korsman) in resistant, moderately resistant and susceptible maize genotypes was studied in the greenhouse under artificial inoculation. The percent spore germination, germ tube growth and formation of mature appressorium on leaves at 24, 36, 48, and 72 h after inoculation did not differ between resistant, moderately resistant, and susceptible maize genotypes (P ≤ 0.05). More germlings were established after penetration on susceptible than resistant and moderately resistant maize genotypes at 72, 96, 120, and 144 h after inoculation. The hyphal wefts in cells of resistant and moderately resistant genotypes were shorter than in susceptible genotypes (P ≤ 0.05). The slow pathogen growth was associated with a reduced number of conidiophores per stroma, spores per unit area and smaller lesions. The reduced pathogen growth after penetration suggests possible involvement of pathogen growth inhibitory substances in maize resistance to C. zeae-maydis type II.

Keywords: Cercospora zeina; host resistance; maize

Grey leaf spot caused by Cercospora zeae-maydis Tehon and Daniels is the most destructive foliar disease of maize worldwide (Ward et al. 1999). Yield losses attributed to grey leaf spot have been reported to be variable (Lipps et al. 1998) with estimated losses as high as 100% when severe epidemics contribute to increased stalk rot and early senescence (Latterall & Rossi 1983). Grey leaf spot is spread mainly through infested maize residues since C. zeae-maydis is capable of surviving in host debris (de Nazereno et al. 1992; Asea et al. 2002). Moderate to high temperatures 20–30°C and prolonged periods of high relative humidity are conditions reported to be favourable for rapid development of the disease (Stromberg 1986).

Studies conducted in the US revealed the existence of two genetically distinct but morphologically similar sibling species of C. zeae-maydis (type I and type II). Type I is widely distributed in US, China and Latin America while the second sibling species (type II) is confined to the eastern US and Africa (Dunkle & Levy 2000). Both species may be found in the field. Dunkle and Levy (2000) compared variability of isolates from Africa with isolates from the US using Fragment Length Polymorphism (AFLP) and Restricted Digests of Internal Transcribed Space (ITS). Group type I was more prevalent in isolates from samples collected in the US. Group type I was not detected from samples from Africa suggesting that Africa probably was the source of C. zeae-maydis group type II found in the US. African and US group type II populations were co-specific with limited variability and type II species grew much slower and did not produce any toxins in culture. Crous et al. (2006) and Meisel et al. (2009) reclassified group type II as distinct species now called Cercospora zeina endemic in African countries. Okori et al. (2003) confirmed the widespread presence of type II biotypes in East Africa (Kenya,
Uganda, and Rwanda) and indicated that gene flow was high among African populations of \textit{C. zeae-maydis}. Ayodele et al. (2000) isolated \textit{C. zeae-maydis} producing toxins in culture with varying levels of aggressiveness from samples collected in Nigeria a characteristics of \textit{C. zeae-maydis} type I. Mathionni et al. (2006) conducted experiments at different locations and concluded that isolates of type II were more aggressive than those of type I and that these types also differed in their degree of fitness in different environments in Brazil.

Methods to manage grey leaf spot include conventional tillage that buries crop residues, crop rotation, fungicides, and utilisation of resistant varieties (Ward et al. 1999). Fungicides are widely used in maize production (Munkvold et al. 2001) but are too expensive for low-income-resource poor farmers in the tropics (Menkir & Ayodele 2005). Utilisation of host resistance is the cost-effective method to manage grey leaf spot (Pratt et al. 1997).

Several sources of resistance to \textit{Cercospora zeae-maydis} type I and type II have been found among temperate and tropical maize germplasms and used in several maize breeding programs (Donahue et al. 1991; Coates & White 1994; Gevers & Lake 1994; Danson et al. 2008). Inbreds with high levels of resistance and agronomically acceptable characteristics such as B68, NC250, Pa875, Va14, Va17, and Va85 have been identified and several hybrids such as B68 × KB1250, KB1250 × Pa875, and NC250 × Pa875 obtained from these inbred lines displayed high levels of resistance and good general agronomic characteristics (Donahue et al. 1991; Gordon et al. 2004). World’s available resistant varieties and inbred lines include Mo18W, NC250, NC250A, NC258, NC290, Pa875, T222, Va14, Va17, Va59, Va85, NC262A, NC264, NC270, NC288, NC290A, NC318, NC320, NC332, NC334, NC334, and NC354 from the USA (Thompson et al. 1987; Ulrich et al. 1990; Bubeck et al. 1993; Saghai-Maroo et al. 1993); KO54W and SOS07W × VO613Y from South Africa (Gevers et al. 1994; Gordon et al. 2004) and CIMMYT resistant lines (CML440, CML443, and CML445).

