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Studies on pathogenicity and host resistance of *Exserohilum turcicum* and *Fusarium* spp. on maize (*Zea mays* L.) cultivated in tropical and temperate climate zones

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"The significance of a fact is relative to [the general body of scientific] knowledge. To say that a fact is significant in science, is to say that it helps to establish or refute some general law; for science, though it starts from observation of the particular, is not concerned essentially with the particular, but with the general. A fact, in science, is not a mere fact, but an instance. In this the scientist differs from the artist, who, if he designs to notice facts at all, is likely to notice them in all their particularity."

Bertrand Russell, 1931 – Book 'The Scientific Outlook'

"Eu prefiro ser essa metamorfose ambulante

do que ter aquela velha opinião formada sobre tudo."

Raul Seixas

Table of Contents

I. Abbreviations

A	leaf CO_2 assimilation
AUDPC	area under the disease progress curve
AUMGC	area under the mycelium growth curve
Ca	air CO_2 concentration
CBE	chlorazol black E staining
Cfa	temperate climate, no dry season and a hot summer (climate classification according to Köppen and Geiger)
Cfb	temperate climate, without dry season and warm summer (climate classification according to Köppen and Geiger)
Ci	intercellular CO_2 concentration
Cwa	temperate climate with dry winter and hot summer (climate classification according to Köppen and Geiger)
DA	discriminant analysis
DAB	3,3-diaminobenzidin
DON	deoxynivalenol (mycotoxin of trichotecenes B group)
3-ADON	3-aceytil-deoxynivalenol (mycotoxin of trichotecenes B group)
15-ADON	15-aceytil-deoxynivalenol (mycotoxin of trichotecenes B group)
dpi	days post inoculation
DA	discriminant analysis
Da	dry weight of the aerial biomass (stem and leaves)
Dax/Dao	relative dry weight of the aerial biomass (stem and leaves)
Dr	dry weight of root biomass
Drx/Dro	relative dry weight of root biomass
Dfb	cold climate without dry season with warm summer (climate classification according to Köppen and Geiger)
E	transpiration
Ex/Eo	relative transpiration
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
FB1, FB2, FB3, FB4	Fumonisins of the B series
FER	Fusarium ear rot or pink ear rot
FGSC	*Fusarium graminearum* species complex
FFSC	*Fusarium fujikuroi* species complex
Fm	maximal fluorescence emissions by light adapted tissues
Fs	steady-state fluorescence emissions by light adapted tissues

Fv	fluorescence increment due to the transition from a dark-adapted state with all-open reaction centers to an all-closed state during a saturating flash of light
GDU	growing degree units
GER	Gibberella ear rot or red ear rot
gs	stomatal conductance
gsx/gso	relative stomatal conductance
H_2O_2	hydroxide peroxide
HR	hypersensitive response
K	grain maize
k	instantaneous carboxylation efficiency (A/Ci)
kx/ko	relative instantaneous carboxylation efficiency
LA	leaf area
LAx/Lao	relative leaf area
MAMPs/PAMPs	microbial- or pathogen associated molecular patterns
MCE	mesophyll colonization efficiency
NB-LRR	nucleotide binding and leucine rich repeat domains
NBT	nitroblue tetrazolium
NCLB	northern corn leaf blight
NIV	nivalenol (mycotoxin of trichotecenes B group)
nPQ	non-photochemical quenching relaxing in the dark
O_2^-	superoxide
PAR	photosynthetic active radiation
PCA	principal component analysis
PCD	programmed cell death
POX	peroxidase
PRR proteins	pattern recognition receptors
PTI	PAMP-triggered immunity
Px/Po	relative CO_2 assimilation
QTL	quantitative trait loci
ROS	reactive oxygen species
QY	effective quantum efficiency of photosystem II
S	silage maize
T max	maximum temperature
T min	minimum temperature
TCA	trichloroacetic acid
VCA	variance component analysis

XCE	xylem colonization efficiency
XPE	xylem penetration efficiency
Ya	average or actual yield
Yg	yield gap
Yo	mean values evaluated in healthy plants
Yp	yield potential
Yx	variable values collected from inoculated plants
ZEA	zearalenone

II. General introduction

A. Maize (*Zea mays*) domestication and production

Maize (*Zea mays* L.), a member of the family Poaceae, was domesticated in Central America around 7000 years ago (Miedaner, 2010). Its name comes from the Taíno-Arawakan word mahiz, which means “life-giver”, and it is result of a single domestication from the wild grass teosinte (*Zea mays* ssp. *parviglumis*) by pre-Hispanic civilizations (Staller, 2010). Molecular studies show high similarities with the *Zea mays* ssp. *parviglumis* population of Balsas River valley, south-western Mexico, indicating that this geographic region might be the center of domestication (Doebley, 1990; Wang *et al.*, 1999; Matsuoka *et al.*, 2002). Archaeological research discovered fossils from maize pollen grains in dry caves in the semi-arid highlands of Mexico, confirming that domestication began about 5100 B.C. (Pope *et al.*, 2001). The domestication by artificial selection promoted rapid changes in phenotype (Wright *et al.*, 2005), especially addressing larger seed sizes and vigor (Dermastia et al., 2009).

Maize was developed into an attractive crop by selecting plants with big ears and seeds. It is a flexible crop as it can be grown for grain or silage production (Miedaner, 2010). Grain maize is mainly produced for human and animal consumption, as well as for ethanol production (Bennetzen, 2009). Silage is used for animal feeding or energy production in biogas systems (Miedaner, 2010). In the least 60 years, global maize production has increased by around six times, reaching 1.14 billion tons in 2018 (Figure 1). Maize production in Europe and South America was about 110 and 130 million tons in 2018, respectively (FAO, 2020). In the least 10 years (2008-2018), the main maize producing countries were the United States with 345 million tones, followed by China (216 million tons), Brazil (71 million tons), Argentina (30 million tons) and Mexico (23 million tons) (FAO, 2020).

Maize breeding in the US Corn Belt evolves two races named northern flint (*Zea mays* var. *indurata*) and southern dent (*Zea mays* var. *indentata*). Northern flint was introduced by Native Americans and was found in northern regions of North America (Troyer, 2001; Hufford, 2016). Southern dent was introduced later from Mexico by the Spanish, after the arrival of Columbus (Troyer, 2001; Hufford, 2016). In general, flint maize has a lower yield (Tamagno *et al.*, 2015), shorter cycle, kernels that are thicker, harder and have a vitreous outer layer. Conversely, dent has a higher yield, longer cycle, kernels that are indented and have higher soft starch content (Troyer, 2001; Unterseer *et al.*, 2016). Additionally, husk leaves (which involve the ear) in dent maize are wider, tighter, greater in number and have a bottleneck (Troyer, 2001).

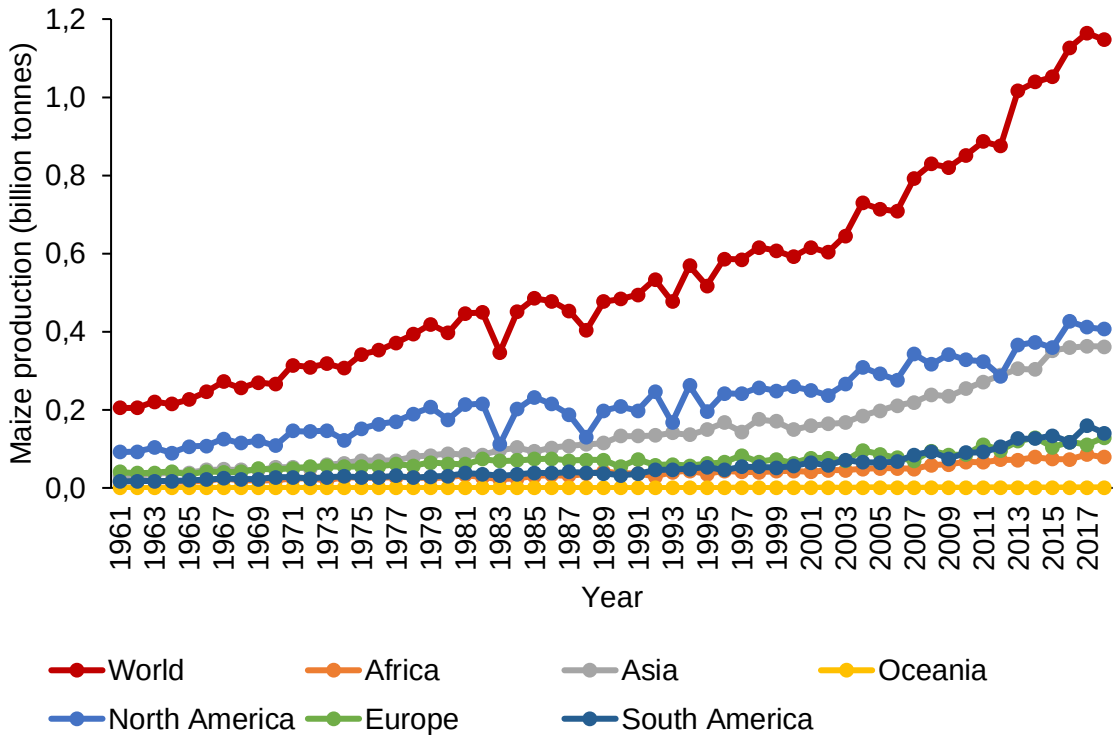


Figure 1. Maize production in the world and per continent from 1961 to 2018 (FAO, 2020).

Estimates show that around two thirds of the maize-producing areas are cultivated with temperate maize and one third with tropical maize (Troyer, 2001). Typically, temperate zones have a more variable and stressful climate compared to tropical zones (Troyer, 2001). Thus, breeding maize for temperate zones requires special attention, such as adaptation within maturity and high yield during a short season (Lee and Tracy, 2009). Besides the maturity and high yield, other agronomical traits need to be considered during selection, such as a good plant architecture which allows mechanical harvest. In this case, the plant must be resistance to lodging factors or dropped ears, such as drought and/or fungal diseases (Lee and Tracy, 2009).

Maize varieties are classified according to the maturity group which consist on the adaptation to particular environments (Lee and Tracy, 2009). A suitable hybrid should maximize full growth during the season. Plants need to flower at the right time, allowing grains to reach the stage of physiological maturity (Lee and Tracy, 2009). In general, maize flowering is affected by the response to day length being triggered close to the equinox, when the day length is short. Therefore, cultivation of tropical maize in temperate zones will promote tall plants, due to the longer days during summer (Troyer, 2001). Commonly, late maturity hybrids have higher yields compared to early maturity hybrids, where the duration of season is limited (Troyer, 2001). The maturity rating designated by the FAO is based on accumulated growing degree units (GDU) during frost-free periods:

$$\mathrm{GDU} = \frac{\mathrm{T\,max} + \mathrm{T\,min}}{2} - \mathrm{T\,min}$$

where $T\,max$ is the maximum temperature, $T\,min$ is the minimum temperature base for maize. If the daily T max is higher than 30°C, the T max will be considered as 30°C in the calculation. If the minimum temperature is lower than 10°C, the T min will be considered as 10°C in the calculation (adapted from Troyer, 2001). In Brazil, GDU are calculated from the date of sowing until female flowering (Zucareli *et al.*, 2010). In Germany, as well as in other countries in Europe, T min is considered as 8°C (Troyer, 2001; DMK, 2020). Maturity groups for maize hybrids cultivated in Germany and Brazil are shown in Table 1.

Table 1. Maize maturity groups for producing silage or grain in Germany, and for producing grain Brazil (Zucareli *et al.*, 2010; DMK, 2020).

	Growing degree units (GDU)	
Maturity group	**Germany**	**Brazil**
Early	S: 170 or K: 220	K: < 830
Middle-early	S: 230 or K: 250	K: 831-899
Middle-late	S: 260 or K: 290	-
Late	S: 300 or K: 350	K: > 900

S – Silage maize; K – grain maize

The cultivation of maize hybrids from a maturity group that is suitable to the environment allows a maximum benefit of genetic capacity of a crop. A yield resulted from selection of suitable genetic material for a region with optimum water and nutrients supply, and under controlled biotic stresses is defined as yield potential (Yp) (Oerke, 2006; van Ittersum *et al.*, 2013). In fact, the yield achieved on the field of farmers is influenced by many other factors, so it is called the average or actual yield (Ya). Biotic and abiotic stresses, such as pests, pathogen attacks and weed competition strongly affect Ya. A good crop management system aims to reduce the yield gap (Yg) between the Yp and Ya (van Ittersum *et al.*, 2013). Overall, yield losses on arable crops caused by weeds are estimated by 34%, followed by pests and pathogens with 18% and 16%, respectively (Oerke, 2006). Indeed, weeds compete with maize for light, water, and nutrients such as nitrogen (Affholder *et al.*, 2003). Additionally, Savary *et al.* (2019) estimated that 22.5% (19.5 - 41.1%) of yield losses are caused by pests and pathogens on maize. Yield losses strictly caused by pathogens on maize represent 8% of all losses (Oerke, 2006). Therefore, some important ear and leaf diseases in maize, with potential to cause yield loss, will be addressed in the following topics.

B. Northern corn leaf blight (*Exserohilum turcicum*)

NCLB was first described in Italy by Passerini in 1876, who named the pathogen *Helminthosporium turcicum*. Later, Alcorn (1988) divided the genera *Helminthosporium* into three: *Drechslera*, *Bipolaris* and *Exserohilum*. *Exserohilum* is segregated into a single genus as conidia are characterized by a protuberant hilum (Leonard and Suggs, 1974). The teleomorph was first described by Luttrell (1958) as *Trichometasphaeria turcica*, and more recently renamed *Setosphaeria turcica* (Luttrell) by Leonard and Suggs (1974). Besides the pathogen nomenclature, the disease also received new denomination during the 1950s. The disease was named NCLB to distinguish it from another corn blight, Southern corn leaf blight (SCLB), which is caused by *Cochliobolus heterostrophus* (teleomorph *Bipolaris maydis*). NCLB is prevalent in northern regions of the United States, whereas SCLB is prevalent in southern regions where higher humidity and temperature are more frequent (Roberts, 1953).

NCLB has been reported worldwide, in all maize-producing areas, from tropical to temperate zones (CABI, 2019). Typical symptoms of NCLB are characterized by elliptical grey-green lesions (Figure 2A), while symptoms of resistance phenotype in plants bearing qualitative resistance, called *Ht* genes (*Ht* for *Helminthosporium turcicum*), are mainly characterized by the presence of chlorosis (Figure 2B) (Galiano-Carneiro and Miedaner, 2017). Yield losses caused by NCLB are related to the level of host resistance, disease severity, plant phenological growth stage during infection, and position of the infected leaves (Levy and Pataky, 1992). Higher levels of resistance have been observed for hybrids containing quantitative and qualitative resistance when compared to hybrids bearing only one type of resistance (Perkins and Pedersen, 1987). Necrotic lesions can reduce CO_2 assimilation by up to 90% at 7 days post-inoculation (dpi), leading to low photosynthetic efficiency (Levy and Leonard, 1990); and consequently reducing seed size (Bowen and Paxton, 1988). The top, middle and bottom thirds of maize plants contributes to photosynthesis in a ratio of 10:5:1. Additionally, yield losses may be increased if the leaf at the ear node shows high disease severity (Levy and Leonard, 1990). High disease severity during reproductive stage, such as two to three weeks after pollination, provoke yield losses between 40% and 70% (Levy and Pataky, 1992). Furthermore, disease severities between 52% and 100% during the full dent stage decrease yields by up to 44% (Bowen and Paxton, 1988). On a global scale, estimates of NCLB yield losses are around 2.5% (Savary *et al.*, 2019).

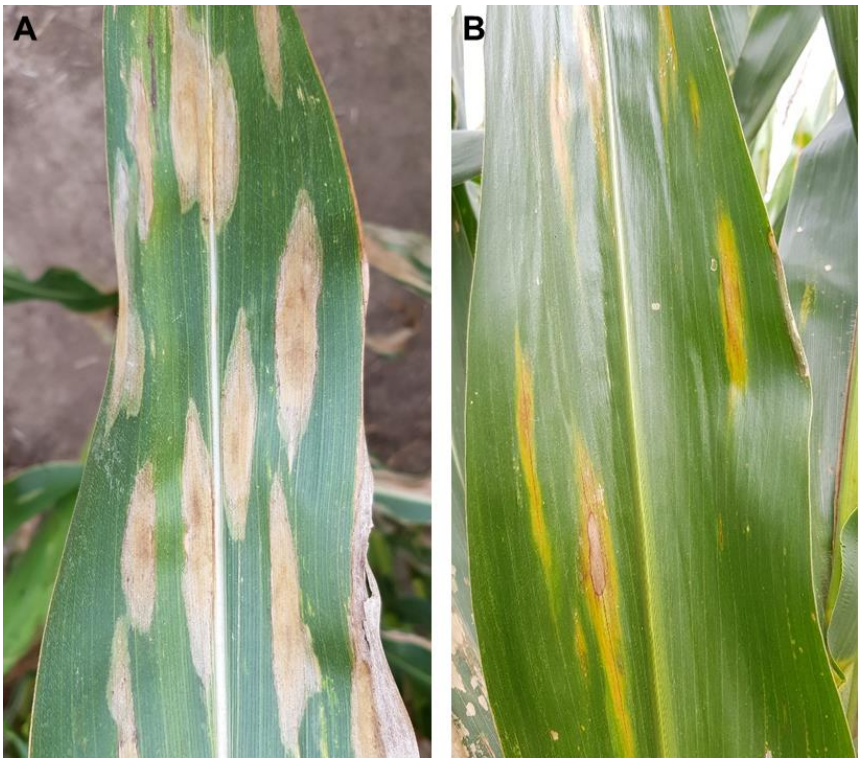
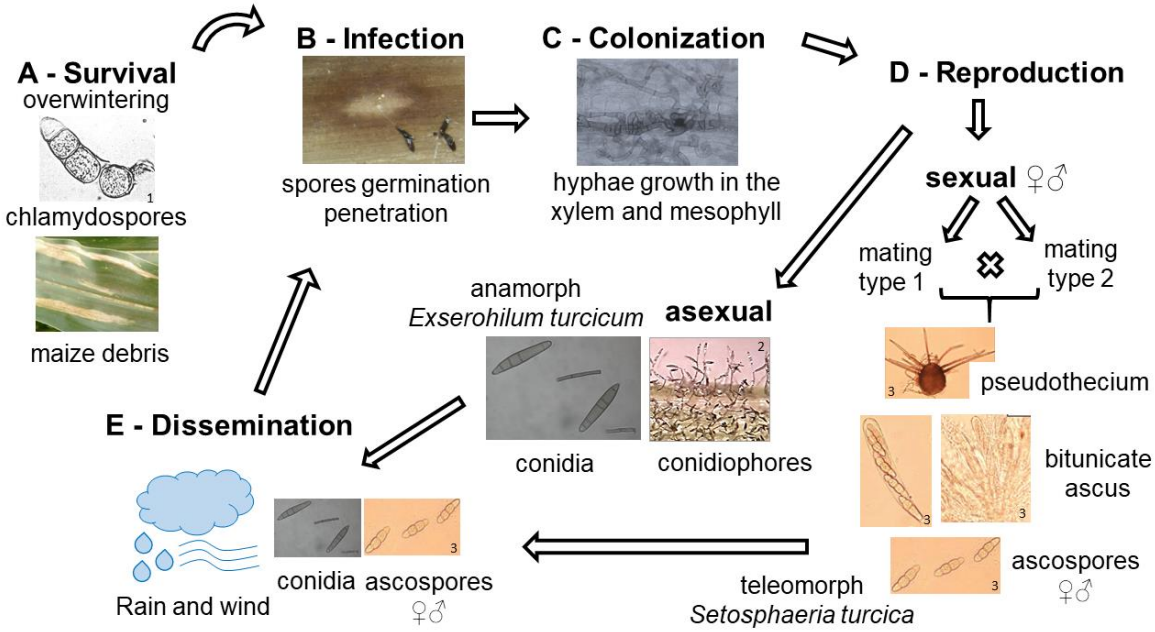


Figure 2. Symptoms of northern corn leaf blight (NCLB) on maize plants in the field. NCLB is characterized by elliptical grey-green lesions (A), whereas symptoms of resistant reaction by plants bearing qualitative resistance are defined by chlorosis (B).

Favorable weather conditions for NCLB development are long dew periods and moderate temperatures, as observed at mid-altitude in tropical regions (Welz and Geiger, 2000). The pathogen can survive on maize debris and overwinter in the form of chlamydospores (Figure 3A) (Boosalis *et al.*, 1967; Levy, 1984, 1995). The formation of chlamydospores is induced by successive nights with relatively low temperatures (<10°C) (Leach *et al.*, 1977). Recently, conidia have been shown to germinate under favorable weather conditions during the successive seasons or as secondary inoculum, and penetrate directly through leaf epidermis (Figure 3B) (Hilu and Hooker, 1964). The pathogen forms a vesicle, colonizing adjacent cell, and posteriorly xylem vessels (Knox-Davies, 1964). A successful colonization (Figure 3C) enables conidiophore formation, sporulation (Figure 3D) (Kotze *et al.*, 2019), and secondary spread (Figure 3E). As *E. turcicum* is heterothallic, sexual reproduction will occur only in the presence of organisms from different mating types. The occurrence of sexual reproduction in the field was recently reported in Thailand (Bunkoed *et al.*, 2014). *E. turcicum* spends most of its life cycle as a haploid organism. The pathogen has a short diploid phase from sexual recombination until meiosis, which results in segregation for ascospores formation. Dissemination occurs by wind and rain splash (Hooda *et al.*, 2017). Moreover, migration on a regional scale is possible via the long-distance dispersal of conidia (Human *et al.*, 2016). *E. turcicum* can survive between crop seasons and be disseminated

by other secondary hosts, such as sorghum (*Sorghum bicolor*) (Ramathani *et al.*, 2011), Johnson grass (*Sorghum halepense*) (Levy, 1984; Levy and Pataky, 1992), and Sudan grass (*Sorghum sudanenses*) (Wathaneeyawech *et al.*, 2015). However, there are reports of host specialization for Indian (Rejeshwar Reddy *et al.*, 2013) and Brazilian isolates (Cota *et al.*, 2010). Thus, the importance of secondary hosts on NCLB epidemics is not well known.



Pictures from Bunkoed *et al.* (2014)[1], Raphael Campos[2], and Bunkoed *et al.* (2014)[3]. Other pictures: own source.

Figure 3. Northern corn leaf blight cycle. *E. turcicum* survives on maize debris and can overwinter as chlamydospore (A). Infection occurs by spore germination and direct penetration on the leaf epidermis (B). Colonization into the xylem vessel and mesophyll allows reproduction (D). Asexual reproduction occurs by conidiophore formation and conidia spread through stomata. Sexual reproduction is only possible when both mating types are available. Then sexual fruiting body (pseudothecium) is formed. Bitunicate ascus evolves ascospores until release. Ascospores and conidia disseminates by rain and/or wind (E).

NCLB control is based upon fungicide application, cultural practices, and the cultivation of resistant hybrids (Welz, 1998). The success of fungicide application on increasing yield is more related to the timing of application. Applications between the mid-stem elongation stage and the flowering stage showed significant increase in production compared to the untreated control (Blandino *et al.*, 2012). In regard to cultural practices, ‘green revolution’ changed many cropping systems, as did the adoption of no-till practices in Brazil and USA in order to reduce soil erosion (Welz, 1998). No-till increases soil humidity and organic

matter and, slows down maize decomposition, maintaining an inoculum source of pathogens (Ono *et al.*, 2011), which consequently may increase disease levels (Cota *et al.*, 2013). Additionally, the plant density had no effect NCLB on fields and disease severity is significantly correlated with the distance to the inoculum source (Adipala *et al.,* 1995). In general, host resistance carried by commercial hybrids against *E. turcicum* is based on qualitative and quantitative resistance (Galiano-Carneiro and Miedaner, 2017). Furthermore, differences in maturity groups and source of resistance may have a contribution to hybrid resistance. An early-maturing hybrid bearing both resistances has shown high resistance levels compared to late-maturing, with yield losses varying from 17% to 43% for the intermediate-maturing group (bearing quantitative resistance) and 63% for the susceptible late-maturing hybrid (Raymundo and Hooker, 1982; Levy and Pataky, 1992). Early-maturing hybrids usually develop fewer secondary cycles of disease (Welz, 1998). A qualitative resistance suppresses sporulation and quantitative resistance reduces the number of lesions caused by *E. turcicum* (Raymundo and Hooker, 1982). Therefore, it is recommended to combine both qualitative and quantitative resistance for NCLB control (Raymundo and Hooker, 1982; Perkins and Pedersen, 1987).

C. Gibberella and Fusarium ear rot

Two types of maize ear rot caused by *Fusarium* spp. are known: Gibberella ear rot (GER or red ear rot) and Fusarium ear rot (FER or pink ear rot). GER is mainly caused by *Fusarium* spp. from the discolor section, or *F. graminearum* species complex (FGSC), which includes *F. graminearum, F. culmorum, and F. cerealis*. FER is caused by *Fusarium* spp. from the liseola section or *F. fujikuroi* species complex (FFGC), represented by *F. verticillioides* (*syn*. *F. moniliforme*), *F. proliferatum* and *F. subglutinans* (Munkvold, 2003b; White, 2010). GER symptoms are usually red or pink mold, which covers large areas of the ear (Figure 4A), whereas FER symptoms are usually white to light pink mold in random kernels, also known as “starburst” (Figure 4B) (Munkvold, 2003b). Sometimes, FER colonization can be symptomless. FER also has the ability to colonize maize plants systematically (Munkvold, 2003b; Gai *et al.*, 2018). GER is usually predominant in areas with cooler temperatures and higher precipitation during the crop season. High humidity during flowering (more precise silking) favors fungus infection. Colonization is stimulated by moderate temperatures and high levels of precipitations during maturity (Munkvold, 2003b). Conversely, FER is observed in warm and dry areas, especially when weather conditions are dry during the grain-filling stage (Munkvold, 2003b; Oldenburg *et al.*, 2017).

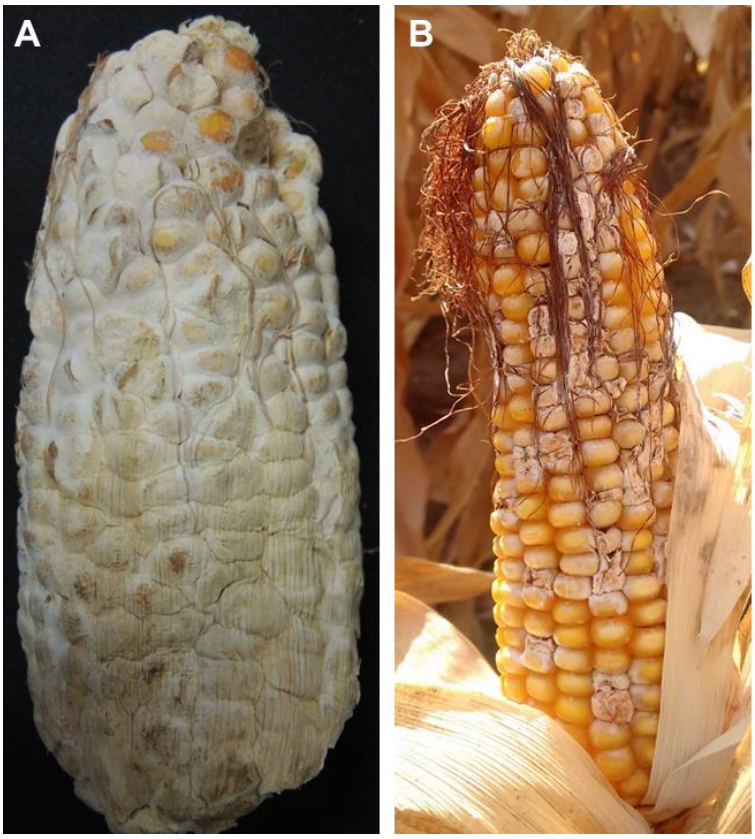


Figure 4. Symptoms of Gibberella ear rot (GER or red ear rot) (A), and Fusarium ear rot (FER or pink ear rot) (B). GER symptoms are gray to pink mold that usually covers large areas of the ear, whereas FER symptoms are usually white mold in random kernels.

In addition to ear rots, *Fusarium* spp. can also attack different maize organs causing stalk, root, and seed rots (White, 2010; Oldenburg *et al.*, 2017). GER infections usually start in the female flowering (Reid *et al.*, 1999). Colonization of further kernels occurs through rachis connection. Conversely, FER infections seem to be more frequent through wounds caused by bird, and insects, such as thrips, earworms, and the European corn borer (*Ostrinia nubilalis*) (Oldenburg *et al.*, 2017; Blacutt *et al.*, 2018). *Fusarium* spp. infections can also occur systemically through the infection of rudimentary ears. Rudimentary ears are a disturbance caused by a fertilization deficiency that stimulates the growth of immature ears below the main harvestable ear. As leaf sheaths and husks are enclosed, high humidity is maintained, which favors spore germination and infection (Oldenburg *et al.*, 2017). Systemic infection may also originate from stalk infections. Stalk rot is related to loading and stalk breakage. Yield losses caused by stalk rot were up to 35% are reported in the second season (winter season or ‘safrinha’ – in Portuguese) in Brazil (Costa *et al.*, 2019). During the winter season, the maize crop is more vulnerable to other abiotic stress, such as water availability, especially in the central regions of the country (Brazilian tropical savanna, ‘cerrado’ – in Portuguese) (Costa *et al.*, 2019), which may increase stalk

susceptibility (Dodd, 1980). Seed infection by *Fusarium* spp. can present disturbances in germination and emergence leading to weaker seedlings or damping-off (Sartori *et al.*, 2004; Machado *et al.*, 2013; Oldenburg *et al.*, 2017). The main symptoms are roots and coleoptile showing brownish to black discoloration (White, 2010). Shoots show physiological alterations when inoculated in the seed stage, due to the acceleration of lignin deposition and modifications of chloroplast orientations in young leaves (Yates *et al.*, 1997). Most seedling infections are caused by inoculum that survived in the soil or residual crops (Bacon and Hinton, 1996; White, 2010).

In summary, *Fusarium* spp. infect distinct maize organs, leading to a complex disease cycle (Figure 5) (Blacutt *et al.*, 2018). The primary inoculum usually comes from the soil (Figure 5A) or is brought from other infested fields by wind and rain in the form of spores (Figure 5C,F). *Fusarium* spp. usually survive in crop residuals in the form of mycelia, conidia or chlamydospores (Khonga and Sutton, 1988). These fungal structures are able to infect seedlings causing seedling blight, or to infect roots causing root rots. *Fusarium* spp. can colonize roots (Yates *et al.*, 1997), cause damping-off, or colonize the plant endophytically (Figure 5B) (White, 2010b; Oldenburg *et al.*, 2017; Gai *et al.*, 2018). The main source of inoculum in stalk and ear infections is brought by wind or by other insects such as caterpillars (European corn borer) (Figure 5C,E). In addition, direct penetration through leaf stomata and trichomes by appressoria-like structures has been reported (Nguyen *et al.*, 2016a, 2016b). However, the main infections occur via silk channels (Munkvold *et al.*, 1997) or via wounded tissue, especially provoked due to insects feeding, (Figure 5C-E) (Blacutt *et al.*, 2018), as susceptibility increases in wounded plant tissue (Schaafsma *et al.*, 1993).

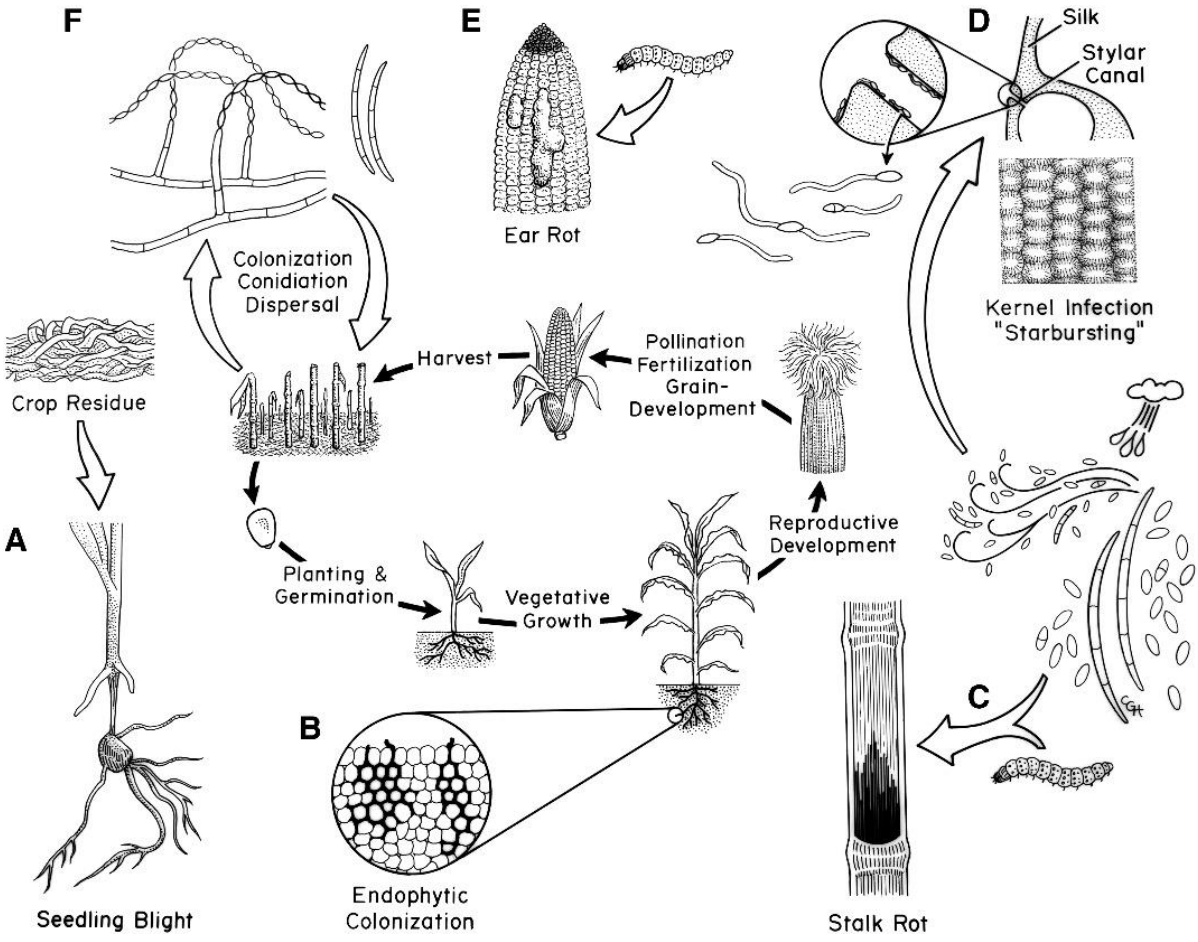


Figure 5. Disease cycle of *Fusarium* spp. in maize (Blacutt *et al.*, 2018). Seedling blight is caused by *Fusarium* spp. infections prevenient mainly from the crop residual or from the soil (A). In some cases, *Fusarium* spp. can colonize endophytically (B). Stalk infections are caused by feeding insect or by mechanical wounds (C). Ear infections are provoked by infections via the silk channel during flowering (D) or by insect feeding (E). The pathogen can sporulate on crop residuals (F) and inoculum is usually dispersed by rain, wind or by insects.

As GER and FER can be caused by many *Fusarium* spp., the species identification can follow different criteria: morphological, biological and phylogenetical (Leslie and Summerell, 2006; Summerell *et al.*, 2010). The morphological criterion is based on macroscopic and microscopic characters (Leslie and Summerell, 2006). The biological criterion is based on the capacity and potential to share the genetic pool; the exchange of genetic material can occur by sexual reproduction or by cross-fertilization. *F. verticillioides* is heterothallic, therefore sexual reproduction is only possible when there are individuals from different mating-types (MAT A-1 and MAT A-2), while *F. graminearum* is homothallic, not requiring individuals from different mating types (Leslie and Summerell, 2006; Blacutt *et al.*, 2018). The same is valid for cross-fertilization; the exchange of genetic material is possible only by

individuals from the same vegetative compatibility group (VCG) (Huang *et al.*, 1997). In this case, two hyphae anastomose (fuse) and form a stable heterokaryon (Kedara *et al.*, 1994; Leslie and Summerell, 2006). Most recently, with the advance of molecular tools, phylogenetic analyses are being applied to avoid the misinterpretation of morphological and biological methods. Phylogenetic methods apply molecular markers and are based on DNA sequences (Leslie and Summerell, 2006). Macroscopic characters are evaluated based on colony characteristics grown on potato-dextrose-agar (PDA) plates, such as color and mycelia features (Figure 6). Microscopic characters are focused on the presence or absence of macroconidia, microconidia, and chlamydospores, and are evaluated from colonies grown in synthetic low nutrition agar (SNA). Important macroconidia features are size, shape, apical and foot cell. For microconidia, the most important features to distinguish *Fusarium* spp. are the size, shape and the phialide (conidiogenous cells) (Leslie and Summerell, 2006).

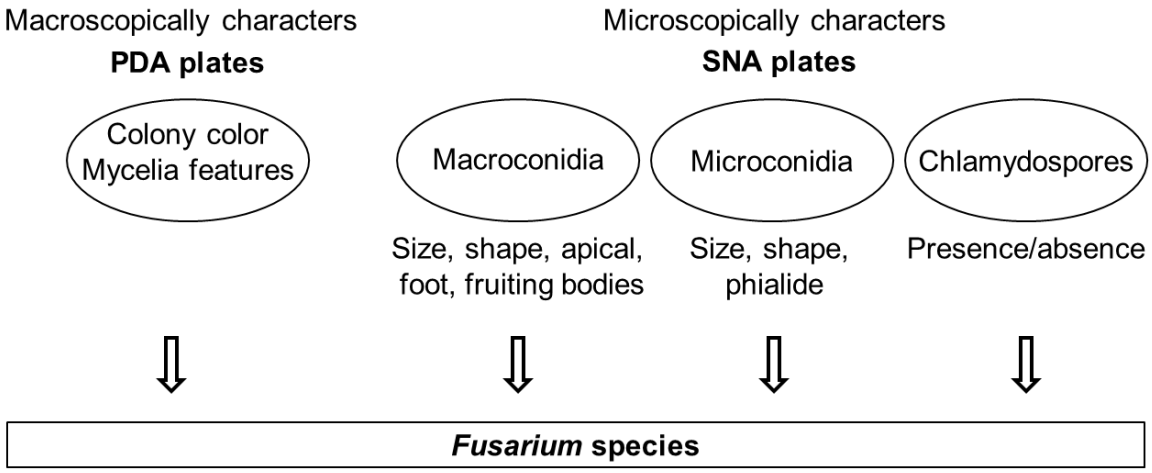


Figure 6. *Fusarium* species identification based on morphological characters. Macroscopically characters evaluated on potato-dextrose-agar (PDA) plates are colony color and mycelia feature. Microscopically characters were evaluated on synthetic low nutrition agar (SNA) plates.

Fusarium spp. distribution is usually related to climate conditions suitable to its host (Summerell *et al.*, 2010). Assessments worldwide shows a broad species spectrum infecting maize ears (Table 2). *Fusarium* spp. monitoring is usually correlated with mycotoxin production, as the chemotype of Fusarium populations may vary. *F. verticillioides*, as with other species from the FFSC, usually producing fumonisins (B series FB1, FB2, FB3 and FB4) (van der Lee *et al.*, 2015; Blacutt *et al.*, 2018). Conversely, *F. graminearum* and FGSC are producers of type B trichotecenes and Zearalenone (ZEA). Nivalenol (NIV), Deoxynivalenol (DON), 3-acetyl- and 15-aceytil-deoxynivalenol (3-ADON

and 15-ADON) are all mycotoxins belonging to the trichotecenes (Logrieco *et al.*, 2002; van der Lee *et al.*, 2015). Fumonisins and trichothecenes can be correlated to the geographic region and weather condition observed for *Fusarium* spp. distribution (Reid *et al.*, 1999; Summerell *et al.*, 2010). Brazilian samples are mainly contaminated with fumonisins (FB1, FB2, FB3) (Almeida *et al.*, 2002; Ottoni, 2008; Lanza *et al.*, 2014), and the same is observed in Tanzania, where the most predominant mycotoxins detected are fumonisins, followed by DON (Degraeve *et al.*, 2016). In Germany, the most commonly detected mycotoxins are DON and ZEA (Goertz *et al.*, 2010). Fusarium populations are dynamic, as observed in Canada, with 3-ADON strains being substituted by 15-ADON (van der Lee *et al.*, 2015).

In general, it is difficult to establish a correlation with climate by analyzing chemotypes within FGSC strains. For instance, 15-ADON producers are mostly prevalent in colder regions, such as North China, while NIV and 3-ADON strains are more common in warmer regions, such as South China (Zhou *et al.*, 2018). In South Brazil, FGSC strains presenting NIV genotype and 3-ADON genotypes (*F. coraderiae*) are more prevalent in areas with high altitude, which are usually colder. Conversely, 15-ADON producer (*F. meriodionale*) are more frequent in lower (warmer) regions (Kuhnem *et al.*, 2016). In South Africa, all FGSC strains isolated from maize are from the trichothecene chemotype 15-ADON (Boutigny *et al.*, 2011). In France, the predominant thichothecene chemotype is 15-ADON (Boutigny *et al.*, 2014).

Table 2. Prevalence of *Fusarium* spp. isolated from ears showing symptoms of GER (Gibberella ear rot) and/or FER (Fusarium ear rot) worldwide.

Region	Country	Disease prevalence	Species prevalence	Reference
South Europe	-	FER	*Fusarium fujikuroi* species complex (FFSC)	Dorn *et al.*, 2009
South Europe	France	-	[1] *F. graminearum, F. boothii*	Boutigny *et al.*, 2017
South Europe	France	-	[2] *F. temperatum*	Boutigny *et al.*, 2017
North Europe	Switzerland	GER	*F. verticillioides*, *F. graminearum*, *F. proliferatum, F. subglutinans, F. cerealis* (*syn. F. crookwellens)*	Dorn *et al.*, 2010
North Europe	Germany	GER	*F graminearum, F. verticillioides, F. temperatum*	Pfordt *et al.*, 2020
North Europe	United Kingdom	GER	*F. graminearum, F. culmorum*	Basler, 2016
North America	USA	GER	*F. verticillioides*	Munkvold, 2003b
North America	Canada	GER	*F. graminearum, F. subglutinans*	Sutton, 1982; Xue et al., 2005
South America	-	FER	*F. verticillioides*	Silva *et al.*, 2007; Iglesias *et al.*, 2010; Stumpf *et al.*, 2013; Lanza *et al.*, 2014
South America	Ecuador	FER	*F. verticillioides*, *F. subglutinans, F. graminearum*	Silva *et al.*, 2007
South America	Mexico	FER	*F. subglutinans, F. verticillioides, F. chlamydosporum, F. poae, F. pseudonygamai, F. napiforme, F. solani*	Morales-Rodríguez *et al.*, 2007
South America	Brazil	-	[1] *F. meridionale, F. graminearum, F. cortaderiae*	Kuhnem *et al.*, 2016
South America	Argentina	-	[1] *F. meridionale, F. boothii*	Sampietro *et al.*, 2011
Africa	Tanzania	GER	*F. verticillioides, F. graminearum, F. poae*	Degraeve *et al.*, 2016
Africa	South Africa	GER	*F. boothii*	Boutigny *et al.*, 2011
Asia	-	GER	*F. asiaticum*	Ndoye *et al.*, 2012
Asia	north China	GER	*F. asiaticum, F. graminearum*	Ndoye *et al.*, 2012
Asia	south China	FER	*F. verticillioides, F. proliferatum, F. meridionale*	Zhou *et al.*, 2018
Asia	South Korea	-	[1] *F. graminearum, F. asiaticum, F. boothii*	Lee *et al.*, 2012
Oceania	New Zealand	GER	*F. graminearum*	Hussein *et al.*, 2002

[1] Studies conducted only on GER (*Fusarium graminearum* species complex - FGSC), [2] Studies conducted only on FER (FFSC)

Besides the frequency and distribution of *Fusarium* chemotypes, the epidemic parameters and toxicology can vary between species. In general, 15-ADON strains produces more perithecia in maize than other chemotype groups such as NIV and 3-ADON (Nicolli *et al.*, 2018). Additionally, a high sexual reproduction corroborates with high a prevalence of 15-ADON in most regions worldwide (Boutigny *et al.*, 2011; Boutigny *et al.*, 2014; Zhou *et al.*, 2018). Moreover, 15-ADON strains are twice more aggressive in wheat than strains from NIV and 3-ADON chemotypes (Kuhnem *et al.*, 2016). Regarding toxicology, NIV is more toxic than DON for humans and animals, as the limit established by the European Scientific Committee for Food for tolerable daily intake is 0.7 µg kg^{-1} body weight and 1 µg kg^{-1}, respectively (van der Lee *et al.*, 2015). Limits permitted by Food and Agriculture Organization (FAO) for DON are 750 µg kg^{-1} per commercialized product, and 1000 µg kg^{-1} for fumonisin B1 (Tola and Kebede, 2016). Since mycotoxins can be a risk to human and animal health, GER and FER control are fundamental to maintaining food security (Savary *et al.*, 2019). In general, diseases caused by *Fusarium* ssp. on maize can lead to yield losses of between 10 and 30% (Logrieco *et al.*, 2002a), especially when considering qualitative yield losses due to mycotoxin contamination (Hallmann and Tiedemann, 2019). Globally, quantitative yield losses caused by stalk and ear rots are estimated as 5% and 2.5%, respectively (Savary *et al.*, 2019). Chemical, cultural and genetic control methods are the most commonly applied methods for GER and FER control. However, the chemical control of *Fusarium* spp. is shown to be inefficient (Munkvold, 2003b; Lanza *et al.*, 2016; Blacutt *et al.*, 2018) and agronomical practices are not strongly correlated to a reduction in disease incidence or severity (Stefanello *et al.*, 2012; Degraeve *et al.*, 2016; Costa *et al.*, 2019; Pfordt *et al.*, 2020). Therefore, host resistance is the most reliable method for FER and GER control (Munkvold, 2003b). Sources of qualitative resistance are almost absent (Reid *et al.*, 1994), thus commercial hybrids bear quantitative resistance (Munkvold, 2003a).

D. Host resistance to plant pathogens

Resistance is the host capacity to avoid pathogen attack or slow down colonization (Miedaner, 2011). In normal conditions, plant pathologists affirm that disease is the ‘exception’; therefore all plants apply mechanisms of basal resistance to avoid the attack of most microorganisms. When the pathogen can break such mechanisms of basal resistance, the disease occurs and the plants turn into hosts (Camargo, 2011b). Host resistance can be divided into two types of resistance: qualitative and quantitative (Camargo, 2011b). Qualitative resistance is a synonym for vertical resistance from the epidemiological perspective being described as discontinuous variation in disease phenotype. Conversely, quantitative resistance, also known as horizontal resistance, is defined by the continuous variation in resistance levels (Vanderplank, 1968). The genotype of qualitative resistance

involves a major gene, the R gene, and is known as monogenic resistance. All of these terms are related to the fact that pathogen recognition is coordinated by one or a few genes (in this case, oligogenic resistance). A race-specific resistance usually has shorter durability of resistance. Conversely, quantitative resistance is known as polygenic, race unspecific or field resistance, because it is best expressed in the field (Table 3) (Miedaner, 2010; Hallmann and Tiedemann, 2019).

Table 3. Differences between vertical and horizontal resistance (Vanderplank, 1968; Miedaner, 2010; Hallmann and Tiedemann, 2019)

Trait	Qualitative resistance/ Vertical resistance	Quantitative resistance/ Horizontal resistance
Resistance effect	yes or no complete	additive effect incomplete
Genotype	monogenic/ oligogenic (major gene or R gene)	polygenic (minor gene)
Specificity	Race specific	Non-race specific
Durability	temporary	durable
Correspondent genes between host and pathogen	yes (gene-for-gene concept)	uncertain
Epidemiological effect	reduces primary inoculum (Q) delay the start of epidemic	reduces apparent infection rate (r) delay the increase of epidemic
Environmental effect	small	high

The invasion of plant pathogens is recognized by the plant immune system, as described in the “zig-zag model” (Jones and Dangl, 2006). Transmembrane pattern recognition receptors (PRR proteins) recognize microbial- or pathogen associated molecular patterns (MAMPs/PAMPs) in a process known as PAMP-triggered immunity (PTI), which is related to basal resistance. When the pathogen releases effectors (in this example can be also called elicitors, when molecules are released by the pathogen), also known as virulence factor, the process results in the process known as effector-triggered susceptibility (ETS). If pathogen recognition stops at ETS, the pathogen will be able to colonize the tissue. Conversely, if pathogen effectors are specifically recognized by PRR-proteins, the result is effector-triggered immunity (ETI). In the case of *Htn1* resistance gene to *E. turcicum*, the PRR-proteins are encoded in the genome at nucleotide binding and leucine rich repeat domains (NB-LRR) (Hurni *et al.*, 2015). At ETI, the induction of a hypersensitive response (HR) may occur (Jones and Dangl, 2006). In cases of qualitative resistance, the incompatible interaction is expressed when the pathogen has an avirulent gene (*Avr* gene) and the host has a resistance gene (R gene). The opposite is a compatible interaction, when the pathogen has no avirulent gene (*avr* gene), or the host has no R gene. Therefore, the

pathogen cannot be recognized by the host, following the gene-for-gene concept (Flor, 1971). A mutation in avirulent genes of a pathogen may suppress several resistance mechanisms. The suppression of a single gene may change the signaling cascade, altering the expression of several genes that might be related to the phenotypical expression of resistance (Camargo, 2011a). Conversely, in cultivars with quantitative resistance, the effect of a single mutation in the pathogen may have small effect on disease levels (Vanderplank, 1968).

The recognition of PAMPs by PRR proteins results in the accumulation of reactive oxygen species (ROS) (Malinovsky *et al.*, 2014). At PTI, ROS can activate protein kinase (MAPK) cascades and transcriptomes will be reprogrammed (Kachroo *et al.*, 2017). Moreover, ROS can play a role in HR. The accumulation of toxic substances such as hydroxide peroxide (H_2O_2) and superoxide (O_2^-) occurs first in the apoplast causing HR. Later, H_2O_2 and O_2^- accumulates in the chloroplasts. Many effectors target electron transport chains in an attempt to stop ROS production. ROS may cause stomatal closure due to their accumulation in guard cells. The damage during photosynthesis process can be observed in resistant plants (Waszczak *et al.*, 2018). The imbalance between ROS production and its detoxification (reduction in the activity of ROS scavenging enzymes) may increase ROS concentration in the host tissue causing programmed cell death (PCD), avoiding pathogen colonization (Apel and Hirt, 2004).

Molecular mechanisms involved in quantitative resistance seems to be poorly understood. Genetically, the quantitative resistance is associated to a quantitative trait locus (QTL) and could be related to several resistance mechanisms (Poland *et al.*, 2009). Resistance mechanism encoded by quantitative resistance can be related to morphology and/or to stages of host development. Another type of resistance could be related with mutation of alleles involved on basal resistance. PRR receptors acting on basal resistance could trigger resistance in distinct stage of pathogen infection and colonization. The third resistance mechanism associated to quantitative resistance is related to biochemical defense processes, such as phytotoxins detoxification and the production of phytoalexins (Poland *et al.*, 2009). The accumulation of callose and phenolic compounds at the penetration sites of *E. turcicum* by maize plants bearing QTLs are another example. QTL could be involved in the defense signal transduction. The regulation of the phytohormones salicylic acid, jasmonic acid and ethylene and on their signaling pathways may vary on resistance levels. The quantitative resistance may be a weaker “form” of the R genes, conferring less effective resistance (Poland *et al.*, 2009). Effectors released by the pathogen can trigger resistance mechanisms encoded by many resistance genes altering how the plant recognizes the pathogen in order to suppress host resistance (Niks *et al.*, 2011, Cowin and Klinberstein,

2017). Thus, quantitative resistance was called minor-gene-for-minor-gene interaction by Parlevliet and Zadoks (1977). The last hypothesis of resistance mechanisms involved on quantitative resistance is related to classes of genes that were not previously reported to have function on resistance. An example is the proline-rich gene, which does not show similarities to the already known defense-related genes, but could trigger pathways indirectly related to resistance (Poland *et al.*, 2009).

The durability of resistance is important from the practical perspective, especially for breeding programs (Galiano-Carneiro and Miedaner, 2017); however, its estimation dependent on other factors. The extensive use of quantitative resistance in large areas, during long periods and under conducive environmental conditions for disease occurrence may affect its durability (Parlevliet, 2002). In general, qualitative resistance triggering mechanisms of hypersensitive reaction is not durable (Parlevliet, 2002). The most durable resistance is based on the additive effect (Table 3) of many genes with small influence on resistance. This characteristic is conferred by resistance of quantitative nature (Parlevliet, 2002). Therefore, erosion of quantitative resistance seems to be difficult being not reported yet. Generally, resistance genes targeted by essential pathogen effectors in the quantitative resistance might confer more durable resistance than resistance genes targeted by non-essential effectors (Pilet-Nayel *et al.*, 2017). Essential pathogen effectors are encoded by genes that are also responsible for other functions of plant development. An example is the *PthXo1* effector excreted by *Xanthomonas oryzae* pv. *Oryzae*; this effector activates a host resistance gene called *Xa13* in rice. However, its allele *xa13* (also named *Os*-8N3), is fundamental for pollen development. Therefore, the presence of *xa13* / *Os*-8N3, which is fundamental for some physiological processes, automatically confers susceptibility in this case (Dangl *et al.*, 2013).

In the host-pathogen interaction between *F graminearum* and maize, effectors are associated with cell wall and membrane degrading enzymes, such as cellulases, pectinase, proteases, xylanases and lipases (Taheri, 2018). Moreover, cutinase and lipase were shown to play a role in the infection of *F. graminearum* (Voigt *et al.*, 2005). ROS can also signal further defense mechanisms, such as lignification and callose deposition (Taheri, 2018). Lignin enforces cell wall resistance against cell wall degrading enzymes and prevent dispersal of phytotoxins in the plant tissue (Vance *et al.*, 1980). In general, mycotoxins may not be considered virulence factors (Desjandins *et al.*, 1995). *In vitro* experiments demonstrate that *F. proliferatum* produces ten times more fumonisins when compared to *F. verticillioides* in maize seedlings (Zhou *et al.*, 2018). Curiously, the most predominant species in the world is *F. verticillioides* (Munkvold, 2003), confirming that highly aggressive

strains are disfavored by natural selection. Thus, less aggressive strains may be favored by arable crops (Iglesias *et al.*, 2010).

E. Aim of the thesis

The introduction of resistance sources obtained from tropical material into the temperate breeding lines is a challenge for breeding programs (Lee and Tracy, 2009). As tropical populations are genetically distantly related to temperate populations, selection for resistance, yield, and maturity is laborious (Miedaner, 2010). Moreover, pathogen populations vary between regions (Miedaner *et al.*, 2010; Hanekamp, 2016), which may influence the efficacy of resistance. The main objective of this work was to compare the pathogen population and host resistance to NCLB, GER and FER in Europe and in South America. Therefore, the aggressiveness, the race distribution from *E. turcicum* and the species frequency of *Fusarium* spp. were compared between regions. Besides studies with pathogen populations, pathogen colonization, host physiology and some epidemiological parameters were characterized for maize plants bearing the *Ht1, Ht2, Ht3* and *Htn1* resistance genes.

In the first chapter, the objective was to characterize the race spectrum and frequency from *E. turcicum* isolates from Argentina and Brazil. Data of race distribution permit an indirect inference of resistance genes introduced in the cultivated maize hybrids. Information about the race frequency may guide breeding programs in these countries.

In the second chapter, once the *E. turcicum* collection of South American isolates was established, the aggressiveness between European and South American isolates was compared under different temperatures. The influence of temperature on pathogen development was demonstrated by *in vitro* and *in vivo* experiments.

In the third, fourth and fifth chapters, the focuses were on host-pathogen interactions in maize plants bearing *Ht* genes. In the third chapter, the pathogen colonization was compared between compatible and incompatible interactions by the quantification of *E. turcicum* DNA and by histological studies. Pathogen colonization was described at five different time points: penetration, first infection stage, late infection stage, first symptom expression, and symptom differentiation.

In the fourth chapter, the physiological and biochemical responses of resistance genes *Ht1*, *Ht2*, *Ht3* and *Htn1* to *E. turcicum* infection were characterized for the incompatible interaction. Photosynthetic variables and biochemical responses were characterized in the incompatible interaction of resistant maize lines and *E. turcicum*. The epidemiological components number of lesions, lesion length and sporulation were quantified.