Resistance to grey leaf spot is inherited quantitatively by genes that act primarily in an additive manner (Donahue et al. 1991; Cromley et al. 2002) and is expressed as the rate disease reducing resistance (Elwinger et al. 1990). Quantitative resistance to grey leaf spot leads to prolonged latent and incubation periods, reduced infection rates, sporulation capacity and the number of lesions (Beckman & Payne 1982; Carson & Goodman 2006). The resistance factors have been mapped to three different chromosomes at least, with some of the quantitative trait loci (QTL) consistently expressed across environments (Clements et al. 2000; Gordon et al. 2004). Carson and Goodman (2006) reported that resistance to grey leaf spot in maize appears to be equally effective against both type I and type II of \textit{C. zeae-maydis} and that aggressive isolates, regardless of which sibling species of \textit{C. zeae-maydis}, should be used to select for grey leaf spot resistance in field trials.

Whilst there are many reported studies on the genetics of resistance to grey leaf spot in maize, little has been reported in the literature on the expression of mechanisms of resistance and host parasite relations particularly at the cellular level between \textit{C. zeae-maydis} type II and maize genotypes differing in susceptibility. Plants defend themselves against pathogens using structural and/or metabolic (biochemical) defence mechanisms existing before infection or induced by the attacking pathogen during the infection process (Agrios 1988). Plants may also carry receptors for pathogen recognition specificity domains that induce the expression of defensins, thionins, oxidative burst and other defence responses (Jones & Dangl 2006; Friedman & Baker 2007; Ting et al. 2008) or evolve resistance (R) genes that enable qualitative resistance mechanisms that control a broad set of disease resistance responses capable of preventing pathogens from further growth. Poland et al. (2009) suggested several hypotheses for a range of mechanisms underlying quantitative disease resistance (QDR) in maize that may utilised similar, but less dramatic, responses similar to those mediated by qualitative resistance factors, but conferring only partial and usually race non-specific response. Bluhm et al. (2008) identified hydrolases and oxidoreductases in \textit{C. zeae-maydis} type I responsible for cellulose degradation and cercosporin biosynthesis, respectively. Similar compounds are frequently associated with the oxidative burst component of plant defence (Low & Merida 1996). The objective of this study was to assess \textit{C. zeae-maydis} type I at different developmental stages, external growth, penetration, and internal colonisation in resistant, moderately resistant and susceptible maize genotypes and associated disease resistance mechanisms.
MATERIAL AND METHODS

Plant material. Locally purchased seeds of maize cultivar that included susceptible (Pannar) and resistant (UH6303) single crossed hybrids and open pollinated moderately resistant cultivar (Staha) were hand sown in 15 cm pots containing sterilised forest soil. Triple superphosphate was applied at the rate of 1.5 g/pot during planting followed by calcium ammonium nitrate at V2 growth stage (Iowa State University Extension Bulletin 2009) at the rate of 2.5 g/pot.