During the investigation on host-pathogen interaction, a different pattern for pathogen colonization and for the epidemiological parameters was observed for plants bearing the *Ht2* gene. Additionally, changes in phenotype were reported for resistant plants maintained under different environmental conditions. In the fifth chapter, our objective was to assess the influence of pre-inoculation temperature on the efficacy of *Ht2*-resistance to *E. turcicum.* For this proposal, disease severity and fungus DNA content were compared between plants susceptible and resistant plants exposed to warm (30/25°C) and moderate (20/15°C) temperature regimes before inoculation.

In the sixth chapter, studies were conducted to compare GER and FER in European and in South America. Therefore, pathogen aggressiveness between isolates collected in Germany and in Brazil and the resistance of European (temperate) and South American (tropical) maize lines were compared under greenhouse conditions. Finally, the effect of pre-inoculation temperatures on resistance of tropical lines was demonstrated for two conditions: warm (30/25°C) and moderate (20/15°C) day/night temperature regimes.

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Chapter 1. Assessment of physiological races of *Exserohilum turcicum* isolates from maize in Argentina and Brazil

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Abstract

Northern corn leaf blight (NCLB) is one of the most important diseases in maize worldwide. It is caused by the fungus *Exserohilum turcicum*, which exhibits a high genetic variability for virulence, and hence physiological races have been reported. Disease control is based mainly on fungicide application and host resistance. Qualitative resistance has been widely used to control NCLB through the deployment of *Ht* genes. Known pathogen races are designated according to their virulence to the corresponding *Ht* gene. Knowledge about of *E. turcicum* race distribution in maize-producing areas is essential to develop and exploit resistant genotypes. Maize leaves showing distinct elliptical grey-green lesions were collected from maize-producing areas of Argentina and Brazil, and 184 monosporic *E. turcicum* isolates were obtained. A total of 66 isolates were collected from Argentina during 2015, 2018 and 2019, while 118 isolates from Brazil were collected during 2017, 2018 and 2019. All isolates were screened on maize differential lines containing *Ht1*, *Ht2*, *Ht3* and *Htn1* resistance genes. In greenhouse experiments, inoculated maize plants were evaluated at 14 days after inoculation. Resistance reaction was characterized by chlorosis, and susceptibility was defined by necrosis in the absence of chlorosis. The most frequent race was 0 in both Argentina (83%) and Brazil (65%). Frequencies of race 1 (6% and 24%) and race 23N (5% and 10%) were very low in Argentina and Brazil, respectively. The high frequency of race 0 isolates provides evidence that qualitative resistance based on the tested *Ht* genes is not being used extensively in Argentina and Brazil to control NCLB. This information may be relevant for growers and breeding programs as the incidence of NCLB is increasing in both countries.

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Keywords: Northern corn leaf blight, qualitative resistance, R genes, race diversity, pathogen population

Introduction

Northern corn leaf blight (NCLB) on maize (*Zea mays*) is caused by the heterothallic ascomycete *Exserohilum turcicum* (Pass.) K.J. Leonard & Suggs (teleomorph *Setosphaeria turcica*). NCLB has spread from tropical to temperate regions in maize producing areas worldwide and is one of the most important foliar diseases of this crop (CABI, 2019; Savary *et al.,* 2019). Long dew periods and moderate temperatures favour disease establishment and development (Welz and Geiger 2000; Galiano-Carneiro and Miedaner 2017). Therefore, regions with favourable environmental conditions usually present high levels of NCLB inoculum (Galiano-Carneiro and Miedaner, 2017). Likewise, the cultivation of susceptible host genotypes, as well as the adoption of new crop management strategies such as shifted sowing dates, irrigation and no-tillage systems, can affect the pathogen life cycle and, consequently, disease severity (Carvalho *et al.,* 2016; Juroszek and von Tiedemann, 2013). In some countries, yield losses up to 40% have been reported, when the host is infected by the fungus within 2 to 3 weeks after pollination (Levy and Pataky, 1992). The main methods of controlling NCLB are host resistance and fungicide application (Galiano-Carneiro and Miedaner, 2017).

Sources of host resistance against *E. turcicum* are quantitative or qualitative. Quantitative resistance is controlled by several race non-specific genes with small to moderate effects, conferring usually an incomplete durable resistance (Parlevliet, 2002; Pilet-Nayel *et al.*, 2017). However, quantitative resistance is more difficult to introgress into breeding lines (Galiano-Carneiro and Miedaner, 2017). Conversely, qualitative resistance is typically race-specific and controlled by *Ht* genes, also termed major or R genes (Galiano-Carneiro and Miedaner, 2017). Usually, qualitative resistance provokes localized cell death, known as a hypersensitive response (HR), which can lead to suppression of pathogen colonization and reproduction (Parlevliet, 2002).

Several *Ht* genes have been identified from different genetic backgrounds and used in breeding programs to improve NCLB resistance (Ferguson and Carson, 2007). The first *Ht* gene reported in the literature was *Ht1*. This gene was found in the maize lines ‘Ladyfinger’ popcorn and ‘GE440’ from Peru and the USA, respectively. The resistance reaction expressed by *Ht1* is described as chlorosis, delay in necrosis and inhibition of sporulation (Hooker, 1963). *Ht2* was the second major resistance gene described for *E. turcicum*. It was found in the Australian maize line ‘NN14B’ and expresses chlorosis as the resistant phenotype. However, *Ht2* was described as having a lower resistance level compared to

Ht1 (Hooker, 1977; Navarro *et al.,* 2020). Apart from *Ht1* and *Ht2*, there is *Ht3*, which was introgressed from a tropical grass, *Tripsacum floridanum*, and expresses chlorosis as the resistant phenotype (Hooker, 1981). *Htn1* is another resistance gene used in breeding programs and was discovered in the Mexican maize variety 'Pepitilla'. The resistance phenotype described for this gene differs from those previously mentioned, as the resistance mechanism is based on a longer latent period (Gevers, 1975). In addition to *Ht1*, *Ht2*, *Ht3* and *Htn1*, other dominant genes have been identified and incorporated into maize hybrids. The gene *Htm1* was discovered in the variety 'Mayorbela' from Puerto Rico and confers resistance by expression of chlorotic lesions (Robbins and Warren, 1993). In Brazil, the resistance gene *HtP* was found in the inbred line 'L30R', which may also confer a chlorotic phenotype or the absence of symptoms, known as full resistance (Ogliari *et al.,* 2005). Another gene conferring full resistance was found in the Indonesian variety 'Bramadi' and is called *HtNB* (Wang *et al.,* 2012). Furthermore, two recessive resistance genes, *ht4* and *rt*, have been reported. The *ht4* gene confers a chlorotic halo and was discovered in the US maize inbred line 357 (BS19) (Carson, 1995). The *rt* gene was found in the Brazilian maize line L40 and confers chlorosis or full resistance (Ogliari *et al.,* 2005).

Physiological races of *E. turcicum* are determined according to virulence to the host *Ht* genes. Studies on the frequency of races are conducted by evaluating the disease phenotype of differential lines carrying a single *Ht* gene inoculated with different isolates. Race 0 isolates are avirulent in plants carrying *Ht* resistance genes. Isolates designated as race 1 are virulent to the *Ht1* gene and race 23N is virulent to the *Ht2*, *Ht3* and *Htn1* genes (Leonard *et al.*, 1989). Following the gene-for-gene concept, each major gene has one corresponding avirulence gene that confers resistance (Flor, 1971). *E. turcicum* excretes protein effectors (virulence factors) that interact with the host resistance proteins, which activate the plant immune system, leading to resistance or susceptibility (Jones and Dangl, 2006). A recent study identified in *E. turcicum* an avirulence gene *AVRHt1* corresponding to the resistance gene *Ht1* (Mideros *et al.,* 2018). *AVRHt1* was expressed *in planta* by a race 23N isolate (Human *et al.,* 2020). Gene effector candidates encoded a hybrid polyketide synthase:nonribosomal peptide synthetase (PKS:NRPS) enzyme (Wu *et al.,* 2015), virulence-associated peptidases leupeptin-inhibiting protein 1 and fungalysin, which represent proteins involved in the biosynthesis of secondary metabolites and cell wall degradation (Human *et al.,* 2020). Moreover, the Ecp6 and SIX13-like protein effectors discovered for *E. turcicum* are similar to the effectors secreted in the xylem by *Fusarium oxysporum* (Human *et al.,* 2020).

Besides the high complexity involved in *E. turcicum* virulence, which instigates the development of molecular studies, monitoring of the distribution of *E. turcicum* physiological

races has been conducted worldwide. A high frequency of race 0 isolates has been observed in most maize producing regions around the world (Abadi *et al.,* 1989; Hanekamp *et al.,* 2014). However, with the introduction of *Ht* genes in commercial hybrids, the frequency of isolates virulent to *Ht1* has increased over the last few decades in the USA and China (Ferguson and Carson, 2007; Dong *et al.,* 2008; Weems and Bradley, 2018; Li et al. 2019). Moreover, some regions in Europe (namely the Netherlands and northern Germany) have presented a high frequency of isolates overcoming Ht3 (Hanekamp *et al.,* 2014). In Brazil, studies conducted with a few isolates have demonstrated that race 0 was the most frequent. In addition, races 1N, 12N and 123N were also identified (Gianasi *et al.*, 1996; Ogliari *et al.*, 2005). For Argentina, there are no reports on *E. turcicum* race diversity.

Despite the reports about the frequency of *E. turcicum* races around the world, there is a lack of information about the presence of *Ht* resistance genes in maize hybrids cultivated in Argentina and Brazil. Information about the race distribution in *E. turcicum* populations may indirectly reveal which are the most cultivated *Ht* genes in these regions. Therefore, race assessment of *E. turcicum* isolates from Argentina and Brazil was conducted to guide breeding programs in these countries.

Material and Methods

Samples collection, isolation and preservation

Maize leaves showing lesions similar to NCLB were collected in maize-producing areas of Argentina and Brazil. Dry leaf pieces were cut from the area between the lesion and the green leaf tissue and disinfected in 2% sodium hypochlorite solution for 30 s. The samples were washed with sterilized distilled water and incubated in the dark at room temperature (24 °C) in petri dishes containing moistened filter paper for 2 to 3 days until grey mycelia were visible. The samples were analysed under a stereomicroscope and single spores were transferred to plates containing synthetic nutrient-poor agar (SNA) medium using a needle. The SNA plates were incubated for 5 days at room temperature until the first mycelia were visible. Then, a young monosporic colony was transferred to a plate containing V8 medium (eight vegetable juice agar) and incubated in the dark at 24 °C. After 14 days, spores were harvested by washing the plate with 25% glycerol solution. The spore suspension was stored at − 20 °C for further experiments.

E. turcicum isolates were grouped according to the country and location of origin. Since temperature and light intensity may influence pathogen development and the phenotype expressed by some *Ht* genes (Thakur *et al*., 1989a, 1989b; Leath *et al.*, 1990), the climate in the sampled area was considered in order to separate isolates according to their region.

Information about the climate was based on the classification proposed by Köppen and Geiger (Peel *et al.*, 2007) and adapted according to climatic information available in national institutions from those countries (IBGE, 2002; ANIDA, 2020). Symptomatic maize leaves were sampled in three climatic regions:

1. Temperate or pampeano climate (Cfa): no dry season and with hot summers—Average minimum temperature between 0 and 18 °C and average maximum temperature higher than 22 °C, annual precipitation around 1200 mm.

2. Sub-tropical from altitute climate (Cfb): no dry season and with warm summers—Average minimum temperature between 0 and 18 °C, with less than 4 months with minimum temperature lower than 10 °C and average maximum temperature lower than 22 °C, annual precipitation around 1500 mm.

3. Tropical climate (Cwa): with dry winter and hot summer—average maximum temperature is higher than 22 °C, annual precipitation around 1200 mm.

In Argentina, leaf samples were collected from regions with a Cfa (temperate or pampeano) or Cwa (Sub-tropical) climate (Figure 1). In Brazil, samples were collected from regions with a Cfa (temperate), Cfb (sub-tropical from altitude) or Cwa (tropical) climate (Figure 1). In both countries, maize breeding programs have developed hybrids adapted to different agroecological regions where maize is produced (Kulka, 2019 – *personal communication*). The map was drawn using the package ggplot2 and sf in R software 3.6.0 (Core Team, 2020).

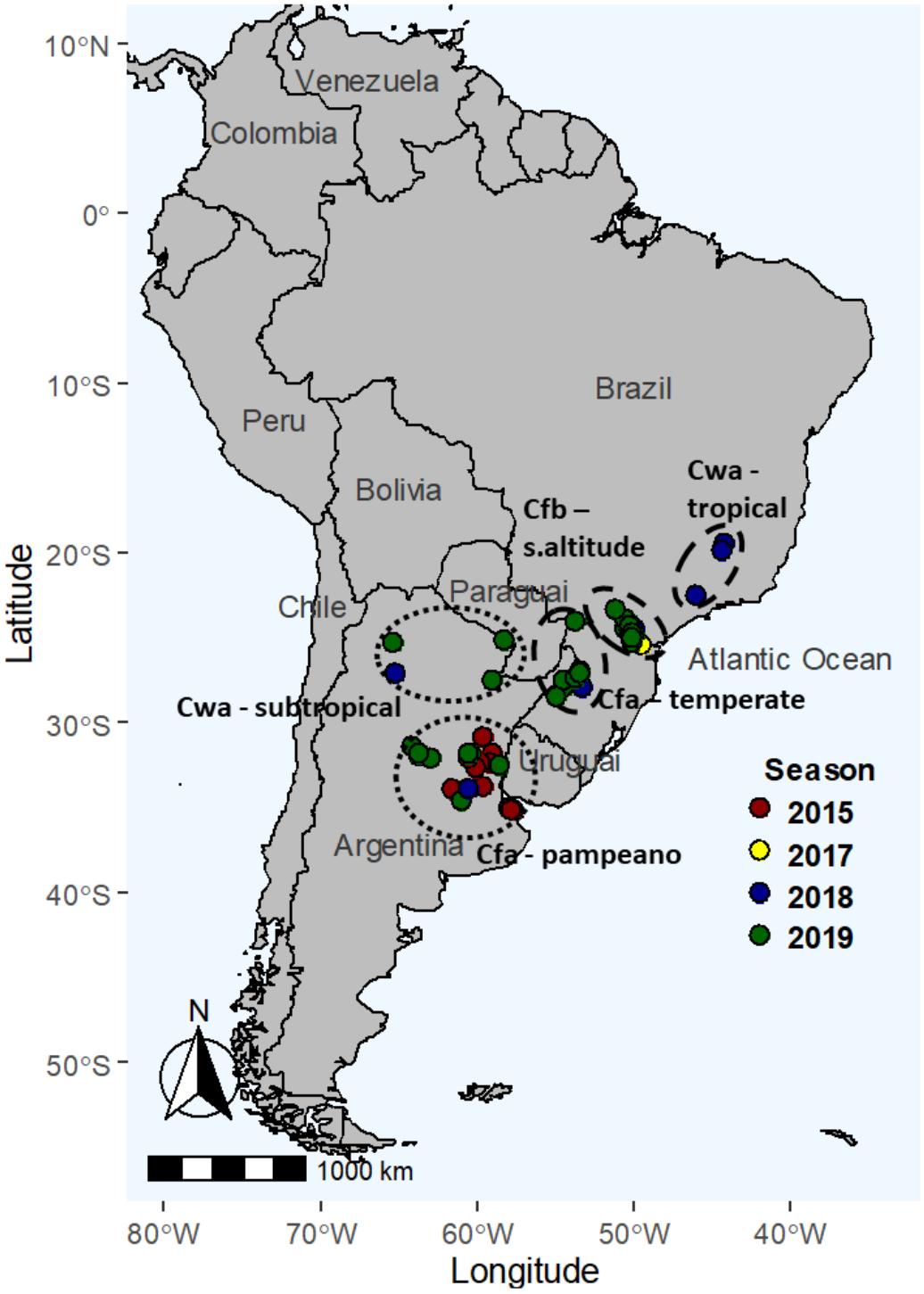

Figure 1. Map of South America with locations were symptomatic leaf samples of northern corn leaf blight were collected for *Exserohilum turcicu*m isolation. Symptomatic maize leaves were collected in 2015, 2017, 2018 and 2019 from maize producing areas in Brazil (dashed lines) and Argentina (dotted lines), according to the Köppen-Gerger climate classification (Peel et al. 2007), adapted using information from Argentinian and Brazilian Institutions (ANIDA 2020; INMET 2020): Argentina—Cfa (temperate or pampeano), Argentina—Cwa (sub-tropical), Brazil—Cfa (temperate), Brazil—Cfb (sub-tropical from altitude) or Cwa (tropical).

Plant material, inoculation and race determination

Maize near isogenic lines derived from the recurrent parent B37 carrying *Ht1*, *Ht2*, *Ht3* and *Htn1* genes and without *Ht* resistance genes (positive control) were used as differential set. Plants were cultivated in a greenhouse (22 ± 6 °C, 70% air humidity, day/night light regime 14/10 h, light intensity of 100 ± 20 µmol m^{-2} s^{-1}). Two seeds per pot (11 × 11 × 10 cm^3) were sown in a soil mixture of compost, clay and sand (3:3:1). Seeds from the differential set were provided by KWS Saat SE (Einbeck, Germany). When the fifth leaf of the maize seedlings unfolded, 1 month after sowing, four plants per isoline were inoculated using a sprayer. Approximately 7 ml of spore suspension adjusted to $3*10^3$ spores ml^{-1} and containing 125 ppm of the surfactant Silwet Gold (Certis Europe B.V., Hamburg) was inoculated per plant. After inoculation, all plants were maintained in a humidity chamber for 24 h and then moved back to the greenhouse. Disease phenotyping was done at 14 and 21 days post-inoculation (dpi), and was based on a diagrammatic ordinal scale used to classify virulence according to the presence or absence of chlorotic and/or necrotic symptoms (Figure 2) (Bigirwa *et al.,* 1993; Hanekamp *et al.,* 2014). The incompatible interaction is predominantly characterized by the presence of chlorosis, whereas compatible interactions consist of necrosis. However, both symptoms are observed in high intensity in the incompatible interaction of *Ht1*. Graphs were plotted using Microsoft Excel 2016.

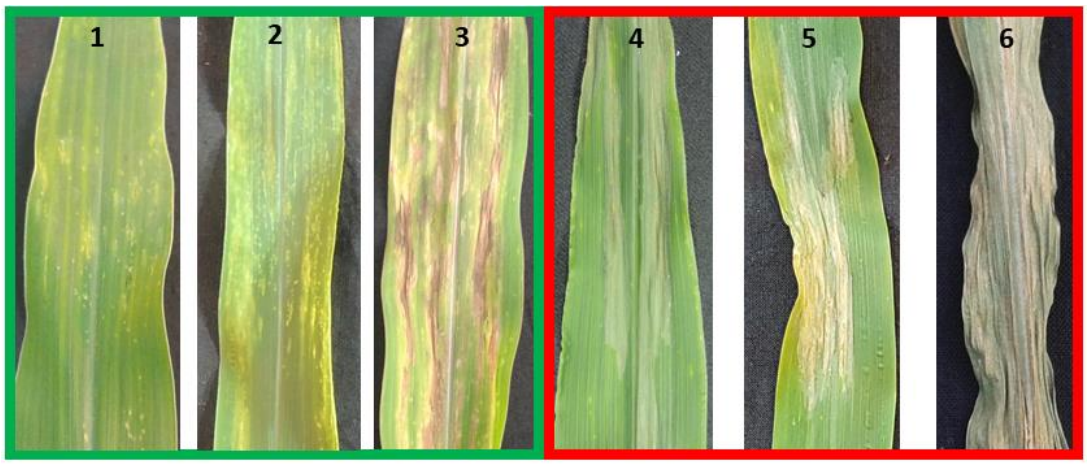

Figure 2. Diagrammatic scale for race phenotyping of northern corn leaf blight caused by *Exserohilum turcicum*, based on Bigirwa *et al.*, (1993) and modified by Hanekamp *et al.,* (2014). Score 1 defines symptoms of chlorotic spots, score 2 describes chlorosis with delimitated necrosis, score 3 represents symptoms of necrotic lesions surrounded by chlorosis, score 4 represents grey elongated lesions, score 5 describes elongated green-grey lesions with necrosis and score 6 is a dead leaf. The absence of chlorosis (> score 4) characterizes a compatible interaction where the pathogen is considered virulent and the host susceptible.

Race diversity indices

The virulence index (I_v) was calculated based on the frequency of isolates and the race complexity using the formula:

$$Iv = \sum (\frac{pi * \mathrm{rc}}{n})$$

where *pi* is the frequency of the *i*th phenotypic race, rc is the race complexity of the *i*th phenotypic race and *n* is the number of isolates in the region (Andrivon and Vallavieille-Pope, 1995). Race complexity (rc) denotes the number of differential lines for which a specific isolate is virulent.

The simple index (I_s) is the simplest diversity index, expressed by the equation:

$$Is = \frac{r}{n}$$

where r is the phenotypic race, and n is the total number of isolates sampled in the region (Weems and Bradley, 2018). The Gleason index (I_g), another diversity index less sensitive to sample size, was calculated by:

$$Ig = \frac{(r-1)}{\ln(n)}$$

where r is the phenotypic race, and *n* is the total number of isolates sampled in the region. The Shannon index (I_w) represents the evenness of race distribution and is calculated by:

$$Iw = -\sum pi \ \ln(pi)$$

where *pi* is the frequency of the *i*th phenotypic race (Groth and Roelfs, 1987). Analysis of correlation between indices was performed using Statistica 13.0 software (Statsoft, Tulsa, USA).

Results

Geographic distribution and frequency of E. turcicum *races*

A total of 184 isolates were obtained from maize fields in Argentina and Brazil between 2015 and 2019 (Table 1). In Argentina, maize leaves were sampled in 2015, 2018 and 2019; a total of 66 isolates were obtained. In Brazil, 118 isolates were obtained from the summer seasons of 2017, 2018 and 2019. Based on the phenotype expressed in the differential set, isolates were categorized into seven physiological races (0, 1, 2, 3, 23N, 3N and 13N). Chlorotic lesions characterized the resistance response. In a susceptible reaction, only necrosis was observed (Figure 2). In total, 132 isolates (71.7%) were avirulent in plants

carrying *Ht* resistance genes, and therefore designated race 0. Race 1 isolates represented 17.4% of the total screened isolates. Fifteen isolates (8.2%) were classified as race 23N. The races 2, 3, 3N and 13N were identified in low frequencies of 1.1%, 0.5%, 0.5% 0.5% and 0.5%, respectively. In Argentina, the highest number of screened isolates was from 2015, and consequently this was the year with the highest number of races. In general, regions with a pampeano/temperate climate (Cfa) exhibited a higher number of races and isolates. In Brazil, most screened isolates were from regions with a subtropical altitude climate (Cfb). It is noteworthy that isolates collected in subtropical and tropical regions (Cwa climate) were mostly race 23N. In Argentina, the vast majority of isolates were race 0, with an isolate frequency of 83.3%, followed by race 1 (6.2%), race 23N (3.5%), race 2 (1.5%), race 3 (1.5%), race 3N (1.5%) and race 13N (1.5%) (Figure 3A). Results were similar in Brazil where 65.3% of the isolates belonged to race 0. However, the frequency of race 1 (23.7%) and race 23N (10.2%) was higher than in Argentina (Figure 3B). Additionally, a race 2 isolate was identified in Brazil, with a frequency of 0.8%.

Table 1. Distribution of *Exserohilum turcicum* isolates according to race assignment by screening on the differential set based on line B37 bearing resistance genes *Ht1*, *Ht2*, *Ht3* and *Htn1*.

		Races							
Country	**Climate**	**0**	**1**	**2**	**3**	**23N**	**3N**	**13N**	**Total**
Argentina	Cfa	44 (86.1%)	1 (2%)	1 (2%)	1 (2%)	3 (5.9%)	-	1 (2%)	51
	Cwa	11 (73.3%)	3 (20.0%)	-	-	-	1 (6.7%)	-	15
	total	55 (83.3%)	4 (6.2%)	1 (1.5%)	1 (1.5%)	3 (3.5%)	1 (1.5%)	1 (1.5%)	66
Brazil	Cfa	29 (61.7%)	14 (29.8%)	1 (2.1%)	-	3 (6.4%)	-	-	47
	Cfb	44 (72.1%)	14 (23.0%)	-	-	3 (4.9%)	-	-	61
	Cwa	4 (40.0%)	-	-	-	6 (60%)	-	-	10
	total	77 (65.3%)	28 (23.7%)	1 (0.8%)	-	12 (10.2%)	-	-	118
Total		132 (71.7%)	32 (17.4%)	2 (1.1%)	1 (0.5%)	15 (8.2%)	1 (0.5%)	1 (0.5%)	184

For climate regions (Cfa, Cwa, Cfb), see text.

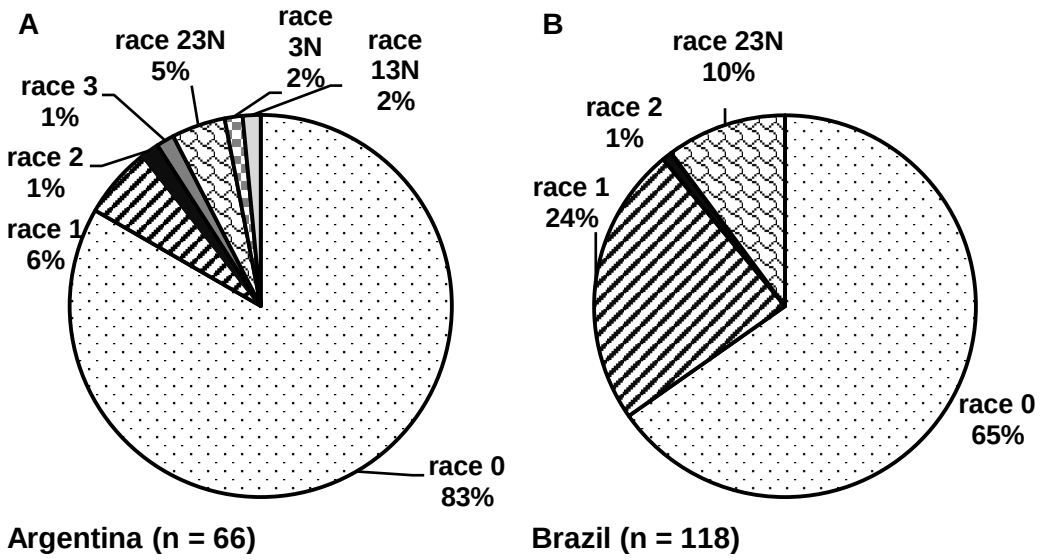

Figure 3. Race frequency of *Exserohilum turcicum* isolates originating from Argentinian samples from 2015, 2018 and 2019 (A) and Brazilian samples from 2017, 2018 and 2019 (B).

Frequency of isolates virulent to a specific Ht *gene and race complexity*

The frequency of virulence to a specific *Ht* gene was compared for all 184 isolates. In general, most screened isolates were avirulent in plants carrying *Ht* resistance genes (race 0), varying from 55 to 75% depending on the year (Figure 4, Supplementary Table 1). An increase in the number of isolates virulent on plants carrying Ht2, Ht3 and Htn1 resistance genes was observed in 2018. In 2019, the decrease in the frequency of avirulent isolates was due to an increase in the frequency of isolates virulent in plants with the Ht1 gene. Race complexity (rc) for the tested isolates was low, since more than 50% of the isolates were avirulent. The only exception was the region with a Cwa climate in Brazil (tropical), where 60% of the isolates were rc 3 (Figure 5). In the other regions of Brazil, between 22 and 33% of the isolates were rc 1. In Argentina, less than 10% of the isolates were rc 1, 1.5% were rc 2 and 6% were rc 3 (Figure 5).

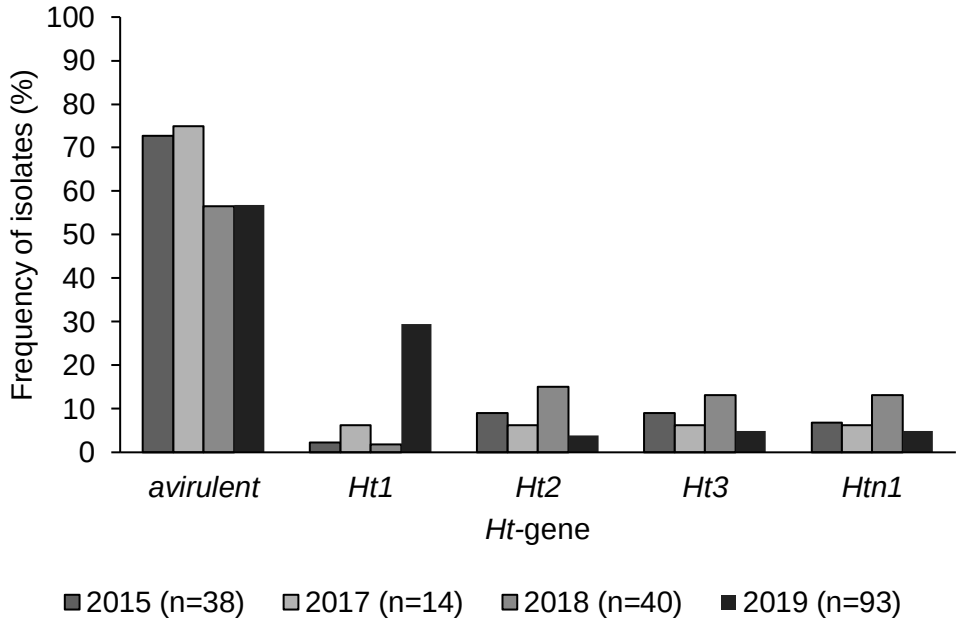

Figure 4. Percentage of *Exserohilum turcicum* isolates virulent to the *Ht* resistance genes *Ht1, Ht2, Ht3* and *Htn1* collected in Argentina in 2015, 2018, and 2019 and Brazil in 2017, 2018, and 2019.

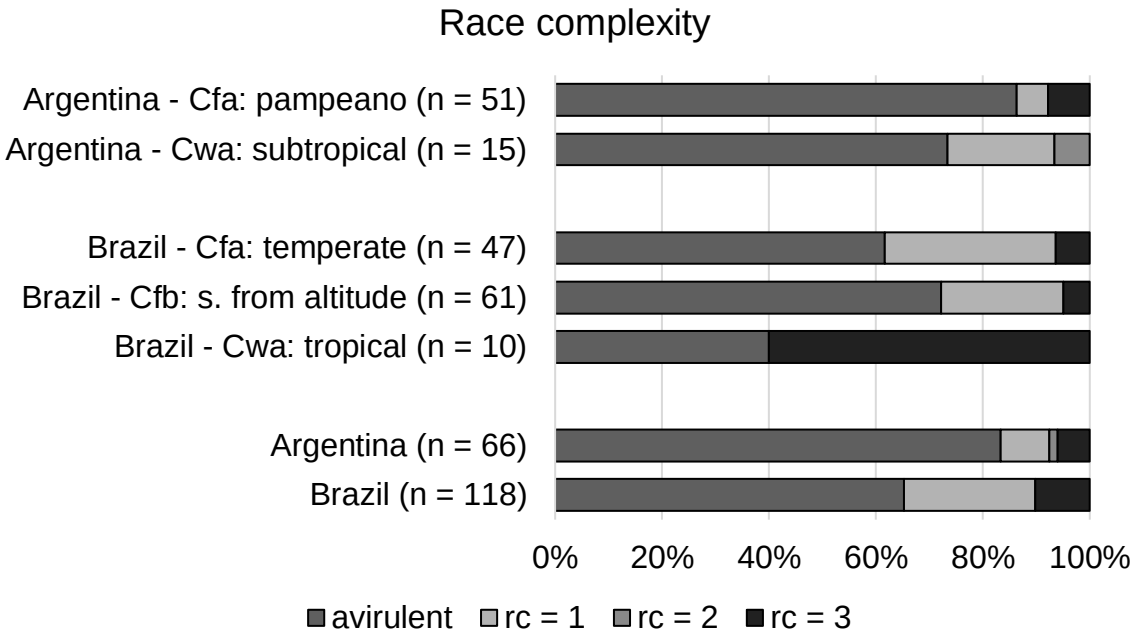

Figure 5. Race complexity of *Exserohilum turcicum* isolates (as a percentage) by country and climatic region based on isolates collected between 2015 and 2019. Race complexity (rc) denotes the number of differential lines for which a specific isolate is virulent (I_v= ∑((pi × rc)/n)), rc 1 = race complexity 1, rc 2 = race complexity 2 and rc 3 = race complexity 3.

Virulence and diversity indices

The virulence and diversity indices are shown in Table 2. The highest number of races was identified in the region of Argentina with a pampeano climate (Cfa). The virulence index was higher for the region with a tropical climate in Brazil (Cwa). The region with a subtropical climate in Argentina (Cwa) presented the highest simple diversity index (I_s) (ratio between races and number of isolates), and between countries, Argentina had the highest I_s. Higher values of I_s are observed in regions with lower number of samples, as observed in both mentioned cases. In addition, the correlation between I_s and the number of samples was negatively significant (Table 3). Conversely, the Gleason index (I_g) was strongly correlated with the number of races and therefore less influenced by the sample size. Argentina had a higher I_g than Brazil at the country level, and the Argentinean region with a pampeano climate (Cfa) had the highest value at the regional level. This indicates a greater diversity of races in these areas (Table 2). I_g is less sensitive to sample size in comparison with I_s, reflected by a strong significant correlation between I_g and the number of races (Table 3). Furthermore, the highest Shannon index (I_w) was observed in Brazil for the region with a temperate climate (Cfa), which indicates the degree to which race distribution within a particular region is equal to that in its respective country. Although the Shannon index represented the uniformity of races, it was not correlated with the other variables and indices (Table 3).

Table 2. Number of isolates, number of races, and virulence and diversity indices for the phenotype of *Exserohilum turcicum* isolates collected in Brazil and Argentina between 2015 and 2019.

Country	Climate	*n*	*r*	I_v	I_s	I_g	I_w
Argentina	Cfa - pampeano	51	6	0,005	0,11	1,26	0,29
	Cwa – subtropical	15	3	0,030	0,23	0,78	0,79
	Cfa – temperate	47	4	0,011	0,09	0,78	0,83
Brazil	Cfb – subtropical from altitude	61	3	0,006	0,05	0,49	0,57
	Cwa – tropical	10	2	0,180	0,20	0,43	0,57
Argentina		66	7	0,005	0,11	1,43	0,32
Brazil		118	4	0,005	0,03	0,63	0,85
Total		184	7	0,003	0,04	1,15	0,75

For climate regions (Cfa, Cwa, Cfb), see text

n = number of isolates, *r* = number of races, I_v = virulence index, I_s = simple index, I_g = Gleason index, I_w = Shannon index

Table 3. Correlation coefficients between number of isolates, number of races, and virulence and diversity indices.

Indices	*n*	*r*	I_v	I_s	I_g	I_w
n	1	0.62	-0.49	-0.78*	0.33	0.24
r		1	-0.58	-0.44	0.93***	-0.40
I_v			1	0.61	-0.51	-0.04
I_s				1	-0.14	-0.11
I_g					1	-0.53
I_w						1

n = number of isolates, *r* = number of races, I_v = virulence index, I_s = simple index, I_g = Gleason index, I_w = Shannon index

Significant coefficients are represented by *** *p*-value ≤ 0.001 and * p-value ≤ 0.05.

Discussion

The race monitoring in Argentina and Brazil revealed a quite homogeneous composition of races across the monitored geographic regions. This indicates that most maize hybrids commercialized in these countries do not carry *Ht* genes and, therefore, virulent isolates are not being selected. It is important to highlight that samples for monitoring of physiological races should be collected from maize hybrids or lines without *Ht* genes (susceptible cultivars). However, breeders are reluctant to share this information for most cultivated hybrids.

Our study indicates that a different race occurrence and distribution can be observed in Argentina and Brazil for the tested isolates, compared to race monitoring data worldwide. For example, in the USA, the frequency of race 0 isolates has decreased from 83% in 1974 to around 50% by the 1990s (Ferguson and Carson, 2007) and to 20% by the 2010s (Weems and Bradley, 2018). The latter study showed that only 26% of isolates were race 1. However, in recent years, the frequency of isolates able to overcome the *Ht1* gene in the country was reported to be 64% (Weems and Bradley, 2018). In Ontario, Canada, the frequency of isolates overcoming *Ht1* was even higher than in the USA, at around 80% from the samples collected between 2012 and 2016. Remarkably, 64% of the isolates were virulent to *Htn1* (Jindal *et al.*, 2019). The increase in frequency of isolates virulent to *Ht1* is a consequence of selection pressure exerted by the widespread cultivation of commercial maize hybrids bearing *Ht1* resistance in the USA (Jordan *et al*., 1983; Ferguson and Carson 2007). It is well known that extensive cultivation of hybrids carrying the same resistance gene enhances the rise of virulent pathogen populations (Mizubuti and Ceresini, 2018).

More recently, the *Htm1* resistance gene was added to the race screening of *E. turcicum* in North America (Weems and Bradley, 2018; Jindal *et al.,* 2019). Surprisingly, 64% of Canadian isolates (Jindal *et al.,* 2019) and 32% of US-tested isolates (Weems and Bradley, 2018) were virulent to *Htm1*. Screening of race M isolates was not easy, due to a limited availability of seeds (Weems and Bradley, 2018). Therefore, these studies used multiple lines, which showed differences in phenotypes for the same resistance gene. For example, although host responses provided by *Ht1*, *Ht2* and *Ht3* usually appeared as chlorosis, as mentioned in the literature (Hooker, 1963, 1977, 1981), strong necrosis was also observed in the line B37*Ht1* (Weems and Bradley, 2018). In line A619, the same authors observed a strong necrosis in plants bearing *Ht2* and *Ht3* genes. Therefore, symptoms of resistance reaction may differ according to the maize line background.

The situation in Europe is different, with 32% of isolates virulent to *Ht3* and 24% to *Ht1* (Hanekamp *et al.,* 2014). Thus, some European hybrids must carry the *Ht3* resistance gene. However, it is important to note that in Europe, the selection pressure exerted by the cultivation of hybrids bearing *Ht* genes is higher than in South America. In Turkey, 68% of isolates were avirulent to *Ht* genes and 16% to the *Ht1* gene (Turgay *et al.,* 2020). Nonetheless, a study from the 1980s reported only race 0 in Israel (Abadi *et al*., 1989). In China, the first study reported a frequency of 40% for race 0, while 41% of isolates were virulent to *Ht1* (Dong *et al.,* 2008). Ten years later, a study with Chinese samples showed that the percentage of isolates from the most frequent races has remained the same (Li *et al.,* 2019). In Brazil, a slight increase in the frequency of isolates virulent to *Ht1* in South America was observed in 2019. In the early 1990s, the most frequent race was 0; however, other races able to overcome *Ht1* were also reported, such as races 1N, 12N and 123N (Gianasi *et al*., 1996; Ogliari *et al*., 2005). Conversely, in most tropical countries, the frequency of isolates virulent to *Ht1* seems to be low, or even absent in some cases. In Kenya, 45% of isolates were virulent to *Ht2* and 29% were identified as race 0 (Muiru *et al.,* 2010). Although race assessments were conducted in Ecuador, Mexico and Zambia, information regarding *E. turcicum* races in these regions is not published in peer-reviewed journals.

Interestingly, in tropical regions, the frequency of isolates virulent to *Ht2*, *Ht3* and *Htn1* was much higher compared to temperate regions. However, qualitative resistance is less used in tropical regions, due to the higher risk of major gene resistance breakdown (Galiano-Carneiro and Miedaner, 2017). As genetic diversity of tropical *E. turcicum* populations is higher than that of temperate populations (Borchardt *et al*., 1998), the pathogen can easily adapt to the *Ht* genes (Galiano-Carneiro and Miedaner, 2017).

The formation of pseudothecia on grasses, such as Johnson grass, has also been observed, indicating that the fungus has the ability to recombine sexually in other hosts (Fallah Moghaddam and Pataky, 1994). However, secondary hosts may also exert selection pressure (Fallah Moghaddam and Pataky, 1994). For instance, *Ht3* is known to be introgressed in maize from the grass *Tripsacum floridanum* (Hooker, 1981) and may be a homologue to *Ht2* in maize (Simcox and Bennetzen, 1993; Fallah Moghaddam and Pataky, 1994). In Uganda, a study with *E. turcicum* strains isolated from sorghum found that 95% of isolates were avirulent on maize (line A619 without *Ht* gene). However, 22% were virulent on plants carrying *Ht1*, 11% were virulent on plants carrying *Ht2* and 5% were virulent on plants carrying *Ht3* (Ramathani *et al.,* 2011).

The unexpected susceptibility of maize plants carrying *Ht* genes, especially to sorghum isolates that are avirulent in maize plants without *Ht* genes, indicates that selective pressure by *Ht2*, *Ht3* and *Htn1* might not be exerted only by maize but also by other grasses or alternative hosts in tropical regions (Fallah Moghaddam and Pataky, 1994). In general, the main sources of fungal genetic diversity are mutations and recombinations (Taylor *et al*., 1999, 2017). Mutations from avirulence to virulence are usually rare; thus, mutation rates are low (McDonald and Linde, 2002). Sexual recombinations may be the source of *E. turcicum* population diversity in the tropics (Borchardt *et al.,* 1998). However, somatic recombinations may also be a source of genetic variability in *E. turcicum* populations, especially in temperate regions (Taylor *et al.,* 1999). In the literature, parasexuality has been described for another ascomycete, *Magnaporthe grisea*, which parasitizes grasses and causes blast disease in rice (Zeigler *et al*., 1997). However, more studies are necessary to prove whether *E. turcicum* has parasexuality, and to identify the contribution of mixed reproduction to *E. turcicum* race diversity.

Qualitative resistance usually leads to a high level of resistance, particularly when the most frequent isolates are avirulent, as observed for Argentina and Brazil. The risk of resistance breakdown due to high genetic flow in populations with mixed reproduction, and the instability of resistance expression due to changes in environmental conditions discourage the use of *Ht* genes in maize breeding programs for tropical regions (Galiano-Carneiro and Miedaner, 2017). Therefore, the use of qualitative and quantitative resistance in tropical and subtropical breeding programs should be accompanied by regular race monitoring to verify if the major genes are still effective in these regions (Perkins and Pedersen, 1987). The introduction of qualitative resistance by recurrent backcrossings is easier and faster for breeders (Pilet-Nayel *et al.,* 2017). Even if qualitative resistance has the disadvantage of shorter durability when compared to quantitative resistance, the introduction of quantitative resistance is more laborious (Galiano-Carneiro and Miedaner, 2017). It is expected that the

durability of major genes is prolonged by pyramiding several major genes in the same cultivars (Pilet-Nayel *et al*., 2017). The information about the race spectrum of *E. turcicum* can support breeders in deciding on the best source of resistance for each region. Moreover, studies on the gene flow between populations from Argentina and Brazil might be conducted to increase knowledge of avirulence to virulence shifts in *E. tucicum* populations, and consequently, the durability of resistance (McDonald and Linde, 2002).

The high frequency of race 0 isolates in Argentina and Brazil leads to the conclusion that most commercialized maize hybrids in these countries do not bear the tested *Ht* resistance genes. This may be due to the fact that most breeding programs have shifted to the use of quantitative resistance after virulence to *Ht* genes had been observed in the 1970s and 1980s (Welz and Geiger, 2000). Therefore, qualitative resistance can be a source of resistance in these countries and, if combined with quantitative resistance, highly effective against NCLB epidemics.

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Supplementary Material

Supplementary table 1. Origin by country, climatic region and sampling year.

			Races							
Year	**Country**	**Climate**	**0**	**1**	**2**	**3**	**23N**	**3N**	**13N**	**Total**
2015		Cfa - pampeano	31	1	1	1	3	-	-	37
		Cwa - sub-tropical	1	-	-	-	-	-	-	1
2018	Argentina	Cfa - pampeano	5	-	-	-	-	-	-	5
		Cwa - sub-tropical	8	1	-	-	-	-	-	9
2019		Cfa - pampeano	8	-	-	-	-	-	1	9
		Cwa - sub-tropical	2	2	-	-	-	1	-	5
		Cfa - temperate	-	-	-	-	-	-	-	-
		Cfb - subtropical from altitude	12	1	-	-	1	-	-	14
		Cwa - tropical	-	-	-	-	-	-	-	-
2017		Cfa - temperate	13	-	1	-	1	-	-	15
2018	Brazil	Cfb - subtropical from altitude	-	-	-	-	-	-	-	-
		Cwa - tropical	4	-	-	-	6	-	-	10
		Cfa - temperate	16	14	-	-	2	-	-	32
		Cfb - subtropical from altitude	32	13	2	-	-	-	-	47
		Cwa - tropical	-	-	-	-	-	-	-	-
all	Total		132 (71.7%)	32 (17.4%)	2 (1.1%)	1 (0.5%)	15 (8.2 %)	1 (0.5%)	1 (0.5%)	184

Supplementary table 2. List of isolates tested on the race assessment.

Isolate	Year	Country	Region	City	Race
A1-1	2015	Argentina	Buenos Aires	Los Hornos	0
A1-2	2015	Argentina	Buenos Aires	Los Hornos	0
A1-3	2015	Argentina	Buenos Aires	Los Hornos	0
A2-2	2015	Argentina	Buenos Aires	Bartolomé Bavio (General Mansilla)	0
A3-1	2015	Argentina	Buenos Aires	Don Joaquín (Magdalena)	0
A3-1	2015	Argentina	Buenos Aires	Don Joaquín (Magdalena)	0
A3-2	2015	Argentina	Buenos Aires	Don Joaquín (Magdalena)	0
A3-3	2015	Argentina	Buenos Aires	Don Joaquín (Magdalena)	0
A3-4	2015	Argentina	Buenos Aires	Don Joaquín (Magdalena)	0
A3-5	2015	Argentina	Buenos Aires	Don Joaquín (Magdalena)	0
A4-1	2015	Argentina	Santa Fe	Santa Isabel	0
A6-1	2015	Argentina	Buenos Aires	Junin	0
A9-1	2015	Argentina	Buenos Aires	San Pedro	0
A10-1	2015	Argentina	Buenos Aires	San Pedro	0
A10-2	2015	Argentina	Buenos Aires	San Pedro	0
A10-3	2015	Argentina	Buenos Aires	San Pedro	0
A10-4	2015	Argentina	Buenos Aires	San Pedro	0
A11-6	2015	Argentina	Buenos Aires	Pergamino	3
A13-1	2015	Argentina	Entre Ríos	La Paz (South)	0
A15-1	2015	Argentina	Entre Ríos	Villaguay	0
A15-2	2015	Argentina	Entre Ríos	Villaguay	0
A15-4	2015	Argentina	Entre Ríos	Villaguay	0
A16-1	2015	Argentina	Entre Ríos	Rosario del Tala	0
A16-2	2015	Argentina	Entre Ríos	Rosario del Tala	2
A16-3	2015	Argentina	Entre Ríos	Rosario del Tala	0
A16-4	2015	Argentina	Entre Ríos	Rosario del Tala	0
A17-1	2015	Argentina	Entre Ríos	Rincón de Nogoyá	0
A17-2	2015	Argentina	Entre Ríos	Rincón de Nogoyá	0
A17-3	2015	Argentina	Entre Ríos	Rincón de Nogoyá	0
A17-4	2015	Argentina	Entre Ríos	Rincón de Nogoyá	0
A18-1	2015	Argentina	Entre Ríos	Victoria	1
A19-1	2015	Argentina	Entre Ríos	Diamante	23N
A19-2	2015	Argentina	Entre Ríos	Diamante	23N
A20-2	2015	Argentina	Entre Ríos	Paraná	23N
A20-3	2015	Argentina	Entre Ríos	Paraná	0
A22-1	2015	Argentina	Entre Ríos	Paraná	0
A22-2	2015	Argentina	Entre Ríos	Paraná	0
A22-3	2015	Argentina	Entre Ríos	Paraná	0

Cont.

Isolate	Year	Country	Region	City	Race
A30	2018	Argentina	Buenos Aires	Pergamino	0
A31	2018	Argentina	Buenos Aires	Pergamino	0
A33	2018	Argentina	Buenos Aires	Pergamino	0
A34	2018	Argentina	Tucumán	Santa Rosa de Leales	0
A35	2018	Argentina	Tucumán	Santa Rosa de Leales	0
A36	2018	Argentina	Tucumán	Santa Rosa de Leales	0
A37	2018	Argentina	Tucumán	Santa Rosa de Leales	0
A38	2018	Argentina	Tucumán	Santa Rosa de Leales	0
A41	2018	Argentina	Tucumán	Santa Rosa de Leales	0
A42	2018	Argentina	Tucumán	Santa Rosa de Leales	0
A43	2018	Argentina	Buenos Aires	Pergamino	0
A44	2018	Argentina	Buenos Aires	Pergamino	0
A46	2018	Argentina	Tucumán	Santa Rosa de Leales	1
A47	2018	Argentina	Tucumán	Santa Rosa de Leales	0
A50	2019	Argentina	Buenos Aires	Junin	0
A51	2019	Argentina	Córdoba	Córdoba	1
A52	2019	Argentina	Córdoba	Oncativo	0
A53	2019	Argentina	Córdoba	Pozo del Molle	0
A54	2019	Argentina	Entre Ríos	Herrera	0
A55	2019	Argentina	Buenos Aires	Pergamino	0
A56	2019	Argentina	Buenos Aires	Pergamino	0
A58	2019	Argentina	Buenos Aires	Pergamino	0
A62	2019	Argentina	Córdoba	Manfredi	3N
A65	2019	Argentina	Chaco	Chaco	0
A66	2019	Argentina	Entre Ríos	Parana	0
A67	2019	Argentina	Entre Ríos	Parana	13N
A68	2019	Argentina	Formosa	Laguna Blanca	0
A69	2019	Argentina	Salta	Valle de Lorna	1
B1-1	2017	Brazil	Paraná	Campo Largo	0
B1-2	2017	Brazil	Paraná	Campo Largo	23N
B1-4	2017	Brazil	Paraná	Campo Largo	0
B1-5	2017	Brazil	Paraná	Campo Largo	1
B2-1	2017	Brazil	Paraná	Campo Largo	0
B2-2	2017	Brazil	Paraná	Campo Largo	0
B2-3	2017	Brazil	Paraná	Campo Largo	0
B2-4	2017	Brazil	Paraná	Campo Largo	0
B2-5	2017	Brazil	Paraná	Campo Largo	0
B3-1	2017	Brazil	Paraná	Castro	0
B3-2	2017	Brazil	Paraná	Castro	0
B3-3	2017	Brazil	Paraná	Castro	0
B3-4	2017	Brazil	Paraná	Castro	0
B3-5	2017	Brazil	Paraná	Castro	0

Cont.

Isolate	Year	Country	Region	City	Race
B7-1	2018	Brazil	Minas Gerais	Sete Lagoas	23N
B7-2	2018	Brazil	Minas Gerais	Sete Lagoas	23N
B7-3	2018	Brazil	Minas Gerais	Sete Lagoas	23N
B7-4	2018	Brazil	Minas Gerais	Sete Lagoas	23N
B9-1	2018	Brazil	Rio Grande do Sul	Tenente Portela	0
B9-3	2018	Brazil	Rio Grande do Sul	Tenente Portela	0
B10-1	2018	Brazil	Minas Gerais	Florestal	23N
B10-2	2018	Brazil	Minas Gerais	Florestal	0
B10-3	2018	Brazil	Minas Gerais	Florestal	0
B12-1	2018	Brazil	Minas Gerais	Estiva	0
B12-2	2018	Brazil	Minas Gerais	Estiva	0
B12-3	2018	Brazil	Minas Gerais	Estiva	23N
B21-1	2018	Brazil	Paraná	Tibagi	0
B21-2	2018	Brazil	Paraná	Tibagi	0
B21-3	2018	Brazil	Paraná	Tibagi	0
B29-2	2018	Brazil	Rio Grande do Sul	Tenente Portela	0
B29-3	2018	Brazil	Rio Grande do Sul	Tenente Portela	0
B30-1	2018	Brazil	Rio Grande do Sul	Palmeira das Missões	0
B30-2	2018	Brazil	Rio Grande do Sul	Palmeira das Missões	23N
B30-3	2018	Brazil	Rio Grande do Sul	Palmeira das Missões	0
B30-4	2018	Brazil	Rio Grande do Sul	Palmeira das Missões	2
B31-1	2018	Brazil	Rio Grande do Sul	Horizontina	0
B31-2	2018	Brazil	Rio Grande do Sul	Horizontina	0
B31-3	2018	Brazil	Rio Grande do Sul	Horizontina	0
B31-4	2018	Brazil	Rio Grande do Sul	Horizontina	0
B34-2	2019	Brazil	Paraná	Ponta Grossa	1
B34-3	2019	Brazil	Paraná	Ponta Grossa	1
B36-1	2019	Brazil	Paraná	Ponta Grossa	23N
B36-2b	2019	Brazil	Paraná	Ponta Grossa	0
B37-1a	2019	Brazil	Paraná	Ponta Grossa	0
B37-1b	2019	Brazil	Paraná	Ponta Grossa	0
B38-1	2019	Brazil	Paraná	Ponta Grossa	0
B38-2	2019	Brazil	Paraná	Ponta Grossa	0
B38-3a	2019	Brazil	Paraná	Ponta Grossa	0
B38-3b	2019	Brazil	Paraná	Ponta Grossa	0
B38-4	2019	Brazil	Paraná	Ponta Grossa	0
B40-2	2019	Brazil	Paraná	Tibagi	1
B40-2	2019	Brazil	Paraná	Tibagi	1
B40-3	2019	Brazil	Paraná	Tibagi	0
B40-4a	2019	Brazil	Paraná	Tibagi	1
B41-2	2019	Brazil	Rio Grande do Sul	Dr. Maurício Cardoso	23N
B41-3	2019	Brazil	Rio Grande do Sul	Dr. Maurício Cardoso	0

Cont.

Isolate	Year	Country	Region	City	Race
B41-4	2019	Brazil	Rio Grande do Sul	Dr. Maurício Cardoso	0
B41-5	2019	Brazil	Rio Grande do Sul	Dr. Maurício Cardoso	0
B42-1	2019	Brazil	Rio Grande do Sul	Santa Rosa	0
B42-2	2019	Brazil	Rio Grande do Sul	Santa Rosa	0
B42-3	2019	Brazil	Rio Grande do Sul	Santa Rosa	0
B43-1	2019	Brazil	Santa Catarina	Iporã	0
B43-2	2019	Brazil	Santa Catarina	Iporã	0
B43-3	2019	Brazil	Santa Catarina	Iporã	0
B44-1	2019	Brazil	Rio Grande do Sul	Rio Grande do Sul	1
B44-2	2019	Brazil	Rio Grande do Sul	Rio Grande do Sul	1
B44-3	2019	Brazil	Rio Grande do Sul	Rio Grande do Sul	1
B44-4	2019	Brazil	Rio Grande do Sul	Rio Grande do Sul	1
B45-1	2019	Brazil	Rio Grande do Sul	Tenente Portela	1
B45-3	2019	Brazil	Rio Grande do Sul	Tenente Portela	1
B45-4	2019	Brazil	Rio Grande do Sul	Tenente Portela	1
B46-1	2019	Brazil	Santa Catarina	Iporã do Oeste	1
B46-2	2019	Brazil	Santa Catarina	Iporã do Oeste	0
B47-1	2019	Brazil	Santa Catarina	Iporã	1
B47-2	2019	Brazil	Santa Catarina	Iporã	1
B47-3	2019	Brazil	Santa Catarina	Iporã	1
B47-4	2019	Brazil	Santa Catarina	Iporã	0
B49-1	2019	Brazil	Rio Grande do Sul	São Luiz Gonzaga	1
B49-2	2019	Brazil	Rio Grande do Sul	São Luiz Gonzaga	1
B49-3	2019	Brazil	Rio Grande do Sul	São Luiz Gonzaga	1
B49-4	2019	Brazil	Rio Grande do Sul	São Luiz Gonzaga	0
B50-1	2019	Brazil	Paraná	Castro/Guantela	1
B50-2	2019	Brazil	Paraná	Castro/Guantela	1
B50-3	2019	Brazil	Paraná	Castro/Guantela	1
B52-1	2019	Brazil	Paraná	Ventania	0
B52-2	2019	Brazil	Paraná	Ventania	0
B52-3	2019	Brazil	Paraná	Ventania	0
B52-4	2019	Brazil	Paraná	Ventania	0
B53-1	2019	Brazil	Paraná	Guantela/Tibagi	0
B53-2	2019	Brazil	Paraná	Guantela/Tibagi	0
B53-3	2019	Brazil	Paraná	Guantela/Tibagi	0
B53-4	2019	Brazil	Paraná	Guantela/Tibagi	0
B54-1	2019	Brazil	Paraná	Ventania/Sapopema	0
B54-2	2019	Brazil	Paraná	Ventania/Sapopema	0
B54-3	2019	Brazil	Paraná	Ventania/Sapopema	0
B54-4	2019	Brazil	Paraná	Ventania/Sapopema	1
B56-1	2019	Brazil	Paraná	Ponta Grossa	0
B56-2	2019	Brazil	Paraná	Ponta Grossa	0

Cont.