Inoculation and experimental design. Forty plants (10 per replication) were inoculated at V6 growth stages by spraying the conidia suspension of C. zeae-maydis type II adjusted to $2 \times 10^4$ conidia/ml using a hand sprayer until runoff. Isolate IGR-1 used in this study was close related to Cercospora zeae-maydis type II based on microscopic characteristics, grew slowly and produced no toxins in culture. Prior to inoculation, pots were arranged in a balanced Complete Randomized Design (CRD) replicated four times. To prepare conidia suspensions of C. zeae-maydis type II, single well separated lesions from samples collected in farmer fields at Mati-Uyole, Mbeya, during 2006 cropping season were cut using sterile blades, surface sterilised in 10% sodium hypochlorite (bleach) for 30 s and incubated in Petri dishes containing moistened filter paper at 24°C for 48–72 h to induce sporulation. Single spores of C. zeae-maydis type II were picked with a sterilized needle verified microscopically and placed in V8-juice agar (350 ml V8 juice, 3 g CaCO$_3$, 20 g agar, and 650 ml distilled water per litre) and cultures were incubated in a light chamber at 28°C with 12 h of darkness and 12 h of cool-white fluorescent light (320 µE/m$^2$/s). The inoculum of C. zeae-maydis type II was prepared by adding 5 ml of sterile distilled water into fresh culture grown in V-8 juice agar. The resulting conidia suspension of C. zeae-maydis type II was strained between two layers of cheesecloth and conidia concentration was adjusted to $2 \times 10^8$ conidia/ml using a haemocytometer prior to inoculation. The inoculated plants were placed in the humidity chamber (243.84 cm length × 121.92 cm width × 152.4 cm height) constructed using polythene sheets (Morogoro Plastics Ltd., Morogoro, Tanzania). Temperature in the chamber was maintained at 28–30°C. The humidity was kept high (approaching 100%) by constantly wetting newspapers spread on the floor of the chambers.

Conditions reported to be conducive for penetration and infection (Beckman & Payne 1982).

Spores, lesion length and conidiophores per stroma measurements. The number of spores per cm$^2$ of leaf surface was estimated using a modified method described by Paul and Munkvold (2004). One square centimetre of leaf tissues with diseased lesions was cut, placed in vials containing 10 ml of sterile distilled water and then hand shaken for 3 min to dislodge the conidia. The number of conidia per cm$^2$ of leaf surface was then estimated using the formula $SA = Sc \times V/A$; where: $SA = \text{conidia/cm}^2$, $Sc = \text{conidia/ml}$, $V = \text{volume of water used (ml)}$, and $A = \text{diseased leaf area}$. Conidia per ml were measured using a haemocytometer. Three leaves selected randomly from each plant were tagged and lesion length was measured using a ruler (scale). Ten leaf pieces per inoculated plant of about 2 cm$^2$ from resistant, moderately resistant and susceptible maize plants were removed using sterile blades and examined under 400× magnification of the light microscope Type 020-507-010 (LeitzBiomed, Munich, Germany). One hundred stromata per replicate were selected randomly and the numbers of conidiophores per stroma were recorded.

Microscopic studies. Ten leaf pieces 10 cm in length were excised from each inoculated resistant, moderately resistant and susceptible maize plant per replicate using a sterile scalpel at 24, 36, 48, and 72 h after inoculation and placed in sterile Petri dishes and transported to the Sokoine University of Agriculture laboratory. Leaves for the study of post-penetration of the C. zeae-maydis type II in host tissue cells were removed at 72, 96, 120, and 144 h after inoculation. A total of 120 leaf pieces were removed for microscopic examination. In the laboratory, the method of Skippe et al. (1974) was used to remove chlorophyll from leaf portions. Leaf portions of 5 mm$^2$ from resistant, moderately resistant and susceptible maize varieties were cut and placed (inoculated surface upwards) into sterile Petri dishes containing two layers of filter paper. An amount of 6 ml of ethanol and 3 ml of acetic acid was added into the Petri dishes and incubated at room temperature (24 ± 1°C) for 48 h in order to remove the chlorophyll. Cleared leaf pieces were stained with lactophenol cotton blue (12 h), mounted on microscope slides with 50% glycerin and observed under 400× magnification of the light microscope (LeitzBiomed Type 020-507-010). Three hundred spores (100 per replicate) were...
measured on susceptible, moderately resistant and resistant maize genotypes for germination, germ tube growth, appressorium formation, penetration (growth of hyphae towards stomata followed by the extension of the germ tube beyond the mature appressorium towards the epidermal cells), establishment (an appressorium producing secondary hyphae greater than 30 µ after penetrating was considered to have established successfully) and hyphal growth in cells. Spore germination, formation of mature appressorium and length of germ tubes were assessed 24, 36, 48, and 72 h after inoculation. The percent germlings established, and the length of extending hyphae were recorded 72, 96, 120, and 144 h after inoculation.