Isolate	Year	Country	Region	City	Race
B56-3	2019	Brazil	Paraná	Ponta Grossa	0
B58-1	2019	Brazil	Paraná	Castrolanda/Castro	1
B58-2	2019	Brazil	Paraná	Castrolanda/Castro	1
B58-3	2019	Brazil	Paraná	Castrolanda/Castro	1
B58-4	2019	Brazil	Paraná	Castrolanda/Castro	1
B59-1	2019	Brazil	Paraná	Londrina	0
B59-2	2019	Brazil	Paraná	Londrina	0
B59-3	2019	Brazil	Paraná	Londrina	0
B59-4	2019	Brazil	Paraná	Londrina	0
B59-5	2019	Brazil	Paraná	Londrina	23N
B60-1	2019	Brazil	Paraná	Ventania	0
B60-2	2019	Brazil	Paraná	Ventania	0
B60-3	2019	Brazil	Paraná	Ventania	23N
B60-4	2019	Brazil	Paraná	Ventania	0
B61-1	2019	Brazil	Paraná	Guantela	0
B61-2	2019	Brazil	Paraná	Guantela	0
B62-1	2019	Brazil	Paraná	Carambei	0
B62-2	2019	Brazil	Paraná	Carambei	0
B62-3	2019	Brazil	Paraná	Carambei	0
B62-4	2019	Brazil	Paraná	Carambei	0

Chapter 2. *In vitro* and *in planta* studies on temperature adaptation of *Exserohilum turcicum* isolates from maize in Europe and South America

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Abstract

Northern Corn Leaf Blight (NCLB) is a fungal leaf disease in maize caused by *Exserohilum turcicum*. NCLB occurs worldwide, from tropical to temperate zones raising the question about plasticity of temperature adaptation of local isolates of the pathogen. Seven isolates of *E. turcicum* originating from South America and seven from Europe were compared for their response to temperature variations *in vitro* and *in vivo* between 15 and 30 °C. *In vitro*, isolates originating from Europe and South America significantly differed in mycelial growth rate at 30 °C and in sporulation at 25 °C and 30 °C. Aggressiveness of *E. turcicum* isolates was evaluated on three susceptible maize cultivars (maize lines B37, Sus1 and the German hybrid Niklas) under different day/night temperature regimes (15/10 °C, 20/15 °C, 25/20 °C, or 30/25 °C) with a photoperiod of 14 h. Aggressiveness, recorded as area under the disease progress curve (AUDPC), of South American isolates was higher than for European isolates at 15 °C, 20 °C and 25 °C, and for sporulation *in vivo* in all temperatures. In general, aggressiveness components were most influenced by temperature. Therefore, multivariate analysis was performed with aggressiveness component data at 30 °C, which expressed the highest number of variables with significant differences between isolate origins. According to their aggressiveness, European and South American isolates can be grouped separately, demonstrating that South American isolates are better adapted to higher temperatures and display a higher level of aggressiveness under similar conditions than European isolates from a cool climate. It is concluded that plasticity of temperature adaptation in *E. turcicum* populations is relatively large and allowed *E. turcicum* to follow the recent expansion of maize cultivation into cool climate zones in Europe. However, our data suggest that adaptation to higher temperature is likely to increase aggressiveness of NCLB on maize in cooler climate zones when experiencing further climate warming. This plasticity in adaptation to

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environmental conditions of *E. turcicum* may also hamper the success of breeding programs as it may decrease the durability of resistance.

Keywords: *Setosphaeria turcica,* aggressiveness, leaf disease*,* climate warming

Introduction

Exserohilum turcicum, the causal agent of Northern Corn Leaf Blight (NCLB), occurs in all maize-producing regions, from tropical to temperate zones (CABI, 2019). The ascomycete *E. turcicum* (teleomorph *Setosphaeria turcica*) causes cigar-shaped green-grey lesions on leaves, which become necrotic in later infection stages and may evolve to blight symptoms, leading to high yield losses in maize. Yield losses caused by NCLB are correlated with host phenological stage, insertion of the infected leaves and host resistance. Infections that occur from 2 to 3 weeks after pollination cause yield losses of up to 40% (Levy and Pataky, 1992). In the vegetative stage, young seedlings usually present higher NCLB susceptibility when compared to 2-month-old plants (Levy and Cohen, 1983a). Lesions on the leaf closest to the cob show a high contribution to yield reduction (Levy and Leonard, 1990).

Maize yield losses caused by *E. turcicum* are up to 40% in South America (Rossi *et al.,* 2010; Cota *et al.*, 2013). In Germany, *E. turcicum* causes yield losses from 10 to 30% in maize production, depending on the host resistance levels (BVL, 2020). Maize-producing regions in South America are classified as Cfa (temperate climate without dry season and with hot summer), Cfb (temperate climate without dry season, with warm summer) or Cwa (temperate climate with dry winter and with hot summer) according to the Köppen-Geiger climate classification (Peel *et al*., 2007). In Europe, maize-producing regions have a mild climate, Cfb, or Dfb (cold climate without dry season, with warm summer) (Peel *et al*., 2007). Furthermore, control methods and cropping systems differ between the regions. Disease control in South America is mainly based on fungicide sprays and resistant cultivars. In Brazil, 28 fungicides are registered for NCLB control (MAPA, 2020). Nonetheless, the cultivation of susceptible genotypes in areas with weather conditions favorable for disease development and the use of no-till practices have increased disease pressure in some regions of South America (Cota *et al.*, 2013). In Germany, the cultivation of resistant cultivars is recommended for NCLB control. In this country, fungicide sprays are unusual, and only one fungicide was registered for NCLB control in 2020 (BVL, 2020).

The range of *E. turcicum* races in Europe seems to be different from that in South America. In 2011 and 2012, 10 races were reported in Europe: 0, 1, 3, 3N, 13, 23, 123, 2, 12, 13N, and 1N (Hanekamp, 2016). In contrast, race 0 is predominant in South America (Navarro *et al*., 2021).

However, isolates capable of overcoming the resistance conferred by *Ht* gene (races 1N, 12N, 123N, 123, 23) were also detected in maize plants during 1993, 1994 and 2005 in Brazil (Gianisi *et al.*, 1996; Ogliari *et al.*, 2005).

Environmental conditions favorable for the occurrence of NCLB in the field are long dew periods and moderate temperatures (Welz and Geiger, 2000). Conidia can germinate from 10 to 35 °C and reach 100% germination from 20 to 25 °C after 2 h of dew (Levy and Cohen, 1983a). As *E. turcicum* penetrates directly through the epidermis (Hilu and Hooker, 1964), the optimal temperature is also required for appressorium formation. Infections occur from 15 to 30 °C, and the optimal temperature is 20 °C. A minimum dew period of 5 h is required for lesion formation. In addition, the minimum dew period required for spore production is 9 h, which is longer than that necessary for infection (Levy and Cohen, 1983a,b). Optimal weather conditions described for disease development are observed in mid-altitude regions in the tropics (Welz and Geiger, 2000). Accordingly, it is hypothesized that the *E. turcicum* center of origin is in the tropical regions, which is also supported by the higher genetic diversity found in those areas (Bochardt *et al.*, 1998). If the pathogen co-evolved with maize (Zea mays), the center of origin should be Central America. If it co-evolved with sorghum (*Sorghum bicolor*) and later jumped to maize, the center of origin should be East Africa (Welz, 1998).

Aggressiveness designates the amount of disease caused by one pathogen isolate on a susceptible host (Vanderplank, 1968). The aggressiveness level is related to the pathogen, but also to the host quantitative resistance and the environmental conditions (Andrivon, 1993). Resistant hosts tend to select more aggressive isolates than susceptible hosts (Delmas *et al.*, 2016). The interaction between host resistance and pathogen genotype plays an important role in the durability of quantitative resistance (Lannou *et al.*, 2012). Pathogen populations with a fast response to selection pressure may erode resistance more rapidly (Delmas *et al.*, 2012).

Aggressiveness is quantified by the evaluation of components related to the disease cycle (Kranz, 2003), such as incubation period, disease severity, and sporulation (Pariaud *et al.*, 2009). These disease components allow quantitative comparisons between pathogen isolates from tropical and temperate climate zones. In tropical regions, high temperatures and short dew periods are unfavorable to conidium survival and germination. As weather conditions in tropical regions are not always favorable for disease spread, tropical pathogens usually have an alternative source of propagation by lesion expansion (Bergamin Filho and Amorim, 1996). Therefore, pathogen development continues by leaf tissue colonization (autoinfection) instead of conidial propagation expansion (Bergamin Filho and Amorim, 1996). Such a strategy is observed for *E. turcicum*, as lesion expansion has been proven to contribute in NCLB epidemics (Vitti *et al.,* 1995, Carson, 2006). However, comparisons between pathogen

populations originating from areas with different environmental conditions are scarce in the literature (Uloth *et al.*, 2015).

Assuming co-evolution with either maize or sorghum (Welz, 1998), *E. turcicum* should be adapted to tropical temperature levels. However, maize production has expanded substantially into cooler climate zones like Germany in the last three decades, exposing potential pathogens to cooler temperature regimes. This raises the question as to whether and how adaptation to cooler temperatures has affected the aggressiveness of *E. turcicum* on maize. A study of aggressiveness with *E. turcicum* isolates from Europe and Africa carried out on detached maize leaves, however, did not confirm different aggressiveness levels according to the isolate origin (Muiru *et al.*, 2010). In the present study, a comprehensive comparison of the aggressiveness of *E. turcicum* isolates under different temperature conditions was performed. Experiments were carried out to verify the effect of temperature on pathogen and disease development, to better understand temperature adaptation of *E. turcicum* isolates originating from tropical and temperate climate zones. The effect of temperature was evaluated on mycelium growth and spore production *in vitro* and on incubation period, disease severity, and pathogen sporulation on maize plants *in vivo.*

Material and Methods

Exserohilum turcicum *isolates*

Isolates selected for aggressiveness comparisons were chosen according to their provenance and race. Isolates were selected in order to have at least one isolate for each race complexity, from a single country. Isolates were obtained from race assessments conducted in Europe (n = 645) (Hanekamp, 2016) and in South America (n = 184) (Navarro *et al*., 2021). Races were determined according to previous works (Hanekamp, 2016; Navarro *et al*., 2021). Briefly, the race determination was conducted by inoculating a differential set of the maize line B37 without resistance genes (control) and B37 carrying the resistance genes *Ht1*, *Ht2*, *Ht3* and *Htn1*, as no molecular methods are established to determine the physiological race of *E. turcicum*. Maize plants were cultivated in a greenhouse (22 ± 6 °C, 70% air humidity, day/night light regime 14/10 h, light intensity 100 ± 20 µmol m^{-2} s^{-1}). The race is determined based on the phenotype 14 days post inoculation. Plants displaying strong chlorosis are classified as resistant, whereas plants showing strong necrosis are susceptible (Bigirwa *et al.*, 1993). Finally, 14 isolates (seven isolates from Europe and seven from South America) were selected for *in vitro* and *in vivo* tests. Race complexity is based on the number of *Ht* resistance genes which an isolate is able to overcome and cause disease (Table 1) (Weems and Bradley, 2018).

Table 1. *Exserohilum turcicum* isolates used for *in vitro* and *in vivo* tests.

Isolate	Continent	Country	Region	County	Race	Rc[1]	Climate[2]
D6-1	Europe	Germany	Bayern	Regensburg	Race 0	0	Dfb
HH138-1	Europe	France	Oberhein Region	Fessenheim	Race 1	1	Cfb
HH80	Europe	Belgium	East Flanders	Beervelde	Race 2	1	Cfb
D10-1	Europe	Germany	Niedersachsen	Meppen	Race 3	1	Dfb
D10-3	Europe	Germany	Niedersachsen	Meppen	Race 3N	2	Dfb
D3-3	Europe	Germany	Bayern	Regensburg	Race 123	3	Dfb
HH123-2	Europe	Turkey	Adana	Adana	Race 23N	3	Csa
B3-2	South America	Brazil	Paraná	Castro	Race 0	0	Cfb
A18-1	South America	Argentina	Entre Ríos	Victoria	Race 1	1	Cfa
B31-2	South America	Brazil	Rio Grande do Sul	Horizontina	Race 2	1	Cfa
A11-6	South America	Argentina	Missiones	Pergamino	Race 3	1	Cfa
B1-5	South America	Brazil	Paraná	Campo Largo	Race 12	2	Cfb
B10-1	South America	Brazil	Minas Gerais	Florestal	Race 23N	3	Cwa
B7-1	South America	Brazil	Minas Gerais	Sete Lagoas	Race 23N	3	Cwa

[1] Race complexity (Rc) denotes the number of differential lines for which a specific isolate is virulent; rc1 = race complexity 1; rc2 = race complexity 2; rc3 = race complexity 3.

[2] The climate from the region where each isolate was collected was classified as Cfa (temperate climate without dry season, with hot summer), Cfb (temperate climate without dry season, with warm summer), Cwa (temperate climate with dry winter and with hot summer), Dfb (cold climate without dry season with warm summer), or Csa (temperate climate with dry summer, with hot summer), according to the climate classification of Köppen-Geiger (Peel *et al.*, 2007).

In vitro *tests*

The *in vitro* experiments were performed for each isolate (Table 1) in order to observe the development of the pathogen under different temperatures. Mycelium growth and spore production were evaluated. All isolates, stored in fresh glycerine (25%) at −20 °C (up to 60 days), were transferred to Petri dishes containing V8 medium (75 mL V8 vegetable juice; 1.5 g $CaCO_3$, 10 g agar-agar). After 28 days, mycelial plugs (3 mm-diameter) were transferred to V8 plates and grown under four temperatures (15 °C, 20 °C, 25 °C, or 30 °C) in the dark for 14 days. Each treatment was replicated four times (4 plates per isolate at each temperature). On each day after inoculation perpendicular measurements of the colony radius were taken from each plate. Finally, the AUMGC was calculated by trapezoidal integration adapted from Berger (1988), according to the following formula:

$$\mathrm{AUMGC} = \sum_{1}^{n-1} \left(\frac{x_i + x_{i+1}}{2} \right) (t_{i+1} - t_i)$$

where x_i is the colony diameter for the measurement number i, t_i is the corresponding number of days of this observation, and n is the number of measurements. AUMGC was calculated until the first plates were totally covered with mycelium, at 7 days post inoculation (dpi). At 15 dpi, conidia were harvested from each plate, by washing with 10 ml of sterile distilled water and stored in falcon tubes at -20°C. Three aliquots from each spore suspension were counted using a hemocytometer; and the conidia production was calculated per plate. Experiments *in vitro* were conducted four times.

In vivo *tests*

Maize plants from the near isogenic line (NIL) B37 (reference line used in race monitoring), Sus1 (highly susceptible line provided by breeders as a positive control), and the hybrid line Niklas® (widely cultivated in Germany) were sown to test the aggressiveness of *E. turcicum* isolates (Table 1). Seeds were provided by KWS Saat SE (Einbeck, Germany). Two seeds per pot (11 × 11 × 10 cm^3) were sown in a mixture of soil with proportions of 3:3:1 (clay: compost: sand). The plants were cultivated in a greenhouse at 24 ± 3 °C, 70% of air humidity, and a light/dark photoperiod of 14/10 h. Maize plants were inoculated about 30 days after sowing when the fifth and sixth leaves were unfolded. In order to prepare the conidia suspension, five plates of each isolate were inoculated and incubated at 25 °C in the dark for 21 days until conidia have developed. Conidia were collected using an aqueous solution containing 125 ppm of the surfactant Silwet Gold® (Certis Europe B.V., Hamburg, Germany), and the suspension was adjusted to 1500 conidia mL^{-1} with a hemocytometer. Approximately 7 mL of conidia suspension were sprayed per plant that were maintained in a humidity chamber for 24 h. All plants were transferred to climate chambers (RUMED® Rubbarth Apparate GmbH, Laatzen, Germany) under the following day/night temperature conditions: 30/25 °C, 25/20 °C, 20/15 °C, and 15/10 °C, with a light/dark photoperiod of 14/10 h, light intensity of 120 ± 10 µmol m^{-2} s^{-1} and relative air humidity of 70%. For each isolate, temperature and maize host, four replicated plants were inoculated. The *in vivo* experiments were repeated two times using four plants as technical replicates.

The comparisons of aggressiveness among isolates were based on the incubation period, disease severity, AUDPC and sporulation. The incubation period was evaluated when the plant showed the first lesion. Disease severity was evaluated every 3 days based on a diagrammatic scale ranging from 2 to 90% (Pataky, 1992). The final disease severity was obtained at 26 dpi. AUDPC was estimated by trapezoidal integration (Berger, 1988) according to the following formula:

$$\text{AUDPC} = \sum_{i=1}^{ni-1} \left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i)$$

where y_i is the disease severity at the i evaluation, t_i is time in days post inoculation at the *ith* evaluation, and *n* is the total number of evaluations.

Sporulation was measured on line B37 plants at 26 dpi. Symptomatic leaf samples of 6 cm^2 (3 cm × 2 cm) from the fifth unfolded leaf were collected at the transition between green and necrotic areas. Samples of the four inoculated plants per treatment were placed on moistened filter paper (Munktell Ahlstrom) to maintain high humidity and stimulate conidia production. Pictures were taken of each sample to quantify the diseased area using Image J1.52a software (Wayne Rasband, National Institute of Health, Bethesda, MD, USA). The disease severity (%) of each sample was estimated using Assess 2.0 software (Lakhdar Lamari, 2008, APS, St. Paul, MN, USA). After 3 days, each sample was placed individually in a Falcon tube containing 4 mL of sterile distilled water amended with 125 ppm of the surfactant Silwet Gold®. Falcon tubes were frozen at −20 °C for further procedures. After mixing of the sample, sporulation was estimated using a haemocytometer. Three aliquots per sample were evaluated, sporulation was estimated from the average of these aliquots and divided by the diseased area, obtaining values of spores×cm^{-2}.

Data analysis

Data analysis of *in vitro* experiments was performed applying mixed models and estimations by the restricted maximum likelihood method using the lmer package of R 3.6.0 software (R Core Team, 2019). The *in vitro* experiments were completely randomized within the temperature treatments. Data of conidia production *in vitro* were analyzed with Box Cox transformation. Data were compared by ANOVA and multiple comparison applying Tukey test between isolates for each temperature (p-value ≤ 0.05).

In the *in vivo* experiments, a variance component analysis (VCA) was performed for the variables incubation period and AUDPC in order to assess the effect of the factors temperature, isolate origin and host genotype. Variance was estimated by the restricted maximum likelihood method and performed using the package VCA in R 3.6.0 software (R Core Team, 2019). Multivariate analysis of variance (MANOVA) was performed for the transformed data using the package car in R 3.6.0 software (R Core Team, 2019). MANOVA was applied to verify the effect of three factors in the three *in vivo* variables together listed in the VCA analysis. Partial eta squared values (η^2) were calculated to obtain the effect of factor on the percentage of total variance. An additional ANOVA was conducted for data of AUDPC and Box-Cox transformed

data of sporulation *in vivo* with the race complexity as main effect and experiment replications was considered as random effect. As the factor temperature was showing the highest percentage of the total variance, an ANOVA was conducted per each temperature considering isolate origin as main effect and experiment replication and host genotypes as random effects. A second ANOVA was conducted per each temperature considering each single isolate as main effect and experiment replication and host genotypes as random effects. In addition, isolates were compared by multiple comparison applying the Tukey test (p-value ≤ 0.05). Data of incubation period and sporulation *in vivo* were analyzed after Box Cox transformation. As the host genotype was contributing to the variance, the effect of host genotype on AUDPC was compared between isolates origin and isolates for each temperature performing an ANOVA with experiment replication as random effect. Another ANOVA was conducted per each temperature considering each single isolate as main effect and experiment replication as random effects. In addition, isolates were compared by multiple comparison applying Tukey test (p-value ≤ 0.05).

Further analyzes were performed using data from the 30 °C experiment; data for the *in vivo* variables were chosen for the reference line B37 at 30/25 °C. The mean of each variable was calculated for each isolate. The relationships between variables were assessed by Spearman's rank correlation between all pairs of variables car in R 3.6.0 software (R Core Team, 2019). A principal component analysis (PCA) was used to explore the associations between variables. Cluster analysis with all variables was performed to identify and to group isolates according to their similarity of aggressiveness. The standardized Euclidean distance was used as a measure of dissimilarity. The following agglomerative hierarchical methods were applied: single linkage, complete linkage, average linkage (also called the unweighted pair groups method using arithmetic average – UPGMA), centroid and Ward (Quinn and Keough, 2002). The method that best fitted to the data was chosen by visual analysis of the grouping pattern. A dendrogram was obtained to visualize the isolates within groups. Cluster analysis was performed using the function hclust in R 3.6.0 software (R Core Team, 2019). Furthermore, stepwise discriminant analysis (DA) was performed to identify which variables contributed most to differences between the two groups of isolates originating from Europe and South America. DA was performed with Statistica 13.0 software (Statsoft, Tulsa, OK, USA) by the forward method. Additionally, a PCA was performed with variables selected by the DA to show isolates position.

Results

Effect of temperature and isolate origin on pathogen development in vitro

Independent of the isolate origin, the optimal temperature for the pathogen growth was 25 °C, followed by the temperatures 20 °C, 30 °C and 15 °C in this order, according to the area under the mycelium growth curve (AUMGC) data (Table 2, Figure 1). Mycelium growth statistically (p-value ≤ 0.05) differed between isolates originating from Europe and South America at 30 °C. At 15 days post inoculation, the optimal temperature for spore production *in vitro* was 20 °C, with a mean of 156,631 conidia per plate, while at 25 °C and 30 °C, the average conidia production was 123,292 and 61,248 conidia per plate. South American isolates sporulated at a higher rate than European isolates at 25 °C and 30 °C (p-value ≤ 0.05) (Figure 1). Considering all temperatures, the average sporulation for South American isolates was 110,064 conidia per plate and for European isolates 105,485 conidia per plate. At 25 °C and 30 °C, the mean sporulation for South American isolates was 126,433 and 73,586 conidia per plate, whereas for European isolates mean values of 120,151 and 48,909 conidia per plate were observed, respectively.

Table 2. Effect of isolate origin, temperature, and their interactions on the area under the mycelium growth curve (AUMGC) and Box-Cox transformed data of sporulation *in vitro* based on a mixed model analysis of variance.

***In vitro* test**			**AUMGC**		**Sporulation *in vitro* (Box-Cox transformed)**	
Effect	**df_n[1]**	**df_d[2]**	***F*-value**	***p*-value**	***F*-value**	***p*-value**
Isolate origin	1	892	8.295	0.004	23.986	< 0.001
Temperature	3	892	396.562	< 0.001	42.731	< 0.001
Isolate origin x temperature	3	892	3.031	0.028	0.419	0.739

[1] Numerator degrees of freedom (df_n), [2] denominator degrees of freedom (df_d); degrees of freedom calculated using the Satterthwaite formula for a mixed model; *F*-value for testing effect and probability (significance) level of *F* -value (*p*-value).

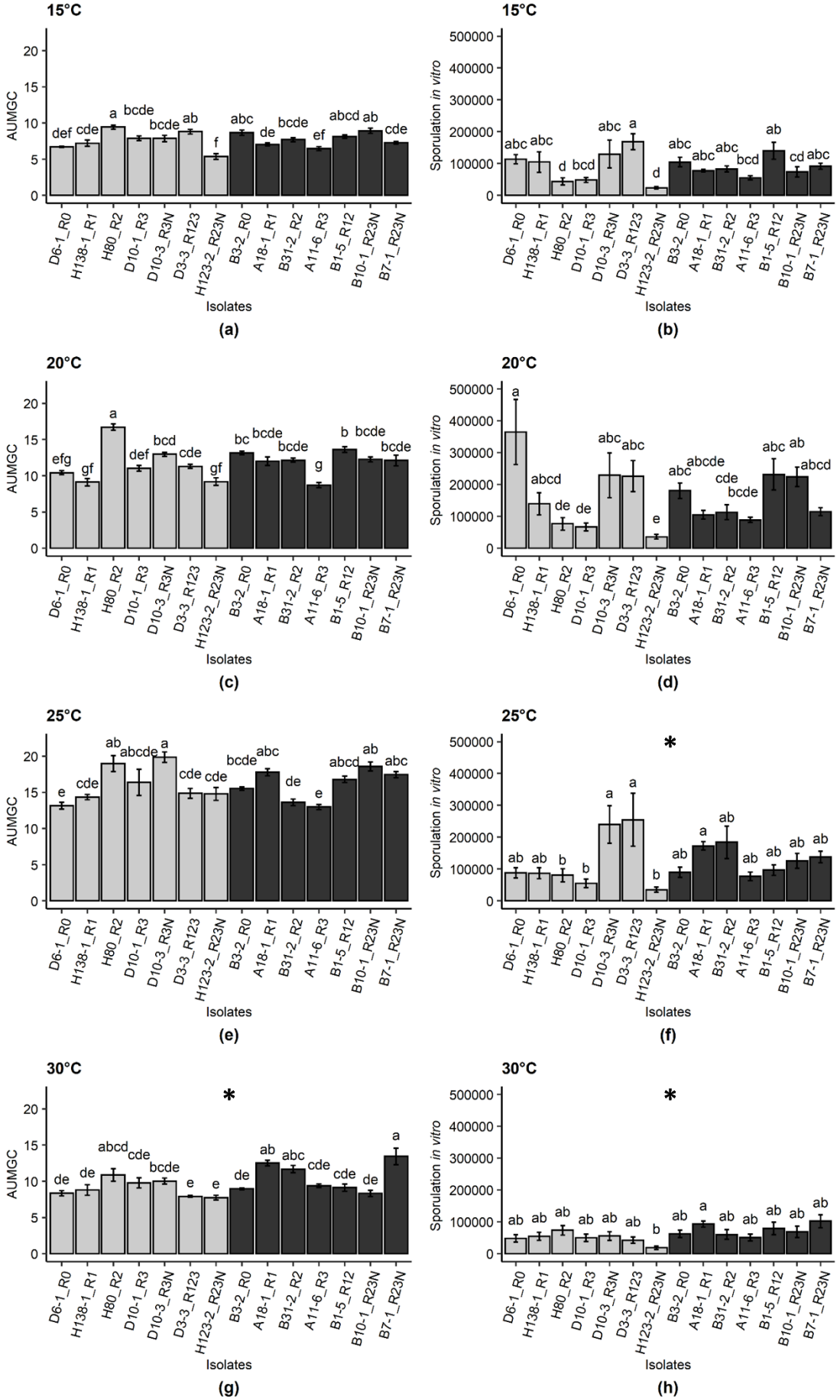
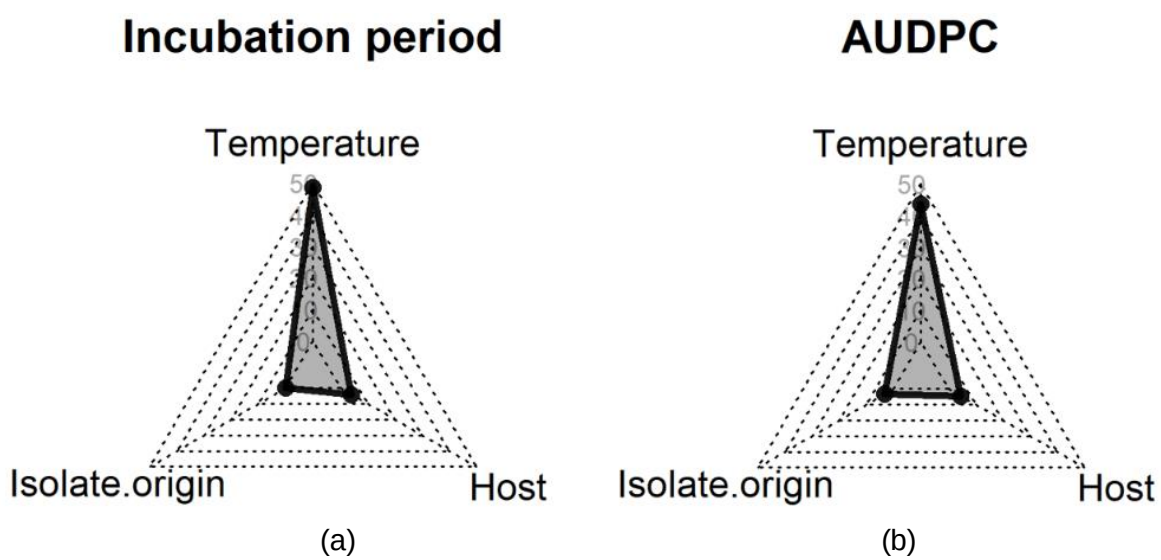

15°C
AUMGC
Isolates
(a)
15°C
Sporulation in vitro
Isolates
(b)
20°C
AUMGC
Isolates
(c)
20°C
Sporulation in vitro
Isolates
(d)
25°C
AUMGC
Isolates
(e)
25°C
Sporulation in vitro
Isolates
(f)
30°C
AUMGC
Isolates
(g)
30°C
Sporulation in vitro
Isolates
(h)
D6-1_R0
H138-1_R1
H80_R2
D10-1_R3
D10-3_R3N
D3-3_R123
H123-2_R23N
B3-2_R0
A18-1_R1
B31-2_R2
A11-6_R3
B1-5_R12
B10-1_R23N
B7-1_R23N

Figure 1. Area under the mycelium growth curve (AUMGC) (a,c,e,g) and spore production in vitro (conidia production per plate at 15 days post inoculation–dpi) (b,d,f,h) for isolates of *Exserohilum turcicum* originating from Europe and South America. Light grey bars represent European isolates, dark bars represent South American isolates. Means sharing the same letter are not significantly different for Tukey test (p-value ≤ 0.05). Graphs labelled with an asterisk (*) indicate that values for the respective variable were significantly higher for South American isolates than European isolates for the analysis of variance (ANOVA, p-value ≤ 0.05). Bars indicate standard errors. Data are pooled from four replicated plates for each isolate which was repeated four times (n = 16 plates per isolate).

Effect of isolate origin, temperature and host genotype on the incubation period, disease severity and sporulation

The effects of temperature, isolate origin, and host were explained for incubation period, and area under the disease progress curve (AUDPC), evaluated in the *in vivo* experiments. These variables showed the highest variance for the factor temperature. The temperature explained 48.6% and 43.7% of the total variance for incubation period, and AUDPC, respectively. All other factors explained less than 5% of the total variance (Figure 2). The effects of temperature and host on incubation period, AUDPC, and disease severity were significant (temperature: Pillai's trace = 1.027, p-value ≤ 0.001; host: Pillai's trace = 0.027, p-value ≤ 0.01; isolate origin: Pillai's trace = 0.004, p-value = 0.14,) for MANOVA with an F-value of 199.7, and degrees of freedom of 24 and 1257. The estimation of the partial eta squared value (η^2) showed that temperature explained 34% of the variance (Supplementary table 1).



(a) (b)

Figure 2. Effect of temperature, isolate origin and host genotype on total variance of incubation period (a), and area under the disease progress curve (AUDPC) (b), evaluated in the *in vivo* experiments in the greenhouse with three maize cultivars. The axis indicates the percentage share of total variance. Variance component analysis (VCA) was estimated by the restricted maximum likelihood method.

The incubation period was longer at the coldest temperature 15/10 °C, with a mean of 15.4 days (Figure 3). At 20/15 °C, 25/20 °C and 30/25 °C, the average incubation periods were 12.7, 11.3, and 11.1 days, respectively. There was no significant difference in incubation periods between isolates originating from Europe and South America (Table 3, *p*-value = 0.072). (Figure 3), showing mean values of 12.5 and 12.7 days, respectively. The AUDPC of South American isolates was significantly higher at 15/10 °C, 20/15 °C and 25/20 °C (Figure 3). In general, South American isolates displayed higher mean AUDPC, when compared to European isolates. The sporulation *in vivo* of South American isolates was higher than of European isolates at all tested temperatures (Table 4, Figure 3), with means of 8768.09 conidia×cm^{-2} vs. 5898.67 conidia×cm^{-2}, respectively.

Table 3. Effect of isolate origin, temperature, host and their interactions on Box-Cox transformed data of incubation period and the area under the disease progress curve (AUDPC) and based on a mixed model analyses of variance.

***In vivo* test**			**Incubation period (Box-Cox transformed)**		**AUDPC**	
Effect	**df_n[1]**	**df_d[2]**	***F*-value**	***p*-value**	***F*-value**	***p*-value**
Isolate origin	1	1299	3229	0.072	42843	< 0.001
Temperature	3	1299	356673	< 0.001	288871	< 0.001
Host	2	1299	52307	< 0.001	42015	< 0.001
Isolate origin x temperature	3	1299	1184	0.314	2522	0.056
Isolate origin x host	2	1299	1938	0.144	6948	< 0.001
Temperature x host	6	1299	13185	< 0.001	3987	< 0.001
Isolate origin x temperature x host	6	1299	1550	0.158	1628	0.135

[1] Numerator degrees of freedom (df_n), [2] denominator degrees of freedom (df_d); degrees of freedom calculated using the Satterthwaite formula for a mixed model; *F*-value for testing effect and probability (significance) level of *F* -value (*p*-value).

Table 4. Effect of isolate origin, temperature, and their interactions on the Box-Cox transformed data of sporulation *in vivo* based on a mixed model analysis of variance.

***In vivo* test**	**Sporulation *in vivo* (Box-Cox transformed)**			
Effect	**df_n[1]**	**df_d[2]**	***F*-value**	***p*-value**
Isolate origin	1	385.16	316.930	< 0.001
Temperature	3	385.14	67.993	< 0.001
Isolate origin x temperature	3	385.05	0.1316	0.941

[1] Numerator degrees of freedom (df_n), [2] denominator degrees of freedom (df_d); degrees of freedom calculated using the Satterthwaite formula for a mixed model; *F*-value for testing effect and probability (significance) level of *F* -value (*p*-value).

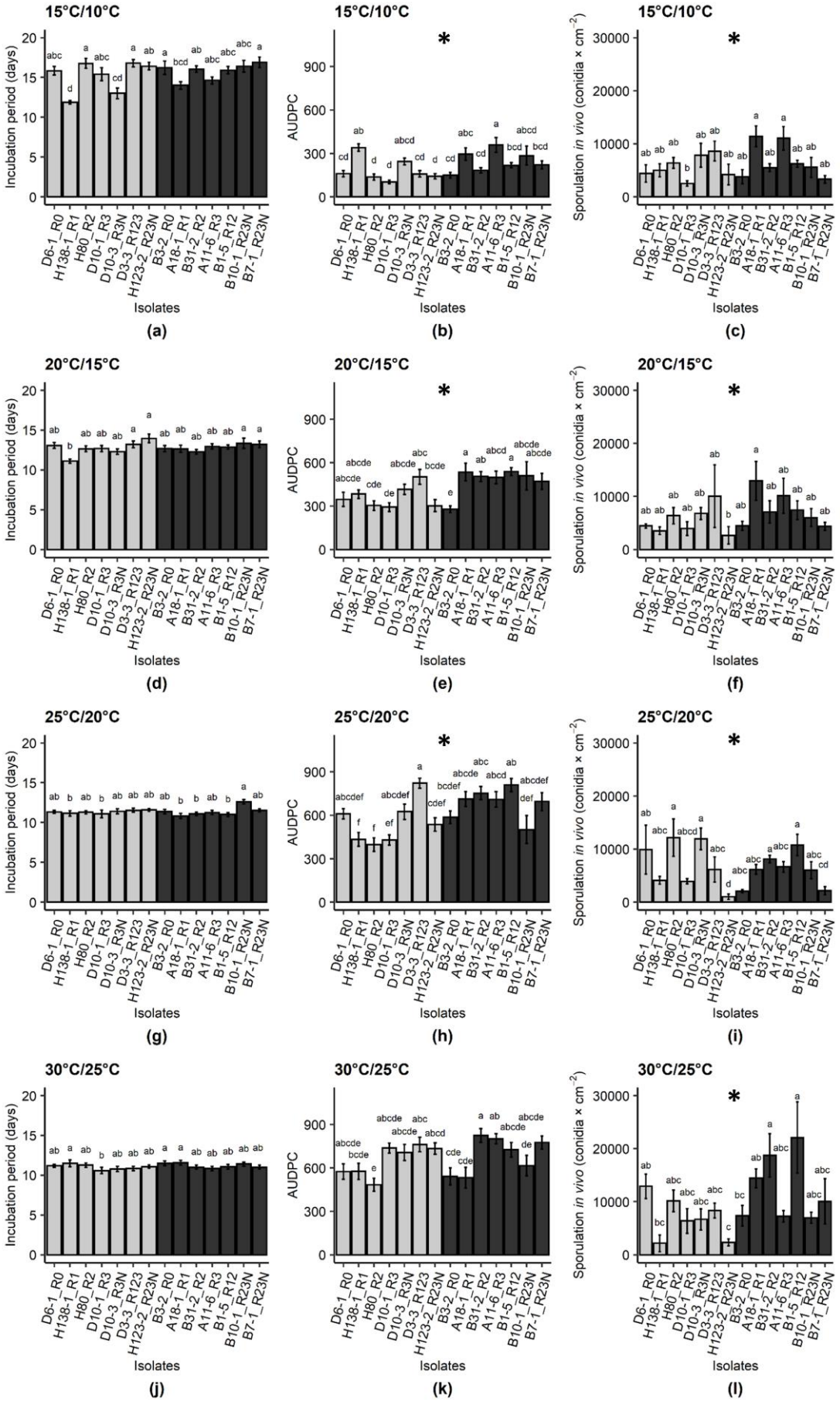

15°C/10°C
20°C/15°C
25°C/20°C
30°C/25°C
Incubation period (days)
AUDPC
Sporulation *in vivo* (conidia × cm^{-2})
Isolates
D6-1_R0
H138-1_R1
H80_R2
D10-1_R3
D10-3_R3N
D3-3_R123
H123-2_R23N
B3-2_R0
A18-1_R1
B31-2_R2
A11-6_R3
B1-5_R12
B10-1_R23N
B7-1_R23N
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)

Figure 3. Incubation period (a,d,g,j), area under the disease progress curve (b,e,h,k) and conidia production *in vivo* [conidia×cm^{-2} of diseased leaf area] (c,f,i,l) for isolates of *Exserohilum turcicum* originating from Europe and South America. Light grey bars represent European isolates, dark bars represent South American isolates. Means sharing the same letter are not significantly different following Tukey test (p-value ≤ 0.05). Graphs labelled with an asterisk (*) indicate that values of the respective variable for South American isolates were significantly higher than for European isolates (p-value ≤ 0.05). Bars indicate standard errors (n = 24 plants). Each experiment was replicated two times.

For the interaction of isolate origin × host genotype, differences in AUDPC between European and South American isolates were observed on the maize line B37 (p-value ≤ 0.01) and the German hybrid line Niklas (p-value ≤ 0.001). With regard to the temperature, higher AUDPC values were recorded for South American isolates at 15/10 °C, and at 20/15 °C for the maize lines B37 and Sus1 (Figure 4, Supplementary figure 1). On the maize hybrid line Niklas, South American isolates caused high AUPDC values under all tested temperatures.

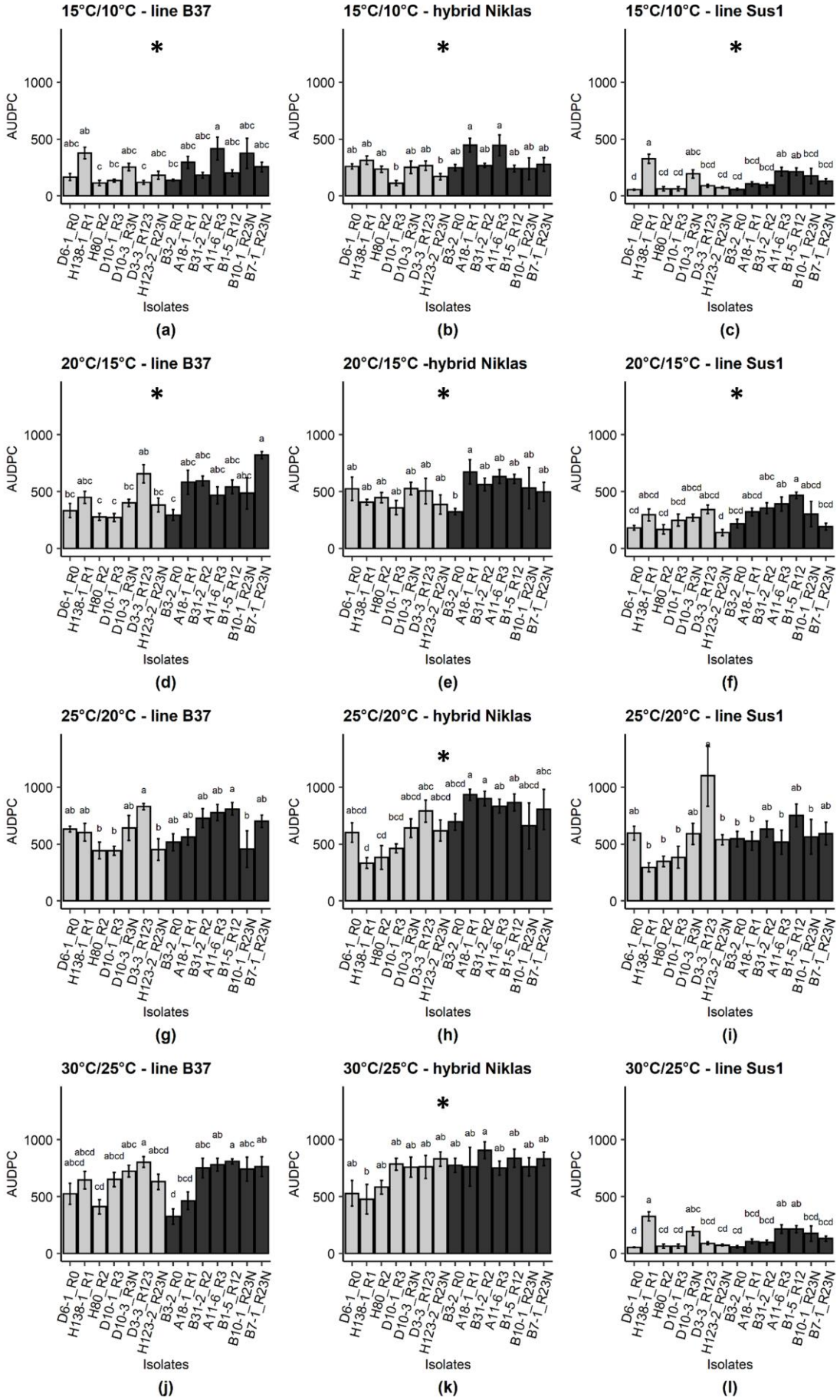
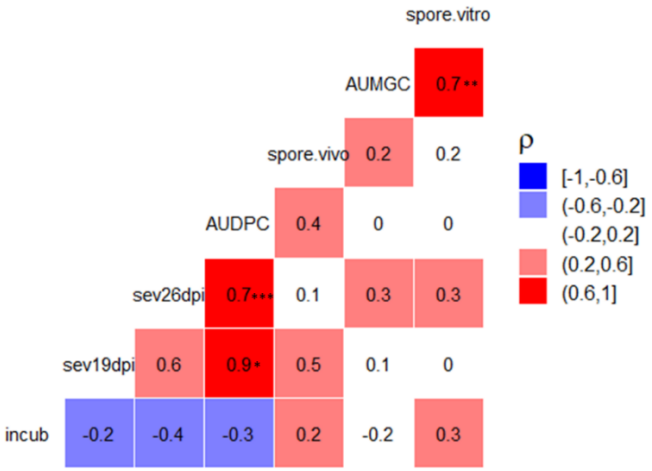

15°C/10°C - line B37
15°C/10°C - hybrid Niklas
15°C/10°C - line Sus1
20°C/15°C - line B37
20°C/15°C -hybrid Niklas
20°C/15°C - line Sus1
25°C/20°C - line B37
25°C/20°C - hybrid Niklas
25°C/20°C - line Sus1
30°C/25°C - line B37
30°C/25°C - hybrid Niklas
30°C/25°C - line Sus1
AUDPC
Isolates
D6-1_R0
H138-1_R1
H80_R2
D10-1_R3
D10-3_R3N
D3-3_R123
H123-2_R23N
B3-2_R0
A18-1_R1
B31-2_R2
A11-6_R3
B1-5_R12
B10-1_R23N
B7-1_R23N
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)

Figure 4. Area under the disease progress curve (AUDPC) of *Exserohilum turcicum* isolates originating from Europe and South America. Effect of host genotypesB37–standard line for race monitoring (a,d,g,j), Niklas®–susceptible hybrid cultivated in Germany (b,e,h,k), and Sus1–susceptible breeding line (c,f,i,l). Light grey bars represent European isolates, dark bars represent South American isolates. Means sharing the same letter are not significantly different following Tukey test (*p*-value ≤ 0.05). Graphs labelled with an asterisk (*) indicate that values for the respective variable of South American isolates were significantly higher than for European isolates (*p*-value ≤ 0.05). Bars indicate standard errors (n = 8 plants). Each experiment was replicated two times.

Relation between aggressiveness components and isolate groups

Further analysis of effects of isolate origin were performed with data of the AUMGC, sporulation *in vitro*, incubation period, AUDPC, sporulation *in vivo*, disease severity at 19 dpi and disease severity at 26 dpi from the reference line B37 maintained at 30/25 °C (30 °C for the experiment *in vitro*) to exclude the effect of host genotype and temperature. The most correlated aggressiveness components were disease severity at 19 dpi and AUDPC (r = 0.88; *p*-value ≤ 0.001). Disease severity at 26 dpi was positively correlated with AUDPC (r = 0.69; *p*-value ≤ 0.05), and disease severities at 19 and 26 dpi were positively correlated with each other (r = 0.56; *p*-value ≤ 0.05). The variables AUMGC and spore production *in vitro*, were correlated to each other (r = 0.68; *p*-value ≤ 0.01) (Figure 5).



*: *p*-value < 0.05; **: *p*-value < 0.01, ***: *p*-value < 0.001.

Figure 5. Spearman correlation coefficients (ρ) for the relation between the means of measured pathogenic traits incubation period (incub), disease severity at 19 dpi (sev19dpi), disease severity at 26 dpi (sev26dpi), area under the disease progress curve (AUDPC), sporulation *in vivo* at 28 dpi (spore.vivo), area under the mycelium growth curve (AUMGC) and spore production *in vitro* (spore.vitro) at 30°C.

A cluster analysis was performed with all variables and the isolates were clustered in five groups by the Ward method. Similarities between isolates from the same group were observed for isolate origin but not for race or race complexity within groups (Figure 6). Additionally, a discriminant analysis (DA) was performed to select the variables that were most contributing to distinguish isolates according to their origin. DA retained the variables incubation period, disease severity at 19 dpi, sporulation *in vivo*, and spore production *in vitro* for isolate discrimination according to their origin. However, even performing DA with the selected variables, the DA misclassified the South American isolate A11-6, which was also observed in the principal component analysis (PCA) conducted with these four DA selected variables (Figure 7). According to the PCA analysis, the components PC1 and PC2 explained 74.14% of the total variability. Except for the South American isolate A11-6, a clear distinction between South American and European isolates was observed.

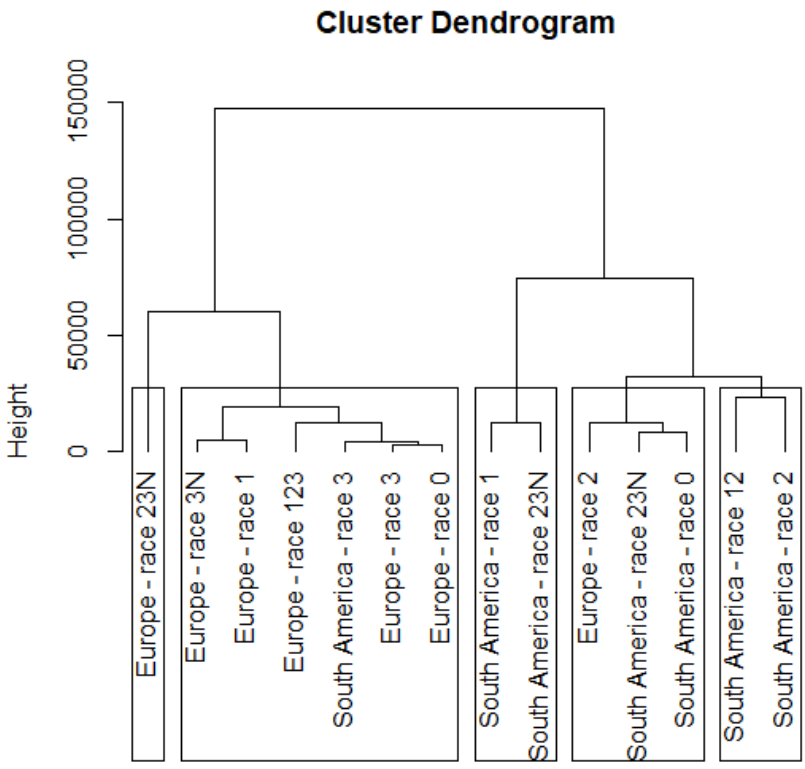


Figure 6. Dendrogram for *Exserohilum turcicum* isolates clustered in five groups according to their aggressiveness level based on standardized Euclidean distance by the Ward method. Cluster analysis was performed using the variables incubation period, severity at 19 dpi, severity at 26 dpi, area under the disease progress curve (AUDPC), sporulation *in vivo*, area under the mycelium growth curve (AUMGC) and spore production *in vitro* with data for the maize line B37 at 30°C.

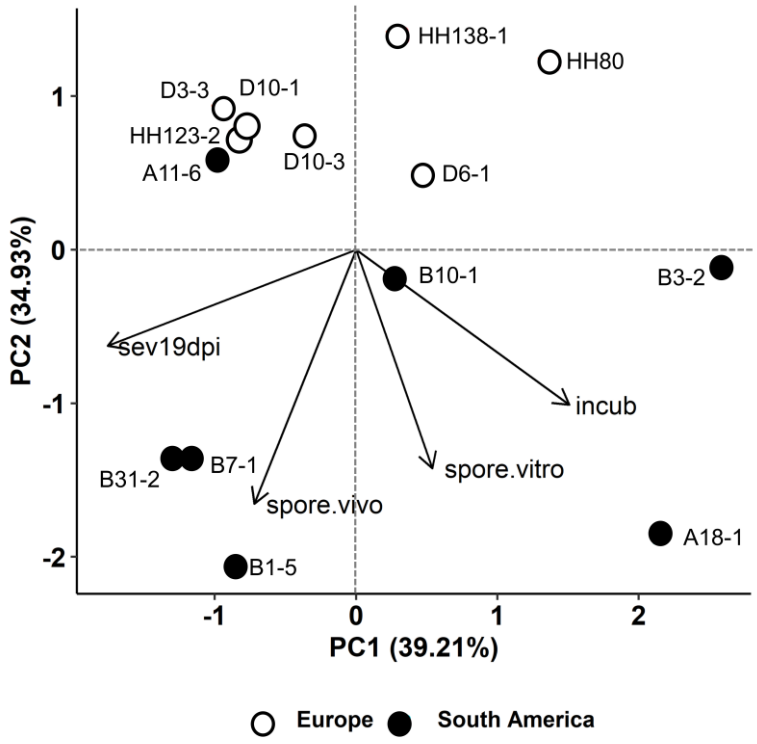


Figure 7. Principal component analysis (PCA) of the variables selected in the discriminant analysis (DA): disease severity at 19 days post inoculation–dpi (sev19dpi), sporulation *in vivo* (spore.vivo), spore production *in vitro* (spore.vitro), and incubation period (incub). Fourteen isolates of *Exserohilum turcicum* originating from Europe and South America were included in the analysis. Variable loadings and isolates scores are represented for the reference line B37 at 30 °C.

Discussion

The underlying concept of this study follows the 'disease triangle' (Agrios, 2005; Scholthof, 2007) considering the factors of temperature, host genotype and isolate aggressiveness putatively determined by the origin. Under controlled conditions, fungal vigor and disease components were most influenced by temperature, which was responsible for the highest variance. In most pathosystems, an increase in temperature is positively associated with aggressiveness (Santini and Ghelardini, 2015; Chen *et al*., 2017). As environmental conditions and host genotype usually strongly correlate with disease (Mariette *et al.*, 2018), the host genotype factor with three levels (reference line B37, susceptible line Sus1, and the hybrid Niklas®) was included in the experimental design. Interestingly, an interaction between isolate origin and host genotype was observed for the variable AUDPC. Disease severity was higher when South American isolates were inoculated in the hybrid Niklas than European isolates (Figure 4, Supplementary Figure 1). It is probable that European hybrids have been selected

by breeding programs according to their responses to European pathogen populations. However, all isolates were virulent in all tested host genotypes, indicating that the tested lines and hybrid do not harbor any known *Ht* genes. Usually, a pathogen population is more aggressive to a host population from the same region (Pariaud *et al.*, 2009), or even more aggressive in cultivars that they were isolated from in the field (Mundt, 2014), indicating adaptive shifts. Thus, aggressiveness can be correlated with genetic background and may have some specificity to the host genotype (Lannou, 2012; Pariaud *et al*., 2009).

Data for mycelium growth represent the effect of temperature on pathogen vigor. The interaction between temperature and isolate origin was significant for the variable AUMGC (Figure 1). South American isolates showed higher mycelium growth at 30 °C indicating that they might be more adapted to higher temperatures than European isolates. A study carried out with *Sclerotinia sclerotiorum* isolates showed a similar result. Isolates collected from warmer areas were better adapted to higher temperatures, and isolates from colder areas were more adapted to colder temperatures (Uloth *et al.*, 2015). In general, plant pathogens adapt to changes in environmental conditions by phenotypic plasticity, migration to areas with more favorable climatic conditions or mutations in their genomes, which all favor pathogen survival. Plasticity consists of the ability to adapt without the need for mutation. Plasticity might be correlated to a population with higher genetic diversity, as tropical *E. turcicum* populations are genetically more diverse (Santini and Ghelardini, 2015; Loladze *et al*., 2014). As reported for most plant pathogens, it is difficult to explain how adaptations occurred in *E. turcicum*. However, it is known that the influence of weather conditions decreases when *E. turcicum* populations are more aggressive (Levy, 1989).

The DA selected the variables incubation period, disease severity at 19 dpi, sporulation *in vivo*, and spore production *in vitro* for classification of isolates according to their origin. DA only misclassified the Argentinian isolates A11-6, leading to the conclusion that the aggressiveness of this isolate is similar to that of European isolates. Climate data from the last 50 years show that Pergamino in Argentina (origin of A11-6) has temperate and very humid weather. Mean precipitation is above 1000 mm per year and the average temperature is 16 °C (Aliaga *et al.*, 2017). In Southeast Brazil, climate data from the last 30 years show average precipitation of around 1500 mm per year and an average temperature close to 21 °C (INMET, 2020). The adaptation to mild temperatures of this Argentinian isolate may, therefore, explain why it was positioned with European isolates.

The increase in disease severity over time is probably due to lesion expansion (Vitti *et al.,* 1995; Carson, 2006), since controlled conditions were not favorable to sporulation, and consequently not favorable to secondary infections. Interestingly, in average sporulation *in vivo*

was higher with South American isolates than for European isolates in all temperatures, while average spore production of South American isolates *in vitro* was higher only at 25 and 30 °C. The more vigorous sporulation of South American isolates provides evidence that they are more effectively propagating at higher temperatures than European isolates. Higher sporulation under high temperatures is usually not expected in nature, since higher temperatures are not favorable for spore survival and germination (Bergamin Filho and Amorim, 1996). However, under high temperature the plant may be affected by heat stress and its defense thus weakened. Therefore, infection and host colonization, and consequently, sporulation might be favored under these conditions (Garrett *et al.,* 2006). A further factor involved might be an increased phytotoxin production, such as monocerin (Robson and Strobel, 1982) and HT-toxin (Wang *et al.,* 2010) by the pathogen, which may suppress host resistance at higher temperatures (Coakley *et al.*, 1999; Tagle *et al*., 2015). A potential mechanism is dysfunction in the detoxification process under such conditions (Pedras *et al.*, 2001). Unfortunately, the effect of high temperature on host resistance in the presence of the pathogen is difficult to analyze under experimental conditions and, therefore, heat stress was neglected in our experiments.