Data analysis and statistical model. Statistical program MStat-c (1989) was used for analysis of variance (ANOVA) and mean separation for all data. A significance level of P ≤ 0.05 was used throughout the study. The statistical model used for the various variables was: 

\[ Y_{ij} = \mu + G_i + \epsilon_{ij} \]

where: \( Y_{ij} \) = observation in the \( i \)th genotype and \( j \)th plot; \( \mu \) = overall mean; \( G_i \) = \( i \)th genotypic effect, and \( \epsilon_{ij} \) = random error.

RESULTS

There were no significant (P ≤ 0.05) differences among genotypes in the number of spores that germinated after 24, 36, 48, and 72 h from inoculation (Table 1). The length of the germ tubes and the percent of germ tubes that formed mature appressorium at 24, 36, 48, and 72 h after inoculation did not differ significantly (P ≤ 0.05) between genotypes (Table 2 and Figure 1). However, there were significant differences (P ≤ 0.05) among genotypes on pathogen units that established successfully after penetration (Table 3). Out of 60 germlings that penetrated the host 144 h after inoculation, 25% produced hyphae longer than 30 µ on resistant genotype UH6303 compared to 78% on susceptible genotype Pannar. At 144 h after inoculation hyphal wefts in cells of resistant genotype UH6303 were significantly (P ≤ 0.05) shorter (14 µ) than in moderately resistant genotype Staha (39 µ) and susceptible genotype Pannar (172 µ) (Figure 2).

Table 1. Percent spore germination of C. zeae-maydis type II 24, 36, 48 and 72 h after inoculation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Spore germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>UH6303</td>
<td>18.3a</td>
</tr>
<tr>
<td>Staha</td>
<td>18.2a</td>
</tr>
<tr>
<td>Pannar</td>
<td>18.5a</td>
</tr>
<tr>
<td>Mean</td>
<td>18.3</td>
</tr>
<tr>
<td>CV (%)</td>
<td>2.01</td>
</tr>
<tr>
<td>SE ±</td>
<td>0.210</td>
</tr>
</tbody>
</table>

Means followed by the same letter within columns do not differ significantly according to DMRT

Figure 1. The length of germ tubes (µm) of C. zeae-maydis type II 24, 36, 48, and 72 h after inoculation on resistant (UH6303), moderately resistant (Staha), and susceptible (Pannar) maize genotypes

Figure 2. The length of extending secondary hyphae (µm) of C. zeae-maydis type II in cells 72, 96, 120 and 144 h after inoculation on resistant (UH6303), moderately resistant (Staha), and susceptible (Pannar) maize genotypes

Means followed by the same letter within columns do not differ significantly according to DMRT.
UH6303 (Table 4). Susceptible maize hybrid Pannar displayed necrotic rectangular lesions after infection while moderately resistant cv. Staha displayed chlorotic lesions (Figures 3 and 4). Resistant cv. UH6303 displayed fleck response (Figure 5). The chlorotic and fleck lesion types displayed low infection units in terms of reduced lesion length, spore numbers and conidiophores per stroma (Table 4).

**DISCUSSION**

Lack of significant differences among genotypes during early developmental stages of *C. zeae-maydis* type II infection (spore germination, germ tube growth, and appressorium formation) is consistent with the reports of other researchers (Beckman & Payne 1983; Thorson & Martinson 1993) where spore germination and penetration of *C. zeae-maydis* type I were not influenced by genotypes.

Table 4. Lesion size, number of conidiophores per stroma and sporulation of *C. zeae-maydis* type II 16 days after inoculation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LL (cm)</th>
<th>No. conidiophore/stroma</th>
<th>SPN × 10^4 per cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH6303</td>
<td>0.7c</td>
<td>3.6c</td>
<td>2.7c</td>
</tr>
<tr>
<td>Staha</td>
<td>1.6b</td>
<td>4.8b</td>
<td>4.0b</td>
</tr>
<tr>
<td>Pannar</td>
<td>4.6a</td>
<td>9.6a</td>
<td>6.1a</td>
</tr>
<tr>
<td>Mean</td>
<td>2.3</td>
<td>6.0</td>
<td>4.3</td>
</tr>
<tr>
<td>CV (%)</td>
<td>12.34</td>
<td>8.43</td>
<td>10.25</td>
</tr>
<tr>
<td>SE ±</td>
<td>0.166</td>
<td>0.293</td>
<td>0.254</td>
</tr>
</tbody>
</table>