A shorter incubation period is usually correlated to higher aggressiveness. *Magnaporthe oryzae* isolates which showed a shorter incubation period had higher values in other aggressiveness components. Therefore, for this pathosystem, isolates which start epidemics early are more aggressive (Ghatak *et al.,* 2013). In the present study, a strong correlation was observed between AUMGC and spore production *in vitro* (Figure 5). However, no correlation was established between the *in vitro* and *in vivo* variables. In the *in vivo* experiment, the factor host genotype was added. As distinct host genotypes have distinct resistance backgrounds, the response to the environment and pathogen isolate can be different (Lannou, 2012). However, maize lines and hybrids used in this study had similar levels of susceptibility. Thus, the host genotype effect was weak, as observed in the VCA.

Cluster analysis of all variables describes aggressiveness grouped isolates in five sub-clusters aggregated under two main clusters, which were strongly associated to the isolate origin in Europe and South America. However, there were no similarities according to race or race complexity (Figure 6). Race complexity reflects the number of different resistance genes that one isolate can overcome (Zhan *et al.*, 2012). In nature, the emergence of complex races is unlikely to occur, unless there is a selection for more virulent populations by the cultivation of multi-resistant varieties. However, isolates bearing more virulence genes may not always be the most aggressive (Pariaud *et al.*, 2009), and may or may not have fitness costs (Zhan *et al.*, 2012). Nonetheless, it is not possible to make the same association for maize–*E. turcicum*,

as aggressiveness is not correlated with pathogen fitness. A high sporulation rate does not imply a higher survival rate (Pariaud *et al.*, 2009). *Cochliobolus carbonum* and *C. heterostrophus* are pathogens that represent a trade-off between aggressiveness and fitness. Low aggressiveness levels and high survival ability were observed for *C. carbonum* which was the opposite of what has been observed for *C. heterostrophus* (Pariaud *et al.*, 2009).

In the PCA, 86% of the European isolates were positioned together in the upper part of the graph and South American isolates were more spread out (Figure 7). The PCA result can be related to the genetic diversity of temperate and tropical *E. turcicum* populations (Borchardt *et al.*, 1998). Populations from temperate zones were less genotypically diverse than populations from tropical zones, potentially because sexual reproduction in temperate zones is rare. In addition, the European population might come from Central American isolates, as the genetic distance between European and Mexican populations is relatively small (Borchardt *et al.*, 1998). According to their aggressiveness, European isolates were positioned closely to each other, whereas South American isolates were spread (Figure 7), supporting this theory (Borchardt *et al.*, 1998).

Overall, our study provides evidence for a strong impact of temperature regimes on vigor and aggressiveness of *E. turcicum* which in turn was related to the origin of isolates from a warmer or cooler climate. Isolates from warmer climates, corresponding to the optimal conditions for the host plant, when tested under equal conditions and on similar host genotypes, grew and sporulated more vigorously *in vitro* and were more aggressive on their host plant. This may indicate a longer and thus more successful adaptation to their host plants in warmer than in cooler conditions corresponding to the history of maize cultivation in tropical and moderate climates. Such adaptive shift to more aggressive fungal isolates may imply that maize cultivation in cooler climates will face more aggressive isolates under continued climate warming.

Conclusions

South American *E. turcicum* isolates grew more vigorously and were more aggressive than European isolates, since the values of most of the tested aggressiveness components (AUDPC, sporulation *in vivo*, AUMGC and spore production *in vitro*) were higher for South American isolates, especially at higher temperatures. The fact that *E. turcicum* isolates originating from regions with warmer temperatures are more aggressive than those from regions with milder temperatures implies a putative effect of longer co-evolution of pathogen and host under warmer conditions promoting adaptive shifts to more aggressiveness. Accordingly, temperature was the factor with the greatest influence on pathogen

aggressiveness, since the tested temperature range was broad, from 15 to 30 °C. Although the host genotype is known to have a large effect on aggressiveness (Pariaud *et al.*, 2009), in our study, the host genotype did not explain the variance because all three hosts were moderately susceptible to *E. turcicum*. The results from *in vitro* and *in vivo* experiments indicate that *E. turcicum* populations display considerable plasticity (Santini and Ghelardini, 2015) and may adapt to the environmental conditions they are exposed to (Delmas *et al.,* 2016). Adaptability to environmental conditions is an advantage for pathogen populations, in case of temperature increases due to climate warming or range expansion of the host crop. The latter has happened with maize in the last few decades when expanding to cooler climates in Europe where a warming climate may thus induce pathogen populations with increased aggressiveness.

Acknowledgments

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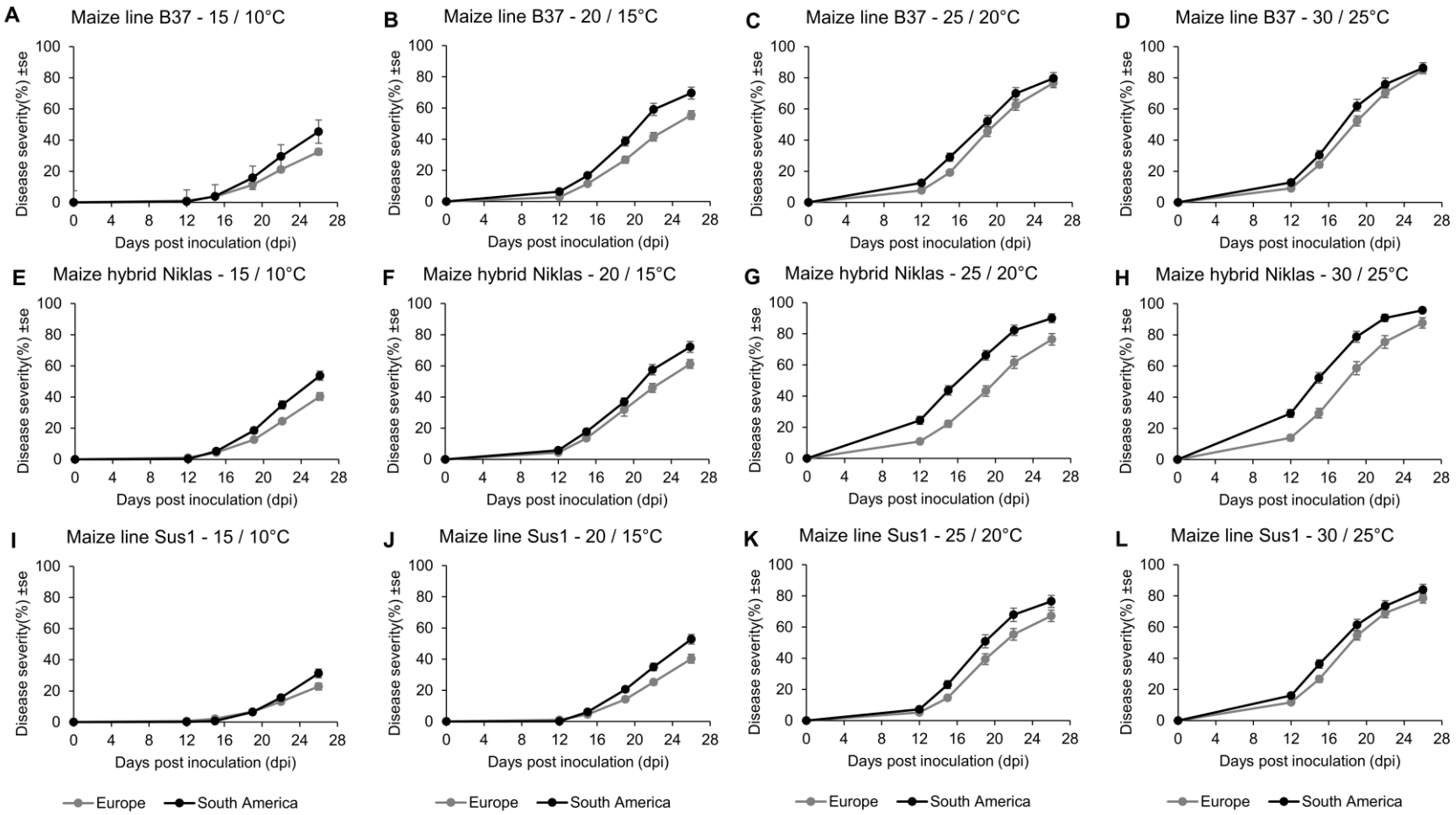
Supplementary Material

Supplementary table 1. MANOVA of *in vivo* variables, incubation period, severity (AUDPC) and disease severity at 26 dpi according to temperature, isolate origin and host.

MANOVA	Pillai's trace	approx. *F*-value	Df.	*p*-value		partial η^2
Temperature	1.0270	163.59	12, 3771	<0.001	***	0.3423
Isolate origin	0.0043	1.82	3, 1255	0.14		0.0043
Host	0.0272	5.77	6, 2512	0.005	***	0.0135
Temperature x isolate origin	0.0059	0.82	9, 3771	0.58		0.0019
Temperature x host	0.0440	3.12	18, 3771	0.009	***	0.0014
Isolate origin x host	0.0015	0.32	6, 2512	0.92		0.0007
Temperature x isolate origin x host	0.0171	1.20	18, 3771	0.25		0.0056



Supplementary figure 1. Disease severity of *Exserohilum turcicum* isolates from Europe and South America inoculated on three different maize hosts: B37–standard line for race monitoring (A–D), Niklas®–susceptible hybrid cultivated in Germany (E–H), and Sus1–susceptible maize line in breeding programs (I–L), and maintained under different day/night temperatures after inoculation: 30/25 °C (A,E,I), 25/20 °C (B,F,J), 20/15 °C (C,G,K), and 15/10 °C (D,H,L). Graphs represent the standard error of seven isolates in two experiments (n = 56).

Chapter 3. Diversity of expression types of *Ht* genes conferring resistance in maize to *Exserohilum turcicum*

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Abstract

Northern corn leaf blight (NCLB) is an important leaf disease in maize (*Zea mays*) worldwide and is spreading into new areas with expanding maize cultivation, like Germany. *Exserohilum turcicum,* causal agent of NCLB, infects and colonizes leaf tissue and induces elongated necrotic lesions. Disease control is based on fungicide application and resistant cultivars displaying monogenic resistance. Symptom expression and resistance mechanisms differ in plants carrying different resistance genes. Therefore, histological studies and DNA quantification were performed to compare the pathogenesis of *E. turcicum* races in maize lines exhibiting compatible or incompatible interactions. Maize plants from the differential line B37 with and without resistance genes *Ht1, Ht2*, *Ht3* and *Htn1* were inoculated with either incompatible or compatible races (race 0, race 1 and race 23N) of *E. turcicum*. Leaf segments from healthy and inoculated plants were collected at five different stages of infection and disease development from penetration (0-1 days post inoculation - dpi), until full symptom expression (14-18 dpi). Symptoms of resistance responses conveyed by the different *Ht* genes considerably differed between *Ht1* (necrotic lesions with chlorosis), *Ht2* (chlorosis and small lesions), *Ht3* (chlorotic spots) and *Htn1* (no lesions or wilt-type lesions). In incompatible interactions, fungal DNA was only detected in very low amounts. At 10 dpi, DNA content was elevated in all compatible interactions. Histological studies with Chlorazol Black E staining indicated that *E. turcicum* formed appressoria and penetrated the leaf surface directly in both types of interaction. In contrast to incompatible interactions, however, the pathogen was able to penetrate into xylem vessels at 6 dpi in compatible interactions and strongly colonized the mesophyll at 12 dpi, which is considered the crucial process differentiating susceptible from resistant interactions. Following the distinct symptom expressions, resistance mechanisms conferred by *Ht1, Ht2, Ht3* and *Htn1* genes apparently are different. Lower disease levels and a

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delayed progress of infection in compatible interactions with resistant lines imply that maize R genes to *E. turcicum* are associated with or confer additional quantitative resistance.

Keywords: R genes, *Setosphaeria turcica*, northern corn leaf blight, qualitative resistance, histology

Introduction

Northern corn leaf blight (NCLB) caused by the ascomycete *Exserohilum turcicum* [(Pass.) Leonard and Suggs], synonym *Setosphaeria turcica* [(Luttrel) Leonard and Suggs] has spread worldwide into regions where maize is cultivated. Yield losses up to 44% were recorded in susceptible hybrids at high disease severity levels between 52 and 100% during the full dent stage (Bowen and Paxton, 1988). Yield losses depend on the level of host resistance, disease severity, plant phenological growth stage during infection, and position of the infected leaves (Levy and Pataky, 1992). Two to 3 weeks after pollination, high levels of disease severity caused yield losses between 40 and 70% (Levy and Pataky, 1992). In addition, high disease severity of the leaf at the ear node is correlated with high yield losses (Levy and Leonard, 1990).

The pathogen can survive as chlamydospore in plant debris (Levy, 1995) and inoculum can be spread by rain and wind (Galiano-Carneiro and Miedaner, 2017). Under conditions of high humidity, conidia are able to germinate after one-hour in a broad temperature range (20–30 °C) (Levy and Cohen, 1983). Conidia germination is bipolar and an appressorium is usually formed at the end of germ tubes (Jennings and Ullstrup, 1957). Appressoria formation starts about 3 h after inoculation (Levy and Cohen, 1983). Infection by *E. turcicum* is usually initiated by direct penetration through the cuticle and epidermis. Penetration through stomata has been observed at 10% of penetration sites (Hilu and Hooker, 1964). As a hemibiotroph, after penetration of the epidermis, hyphae invaginate the membrane in the first stages of infection and a spherical intracytoplasmic vesicle is formed (Hilu and Hooker, 1964; Knox-Davies, 1974). After the primary stage of infection, hyphae start colonization of adjacent cells in the mesophyll (Knox-Davies, 1974) until xylem vessels are reached (Muiru *et al*., 2008; Kotze *et al.,* 2019). In later stages of infection, the pathogen may leave the xylem, colonize mesophyll cells, and form conidiophores on the leaf surface, which will disperse the conidia (Kotze *et al.,* 2019). The sexual stage was first reported in fields in Thailand. Sexual reproduction only occurs in populations with both mating types. Moreover, perithecia induction and maturation requires specific climatic conditions (Bunkoed *et al*., 2014).

Typical symptoms of NCLB are gray-green elongated necrotic lesions (Galiano-Carneiro and Miedaner, 2017). Disease levels may range from small lesions to necrosis of whole

leaves (Welz and Geiger, 2000). Seedlings are more susceptible to disease than young plants (Levy and Cohen, 1983). Fungicide application and host resistance are typically applied for NCLB control (Galiano-Carneiro and Miedaner, 2017). However, resistant cultivars are more frequently used in maize fields worldwide. Host resistance is based on qualitative and/or quantitative resistance. In breeding programs, qualitative resistance can be a faster strategy to improve resistance on new hybrids (Galiano-Carneiro and Miedaner, 2017). Resistance mechanisms and, consequently, phenotypes might differ in plants bearing different resistance genes. The resistance phenotype typically expressed by the resistance genes *Ht1*, *Ht2*, and *Ht3* is a chlorosis while the resistance mechanism described for plants harboring *Htn1* is an extended latent period (Levy and Pataky, 1992).

Ht1 was first found in two lines, “GE440,” from the United States, and “Ladyfinger,” a popcorn variety from Peru (Hooker, 1963). The reaction on hybrids bearing this resistance gene are characterized by chlorotic lesions, a delay in necrosis, and inhibition of fungal sporulation. *Ht2* was discovered in a line from Australia, “NN14B,” which displayed chlorotic lesions (Hooker, 1977). In the first description of the *Ht2* gene, lower resistance levels were mentioned when compared to the *Ht1* gene. The third R gene (*Ht3*) described for *E. turcicum* also expressed resistance by chlorotic lesions and was introgressed from a grass, *Tripsacum floridanum*, native to Cuba and Florida (Hooker, 1981). Another resistance gene used in breeding programs is known as *Htn1* and is derived from the Mexican maize variety “Pepitilla” and the resistance mechanism described is a delay in infection (Gevers, 1975).

The introduction of qualitative resistance in commercial hybrids may promote the selection of new physiological races. The race nomenclature for *E. turcicum* in maize is based on the resistance gene(s) which the isolate can overcome (Leonard *et al.,* 1989). Race 0 only infects plants without any resistance genes. Conversely, race 23N isolates are virulent on plants carrying resistance genes *Ht2*, *Ht3*, and *Htn1*. Several race monitoring studies using *E. turcicum* populations from different regions of the world have identified races that have overcome all major resistance genes. In the United States, the frequency of isolates virulent on maize lines containing *Ht1* was higher than the frequency of race 0 isolates due to widespread cultivation of commercial hybrids with the *Ht1* resistance gene in recent years (Ferguson and Carson, 2007; Weems and Bradley, 2018).

Ht resistance genes have been widely used in breeding programs (Welz and Geiger, 2000; Galiano-Carneiro and Miedaner, 2017). It has been hypothesized that fungal colonization, especially xylem penetration may differ between compatible and incompatible interactions. In an incompatible interaction, hyphae are restricted to xylem vessels (Muiru *et al.,* 2008;

Kotze *et al*., 2019). Therefore, the aim of this work was to characterize and quantify fungal colonization in plants carrying the resistance genes *Ht1*, *Ht2*, *Ht3*, and *Htn1* with isolates displaying compatible and incompatible interactions. The *in situ* characterization of fungal growth in host tissue was based on five different time points from initial penetration through symptom differentiation between interactions. Fungal DNA quantification and histological studies were performed with the differential set of near isogenic inbred lines of the recurrent parent B37 without resistance genes and near isogenic lines harboring different *Ht* resistance genes.

Material and Methods

Plant material, fungal strains and inoculation

Maize plants from the differential set based on near isogenic inbred lines of the recurrent parent B37 with no qualitative resistance gene and with resistance genes *Ht1, Ht2, Ht3,* and *Htn1* were cultivated in the greenhouse at 24 ± 3°C, 70% relative humidity, a day/night light regime of 14/10 hours and light intensity of 120 ±10 µmol m^{-2} s^{-1}. Two seeds per pot (11 cm x 11 cm x 10 cm) were sown in a mixture of compost, clay, and sand in the proportion 3:3:1. Seeds were provided from KWS Saat SE (Einbeck, Germany). Maize plants were inoculated using a sprayer when the fifth and sixth leaves were unfolded, about 30 days after sowing. Incompatible interactions were induced by inoculating race 0 on near isogenic lines B37*Ht1*, B37*Ht2*, B37*Ht*3 and B37*Htn1*, whereas the compatible interaction was studied by inoculating the same race 0 isolate on B37 without resistance genes. Furthermore, compatible interactions were analyzed by inoculating race 1 on B37*Ht1*, and race 23N on B37*Ht2*, B37*Ht3*, and B37*Htn1* lines (Supplementary table 1). The origin of isolates and race determination were described previously (Hanekamp, 2016). Each plant received seven ml of a conidia suspension at a concentration of 3,000 conidia ml^{-1} and was maintained in a humidity chamber for 24 h.

Fourteen days post inoculation (dpi), four plants per treatment were evaluated to confirm compatible and incompatible interactions between the differential lines and isolates (Supplementary table 1). Leaf samples were collected from the inoculated area with visual symptoms. Disease phenotyping, disease rating, fungal DNA quantification, and microscopic studies on fungal colonization were based on five different time points: penetration (0–1 days post inoculation, dpi), first stages of infection (2–4 dpi), pre-symptomatic disease stage (5–7 dpi), first symptom expression (10–12 dpi), and occurrence of symptoms distinguished between interactions (14–18 dpi). Before sampling at the last time point, disease severity was evaluated in ten plants based on a diagrammatic

scale (Pataky, 1992). Disease severity of the replicated experiment is presented in Supplementary figure 1.

DNA quantification of Exserohilum turcicum *in infected leaves*

Leaf samples for DNA quantification were harvested right after inoculation (0 dpi), and three, six, ten, and fourteen-days post inoculation (dpi). Nine plants were harvested per treatment and timepoint. The fourth and fifth leaves from three plants were harvested and pooled together in one biological replicate. Three biological replicates were used per treatment and timepoint. For the DNA standard curve, the race 0 isolate was grown in liquid Czapek Dox Medium at 22°C in the dark. The mycelial culture was shaken at 100 rpm for 14 days and then filtered by vacuum suction. The mycelium was frozen, lyophilized, ground, and homogenized with a mixer mill (Retsch® MM400, Haan, Germany). Genomic DNA (gDNA) extraction was performed with the CTAB method (Brandfass and Karlovsky, 2008), where 1 ml of CTAB-buffer (20 mM Na-EDTA, 0.13 M sorbitol, 30 mM N-laurylsarcosine, 20 mM CTAB, 0.8 M NaCl, 10 mM Tris – pH 8.0 adjusted with NaOH) were added to 50 mg ground leaf sample. Proteinase K (1 µl from 20 mg ml^{-1} stock solution) was added to each sample. The mixture was treated in an ultrasonic bath for 5 s then incubated for 10 min at 42°C and 10 min at 65°C (tubes were shaken during incubation). After incubation, 800 µl of chloroform-isoamyl alcohol (24:1) were added and tubes were shaken. Samples were incubated for 10 min on ice, then centrifuged at 13,000 × g for 10 min (Hettich Zentrifugen Mikro 220R, Germany). The supernatant was transferred to another tube with 200 µl of 30% (w/v) PEG and 100 µl 5 M NaCl. The pellet was washed with 70% (v/v) ethanol, then dried at room temperature. The dry pellet was dissolved in 100 µl TE buffer pH 8.0 (0.1 M Tris, EDTA 10 mM) and stored at -20°C. After DNA extraction, 1 µl of each sample was placed in an agarose gel (1%) and electrophoresis was performed to verify the DNA extraction procedure.

The amount of DNA was measured by electrophoresis in an agarose gel (1%) and compared with known DNA concentrations of bacteriophage Lambda. Samples with a high genomic DNA (gDNA) concentration were diluted 1:10. The dilution factor was considered in further calculations. A standard curve was obtained by diluting fungal DNA from 1,000 to 0.01 pg $µl^{-1}$ (1,000, 100, 10, 1, 0.1, 0.01 pg $µl^{-1}$) to quantify the target sequence by qPCR. The calibration curve was based on a linear regression of the quantification cycle value versus the logarithmic values of known gDNA. Data were analyzed with the software BioRad CFX Maestro 1.1 (Fa. Bio-Rad).

Quantitative polymerase chain reaction (qPCR) analysis was performed with a primer pair designed to amplify the pathogen specific internal transcribed spacer (ITS) region (Beck

1998). The primer pair used was (forward) JB 586 (5'-TGGCAATCAGTGCTCTGCTG-3') and (reverse) JB 595 (5'-TCCGAGGTCAAAATGTGAGAG-3'), resulting in an amplicon size of 485 base pairs. PCR reactions were performed with 5 µl of the premix qPCR BIO SyGreen Mix Lo-ROX (PCR Biosystems, London, United Kingdom) with a primer concentration of 0.4 µM and 1 µl from the DNA sample. The final volume of the reaction was 10 µl. The optimal thermal cycling conditions (CFX384 Thermocycler - Biorad, Rüdigheim, Germany) were 94°C for 3 min, followed by 40 cycles of 94°C for 5 s (denaturation), 63.5°C for 15 s (annealing), 72°C for 15 s (elongation), and 72°C for 5 min for final elongation. Three technical replicates were performed for each biological replicate.

Fungal DNA contents were compared between lines in the compatible interaction within every timepoint, for 10 dpi and 14 dpi by analysis of variance (ANOVA) and multiple comparison applying post hoc Tukey test (p-value ≤ 0.05) performed in the R software 3.6.0 (Core Team 2019) and graphics were generated in the software Microsoft Excel 2016. Data of the replicated experiment is presented in Supplementary figure 2.

Histological studies

Leaf segments for the histological studies were collected at 1, 3, 6, 12 and 18 dpi. The fourth and fifth leaves from two plants were collected, resulting in four biological replicates per treatment and sampling time point. For every sampled leaf, six square centimeter (2 x 3 cm^2) leaf segments were cut and fixed in FAA-solution (90 ml of ethanol 70%, 5 ml formaldehyde 36%, and 5 ml acetic acid 99%) and stored at room temperature. Leaf pigments were removed in two subsequent steps. First, they were incubated in 70% ethanol for two hours at room temperature and then washed with water. This step was followed by incubation in a water bath at 90°Cfor 2.5 h in closed flask containing 2 M potassium hydroxide (KOH) in a 90°C water bath for 2.5 hours. After bleaching, samples were washed with tap water and stained with Chlorazol Black E (CBE) (Sigma Aldrich) solution (0.03% [w/v] chlorazol black E; lactic acid, glycerin, distilled water in the proportion 1:1:1) at 60°C in a water bath overnight (adapted from Wilcox and Marsh, 1964). After staining with CBE, leaf segments were transferred in 50% glycerin and analyzed with light microscopy within the next 48 h.

Samples were analyzed using 50% glycerin as mounting fluid. Three parameters were evaluated during light microscopy analysis: xylem penetration efficiency (XPE), xylem colonization efficiency (XCE), and mesophyll colonization efficiency (MCE). Effective xylem penetration was considered when hyphae were able to penetrate the xylem vessel (Figure 1A-B). Xylem colonization occurred when two or more hyphae were visible inside the xylem vessel (Figure 1C). Mesophyll colonization was considered when hyphae left the

xylem and colonized mesophyll tissue in a region different from the penetration site (Figure 1D-F). Ten penetration sites were evaluated per sample, resulting in forty penetration sites being studied per treatment and time point. XPE, XCE and MCE were calculated by dividing the number of successful penetrations or colonizations to the number of evaluated penetration sites and transformed to percentage. Data were analyzed with the software Microsoft Excel 2016 and Statistica 13.0 (Statsoft, Tulsa, US). Data from each resistant line were compared with B37 using a Chi-square test (* p-value ≤ 0.05, ** p-value ≤ 0.01, *** p-value ≤ 0.001). Fungal colonization was illustrated using the Corel Draw graphics suite X8 software (Corel Corporation, Ottawa, Canada).