Means followed by the same letter within columns do not differ significantly according to DMRT; LL – lesion length; SPN – spore number

Means followed by the same letter within columns do not differ significantly according to DMRT; 1percent germnings with extending hyphae > 30 µ in cells

Table 3. Percent germlings of *C. zeae-maydis* type II established in cells 72, 96, 120, and 144 h after inoculation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Germlings established (%)^1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 h</td>
</tr>
<tr>
<td>UH6303</td>
<td>12.3^c</td>
</tr>
<tr>
<td>Staha</td>
<td>26.7^b</td>
</tr>
<tr>
<td>Pannar</td>
<td>55.4^a</td>
</tr>
<tr>
<td>Mean</td>
<td>31.5</td>
</tr>
<tr>
<td>CV (%)</td>
<td>2.46</td>
</tr>
<tr>
<td>SE ±</td>
<td>0.448</td>
</tr>
</tbody>
</table>

Means followed by the same letter within columns do not differ significantly according to DMRT; 1percent germnings with extending hyphae > 30 µ in cells

Figure 3. Necrotic rectangular lesions on susceptible cv. Pannar.
al. (1987) found no difference between maize hybrids of varying resistance in penetration of *C. zeae-maydis* type I in inoculated leaf discs and speculated that the relative resistance among hybrids may involve differences in the pathogen growth within tissues. Moreover, plant cells may contain hydrolytic enzymes such as glucanase and chitinases which can cause the breakdown of pathogen cell wall components limiting growth in host cells (Agrios 1988) or releases of toxic phenolic compounds and phytoalexins in cells upon infection capable of inhibiting pathogenic fungi (Farkas & Kiraly 1963; Bell 1981). Numerous genes and/or proteins have been identified that mediate plant defence signal transduction (Hammond-Kosack & Parker 2003). Some of the key endogenous chemical mediators of plant defence signal transduction include salicylic acid, jasmonic acid or jasmonate, ethylene, reactive oxygen species, and nitric oxide (Jones & Dangl 2006; Friedman & Baker 2007; Ting et al. 2008). A change in the cell chemical environment in relation to host resistance following infection by *C. zeae-maydis* type II is poorly known. However, the absence of structural barriers that could inhibit *C. zeae-maydis* type II suggests that other disease resistance mechanisms probably of chemical nature may be involved in the resistance of maize against *C. zeae-maydis* type II. *Cercospora zeae-maydis* type II is reported not to produce cercosporin in culture; phototoxin is reported to be a virulent factor in the development of grey leaf spot in maize but it may be produced in host cells (Shim & Dunkle 2002). However, the host resistance to *Cercospora* diseases including grey leaf spot of maize has not been correlated with resistance to cercosporin or interference with cercosporin production or action, except for one report in rice (Batchvarova et al. 1992) suggesting that several disease resistance mechanisms may be involved. The slow pathogen growth was associated with a reduced number of conidiophores per stroma, spores per unit area, and smaller lesions suggesting that most of the pathogen development structures were also affected that lead to reduced pathogen units available to initiate and propagate the disease. However, more studies are required on cell chemical changes within the cell environment following infection by *C. zeae-maydis* type II in relation to resistance in maize. This is the first systematic analysis of infection biology of *C. zeae-maydis* type II (syn. *C. zeina*) widely spread sibling species in Africa. Carson and Goodman (2006) evaluated US *C. zeae-maydis* type I and type II to determine whether grey leaf spot resistance is species specific; isolates within the two sibling species of *C. zeae-maydis* were highly variable in aggressiveness on maize hybrids and resistance in maize to grey leaf spot was equally effective against both type I and type II of *C. zeae-maydis* suggesting that aggressiveness of isolates, regardless of the sibling species of *C. zeae-maydis*, should be used to select for grey leaf spot resistance in field trials.

**References**


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