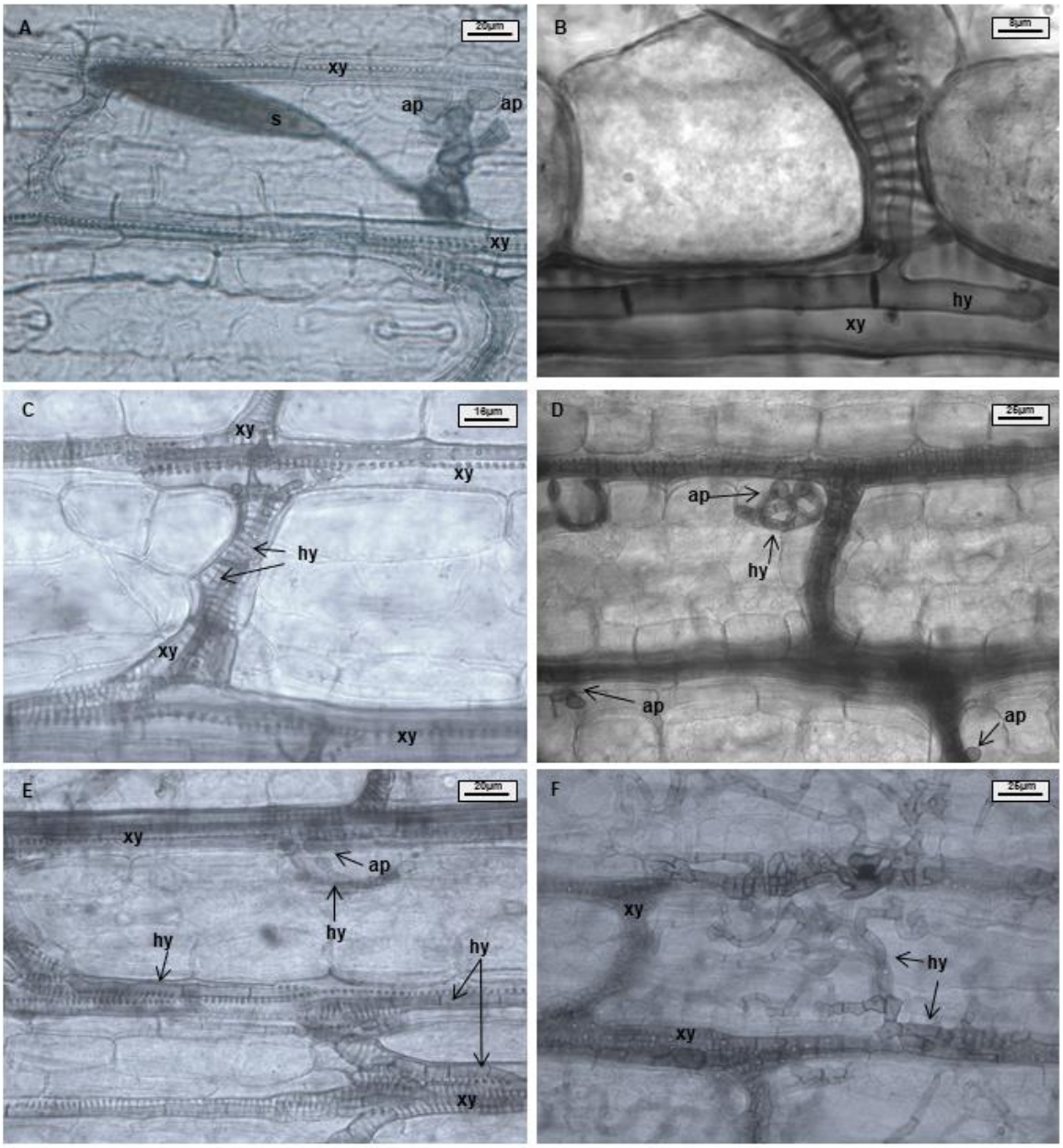

Figure 1. Maize leaves infected with *Exserohilum turcicum* after staining with Chlorazol Black E (CBE). Light microscopical images of the mesophyll. Appressorium-like structures formed from hyphae in the mesophyll. Hyphae originating from a germinated spore penetrate the epidermis and colonize the mesophyll (A). One hypha colonizing a xylem vessel (B). Hyphae growing in a xylem vessel (C). Mesophyll penetration/colonization takes place at a different location than initial penetration (D). Appressorium-like structure formed from hyphae inside a xylem vessel (E). Xylem vessels and mesophyll colonized by the fungus (F). B37*Ht2* incompatible interaction at 6 days post inoculation (dpi) (A), B37 at 6 dpi (B, C, E), B37 at 12 dpi (D), and B37 at 18 dpi (F). Hyphae (hy), xylem (xy), appressoria (ap), and spore (s).

Results

Symptomology

Disease symptoms in incompatible interactions were mostly characterized by chlorosis, while chlorosis was absent in compatible interactions. In the compatible interaction, symptoms of necrosis developed and were characterized by strong leaf blight. At 1 dpi, most plants showed no symptoms (Figure 2A). It was possible to observe slightly water-soaked spots on some leaves when illuminated from the backside. The first chlorotic spots were found in both interactions at 3 dpi (Figure 2B). Six-days post inoculation, all plants and interactions still presented chlorotic spots, except for the incompatible interaction B37*Ht1*, where yellow spots had developed into elongated soaked lesions (Figure 2E). Ten-days post inoculation, the first grey necrotic lesions were observed in compatible interactions (Figure 2C). Differences in symptoms between compatible and incompatible interactions were clearly distinguishable in almost all plants at 14 dpi (Figure 2D,F-J).

Fourteen days post inoculation, B37 presented typical grey necrotic lesions (Figure 2D) and sometimes the leaf was completely dried (Figure 2L). In the incompatible interaction, each *Ht*-resistance gene expressed different symptoms of resistance indicating differences in the underlying resistance mechanisms. B37*Ht1* presented chlorosis with strong necrosis and developed a completely dry leaf (Figure 2F). In B37*Ht2*, a distinction between compatible and incompatible interaction based on chlorosis and necrosis was not clear (Figure 2G,H). Some plants expressed chlorosis, while others expressed small grey lesions, even in inoculations with the same isolate and in the same experiment. In contrast, symptom expression by B37*Ht*3 was quite uniform compared to the other resistant lines. B37*Ht*3 consistently formed yellow spots at penetration sites (Figure 2I). Older leaves from B37*Htn1* developed small wilt-type spots, independent of the kind of inoculation (Figure 2K). Furthermore, the disease levels in the compatible interactions varied between B37 and differential lines with resistance genes. B37*Ht1* showed higher disease severity than B37, with an average of 63% and 43%, respectively. Lines B37*Ht2*, B37*Ht3,* and B37*Htn1* displayed fewer symptoms than B37. In the incompatible interaction, B37*Ht3* and B37*Htn1* did not develop any necrosis, in contrast to B37*Ht1* which showed strong necrosis, resulting in an average disease severity of 42%. In the incompatible interaction, B37*Ht2* some leaves showed small grey lesions, but disease severity was lower than 5% (Figure 3).

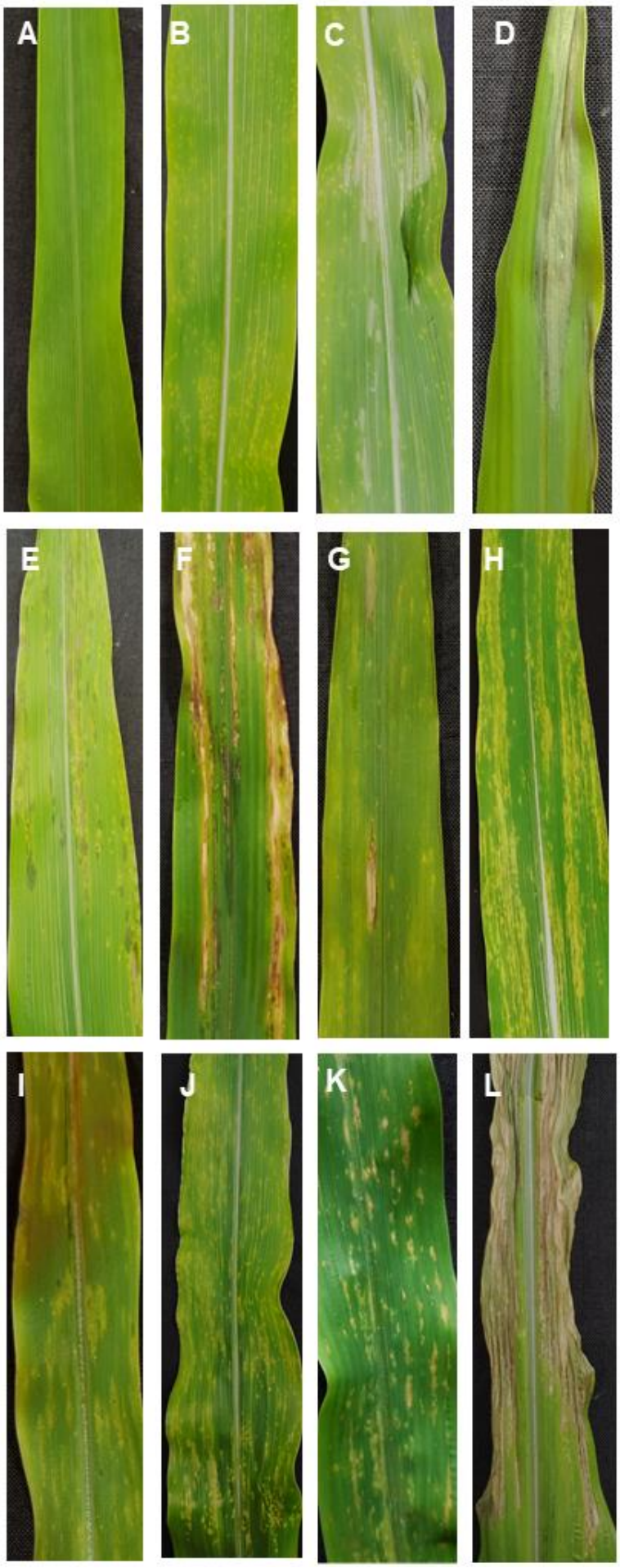
A
B
C
D
E
F
G
H
I
J
K
L

Figure 2. Symptomology of *Exserohilum turcicum* leaf infection on maize differential lines B37 without resistance genes and with the resistance genes *Ht1, Ht2, Ht3,* and *Htn1*. Symptom development in a compatible interaction (race 1 or race 23N) one day post inoculation (dpi) (A), 3 dpi (B), 10 dpi (C), and 14 dpi (D). In the incompatible interaction (race 0 isolate), B37*Ht1* small soaked lesions are present at 6 dpi (E) and strong necrosis surrounded by chlorosis occurs at 14 dpi (F). Symptoms of an incompatible interaction at 14 dpi on B37*Ht2* (G, H), B37*Ht3* (I), and B37*Htn1* (J). Healthy plants of B37*Htn1* display yellow spots on older leaves (K). Strong necrosis with brownish lesions was observed in the compatible interaction with B37*Htn1* (L).

DNA quantification of Exserohilum turcicum

Fungal DNA was detected for both interactions in all inoculated samples at 3, 6, 10, and 14 dpi. DNA content increased over time until the last sampling time points at 10 and 14 dpi. The compatible interaction displayed a higher fungal DNA content after 10 dpi compared to the incompatible interaction at a time point where first symptoms became visible. Moreover, at 14 dpi, B37 presented the highest amount of fungal DNA, followed by the compatible interaction on B37*Ht1*. The compatible interaction of B37*Ht1* (inoculated with race 1) presented a higher DNA content at 10 dpi, due to early symptom expression and higher disease severity (Figure 3), when compared to the other lines. At 14 dpi, high fungal DNA contents were recorded in all compatible interactions, ranging from 700 to 3100 ηg DNA/g dry weight. In the compatible interaction, B37*Htn1* showed lower fungal DNA-content compared to the other resistant lines, which was in correspondence with disease severity (Figures 3 and 4).

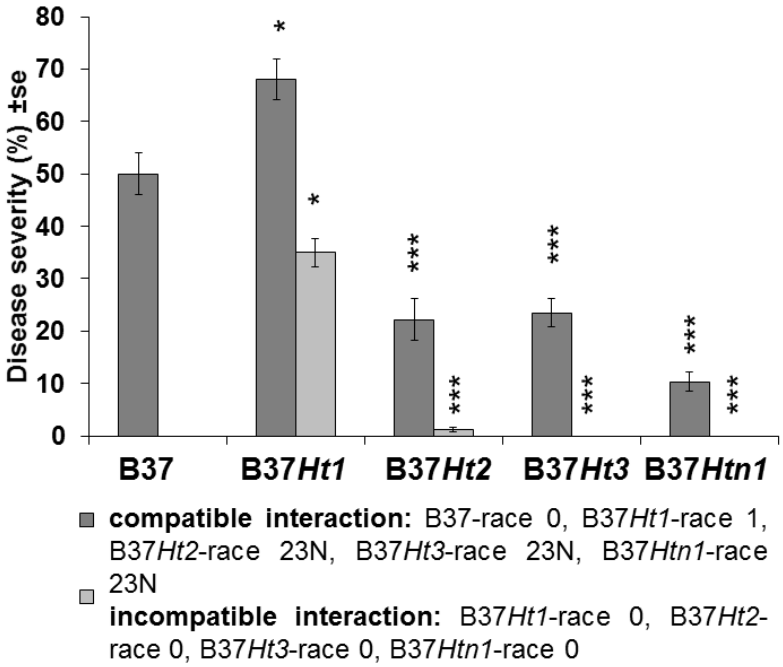

Figure 3. Disease severity and standard error for compatible and incompatible interactions between *Exserohilum turcicum* and the maize lines B37 without resistance genes and with resistance genes *Ht1*, *Ht2, Ht3,* and *Htn1*. Disease severity was evaluated 14 days post inoculation (dpi). Ten plants were evaluated per treatment (n = 10 plants). Data from the first replication experiment are presented in the graph. Data from each line with resistance genes was compared with B37 by Mann–Whitney-U test (**p*-value ≤ 0.05, ***p*-value ≤ 0.01, and ****p*- value ≤ 0.001).

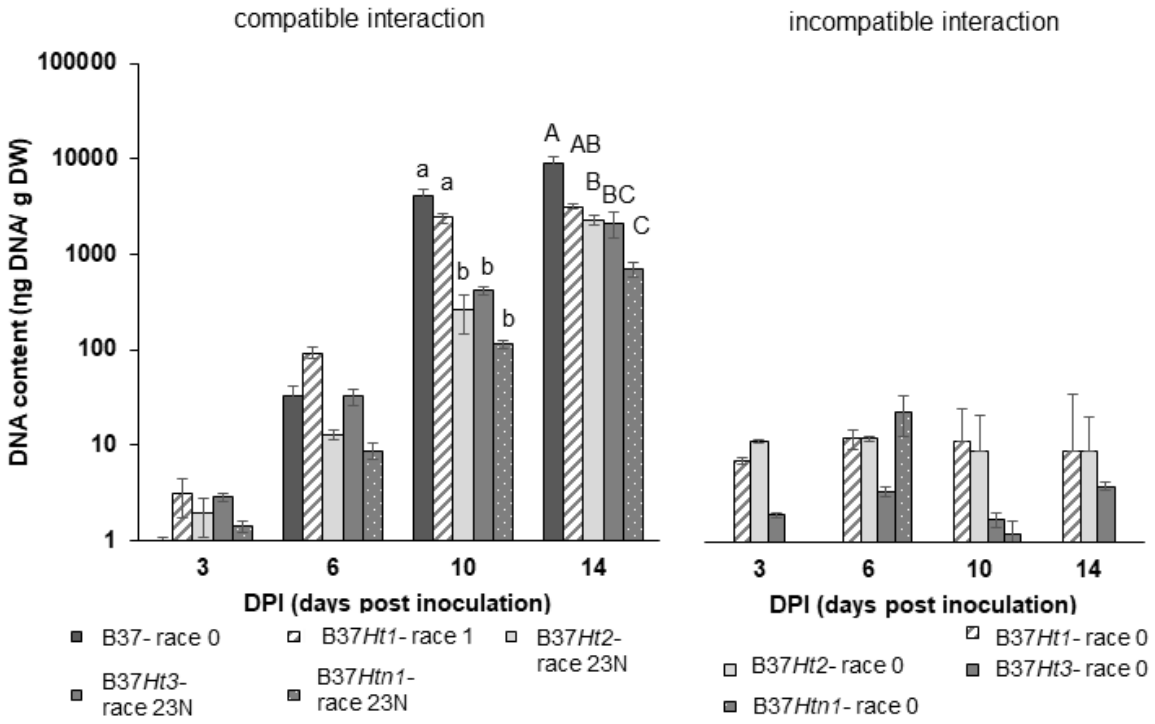

Figure 4. DNA-contents with standard errors for compatible and incompatible interactions between *Exserohilum turcicum* and the maize lines B37 without resistance genes and with the resistance genes *Ht1, Ht2, Ht3*, and *Htn1*. Samples for qPCR analysis were collected 0, 3, 6 10, and 14 days post inoculation (dpi). The DNA content is presented in ηg DNA/g leaf dry weight. The fourth and fifth leaves from three plants were pooled in one biological replicate. In total, nine plants were harvest per treatment and timepoint (n = 3 biological replicates). Data from the first replication experiment are presented in the graph. Lowercase letters indicate significant differences between treatments at 10 dpi. Uppercase letters indicate significant differences between treatments at 14 dpi. Means sharing the same letter were not significantly different following Tukey-adjusted comparisons for data with a log-transformation (p-value ≤ 0.05).

Histological studies

Penetration through the epidermis and into the xylem were observed in both compatible and incompatible interactions (Figure 5). However, in the compatible interaction, the fungus was able to substantially colonize the xylem tissue resulting in the pathogen hyphae growing through the xylem and into the mesophyll. Mesophyll colonization was primarily observed at greater distance from the penetration site. Under favorable environmental conditions, particularly under high humidity, the pathogen developed reproductive structures (Figure 5A).

Xylem penetration efficiency in the incompatible interactions was between 20 and 40% for the lines bearing resistance genes (Figure 5C). However, in the compatible interaction XPE increased for all lines over time (Figure 5D). The XPE in B37*Ht1* and B37*Ht3* was similar to that of B37. At 3 dpi, XPE was lower in B37*Ht2* and B37*Htn1* than in B37. The XCE was evaluated starting at 6 dpi. For the incompatible interaction, the average XCE was around 10% for B37*Ht1* and B37*Ht2* (Figure 5E). In the compatible interaction, XCE was similar to XPE, since they increase with time after inoculation (Figure 5F).

The MCE was evaluated starting at 12 dpi. The MCE for incompatible and compatible interaction was similar to XCE; all incompatible interactions had less than 5% MCE (Figure 5G). In the compatible interactions, the MCE increased over time. B37*Ht2* and B37*Htn1* presented a lower MCE than B37*Ht1* and B37*Ht3* (Figure 5H). The experiments were repeated and the results obtained in the first run were confirmed by the second run (Supplementary figures 1,2 and 3).

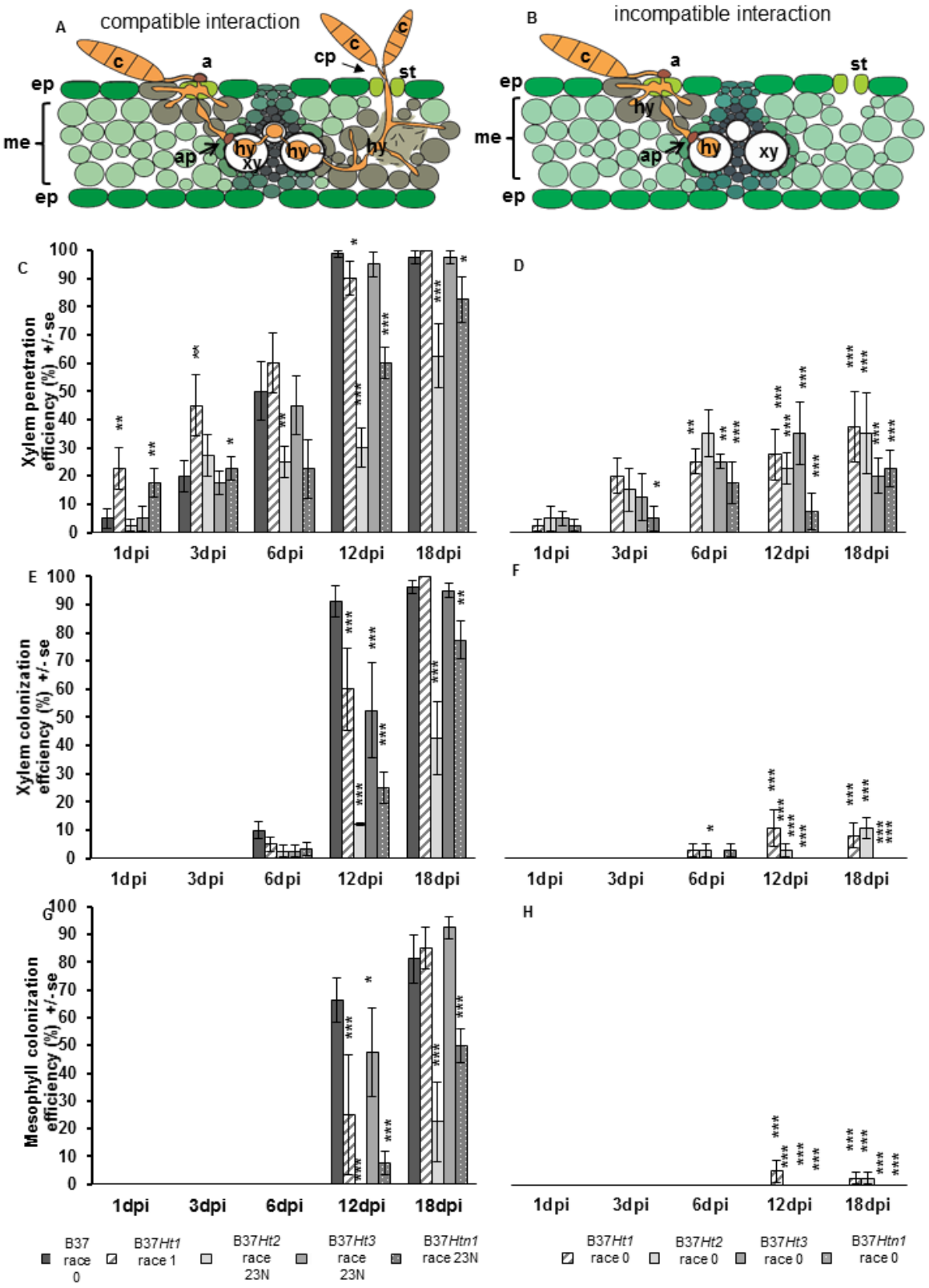
A
compatible interaction
B
incompatible interaction
c
a
cp
st
ep
me
ap
hy
xy
C
D
E
F
G
H
Xylem penetration efficiency (%) +/- se
Xylem colonization effciency (%) +/- se
Mesophyll colonization efficiency (%) +/- se
1dpi
3dpi
6dpi
12dpi
18dpi
B37 race 0
B37Ht1 race 1
B37Ht2 race 23N
B37Ht3 race 23N
B37Htn1 race 23N
B37Ht1 race 0
B37Ht2 race 0
B37Ht3 race 0
B37Htn1 race 0

Figure 5. Schematic representation of compatible and incompatible interactions of maize leaves with *Exserohilum turcicum*. Appressorium-like structures are formed from hyphae in the mesophyll with the objective to penetrate into the xylem vessel. The hyphae derive from a germinated spore and penetrate into the epidermis and colonize the mesophyll (A,B). Colonization of the xylem vessel and spread to a new area of the mesophyll with conidiophore formation through the stomata, characterizing a compatible interaction (A). The effect of resistance genes *Ht1, Ht2, Ht3*, and *Htn1* in the respective B37 maize lines on xylem penetration efficiency (XPE), xylem colonization efficiency (XCE), and mesophyll colonization efficiency (MCE) of *E. turcicum* compared between compatible (A,C,E,G) and incompatible (B,D,F,H) interactions. Four samples were collected 1, 3, 6, 12, and 18 days post inoculation (dpi) per isolate and interaction (n = 4). Data represent the 10 penetration sites evaluated per biological replicate. Data from each line with *Ht* resistance genes was compared with B37 by Chi-square test (**p*-value ≤ 0.05, ***p*-value ≤ 0.01, and ****p*-value ≤ 0.001). Bars indicate standard error. Symbols indicate: c, conidium; a, appressorium; st, stomata; ep, epidermis; me, mesophyll; xy, xylem vessel; ap, appressorium-like structure; hy, hyphae; cp, conidiophore.

Discussion

Symptoms of *Exserohilum turcicum* infection in maize leaves had a differential pattern according to the *Ht* resistance gene (Figure 2). In general, chlorosis was associated with resistance, and therefore classified as incompatible interaction, whereas gray-green necrotic lesions were typically observed in compatible interactions. However, lesion size or number of lesions per leaf differed between compatible responses of lines (Abadi *et al.*, 1989). B37, B37*Ht1* and B37*Ht3* usually had higher disease severity and larger lesions than B37*Ht2* (Figure 3, Supplementary figure 1). In the incompatible interaction, the phenotype of infected plants differed according to the *Ht* resistance gene (Figure 2). The phenotype of plants bearing the *Ht1* gene was always characterized by longer necrotic lesions surrounded by chlorosis (Hilu and Hooker, 1963). Typically, water-soaked lesions developed into brownish lesions in the incompatible interaction. Interestingly, the phenotype observed in the incompatible interaction with B37*Ht2* switched between chlorosis and small lesions. The resistance conferred by *Ht2* was characterized by a lower resistance level, as described by (Hooker, 1977). Conversely, the resistance phenotype expressed conferred by *Ht3* was uniform. Resistant plants always developed chlorotic spots (Hooker, 1981). As described previously, plants bearing the *Htn1* gene switched between lesion-free plants (Gevers, 1975) and plants showing a few wilt-type lesions, similar to small soak spots (Figure 2K). However, plants displaying extended latent period were also observed, as mentioned in the literature (Gevers, 1975).

Molecular studies identified three candidate genes in the *Htn1* locus, which encode wall associated receptor-like kinases (RLKs). These resistance genes produce proteins that are able to recognize pathogen invasion and cell wall disruption (Hurni *et al.*, 2015). Consequently, lower levels of disease severity and fungal colonization in the compatible interaction may be related to this resistance mechanism. The average disease severity on B37*Htn1* was 9.5%, which was low compared to compatible interactions in the other lines (Figure 3). In the qPCR studies, B37*Htn1* inoculated with race 23N (compatible interaction) displayed the lowest fungal DNA content (Figure 4, Supplementary figure 2) and XCE and MCE were also delayed, increasing only slowly over time (Figure 5). Therefore, the low fungal DNA content and disease severity observed in the compatible interaction of B37*Htn1* may be related to the resistance mechanism of an extended latent period (Figure 4). The different patterns in hyphal colonization and DNA content confirm that the *Htn1* gene does not offer a completely effective barrier against fungal infection (Gevers, 1975). This supports the molecular analysis that the *Htn1*-gene locus confers a polygenic quantitative resistance against NCLB (Hurni *et al.*, 2015).

Chlorazol Black E (CBE) staining has not been used before to analyze the *in situ* interaction of *E. turcicum* on maize. Regardless of the type of interaction, line, or the staining used to perform the analysis, a stained halo surrounding the infection site was visible at the penetration sites (Hilu and Hooker, 1964). The histological analysis performed with CBE allowed a clear identification of the cell wall. The black color provided by the staining conferred optimum contrast of plant and fungal cell walls for microscopic analysis. An alternative staining technique with calcofluor and distaining with cellulase was previously described for hyphae detection in plant tissue (Trese and Loschke, 1990). However, the hyphal growth could not be clearly observed and, consequently quantified using calcofluor staining. Conversely, CBE staining enabled to visualize and measure XCE and XME, thus differences between interactions could be identified. Before staining, the clearing of specimens was performed by the use of KOH. In contrast to staining with calcofluor (Trese and Loschke, 1990), CBE provides better distinction between xylem and mesophyll tissue due to its affinity for lignified tissue, such as tracheary elements. Moreover, CBE also stained the fungal cell wall, due to its affinity for chitin. CBE has been previously used for staining mycorrhizae (Brundrett *et al.*, 1984). However, CBE staining, as other light microscopy techniques, is not sufficient for a higher level of detail e.g. the identification of cell wall or cell membrane modifications. Therefore, other techniques enabling a higher resolution, such as transmission electron microscopy, are necessary to identify ultrastructural resistance mechanisms like cell wall thickening (Berliner *et al.*, 1969).

Exserohilum turcicum is a hemibiotroph characterized by a sequence of biotrophic and necrotrophic phases of infection. The biotrophic phase includes xylem penetration and colonization. The necrotrophic phase starts when the hyphae leave the xylem vessel and provoke plasmolysis of the mesophyll cells until the conidiophores are formed through the stomata (Kotze *et al.*, 2019). Even in the incompatible interaction, the pathogen demonstrated the ability to penetrate into the xylem vessels (Muiru *et al.*, 2008). In the compatible interaction, however, hyphae grew and spread into vascular bundle sheath cells. In all *Ht*-resistant lines tested in this study (*Ht1, Ht2, Ht3* and *Htn1*), the resistance expressed at the time point of xylem colonization was crucial for further steps in the pathogenesis. Our quantitative analysis of the infection progress suggests that between 3 and 6 dpi is the critical time, during which the resistance mechanism becomes effective to avoid further xylem colonization. Therefore, alterations in the integrity of xylem tissue may be recognized by the host (Bellincampi *et al.*, 2014) between the establishment of infection and the pre-symptomatic state (5-7 dpi).

Differences in symptom expression and fungal colonization, which were observed for each resistant line, strongly suggest that each *Ht* resistance gene encode for distinct resistance mechanisms. In the early infection stages, the *Ht1* resistance displays big necrotic lesions surrounded by chlorosis. However, necrosis observed in the incompatible interaction was not caused by pathogen colonization, since the fungal DNA content was not high. Disease severity was around 50%, but the efficiency of fungal colonization in the mesophyll was low and did not correlate with symptom expression. Therefore, the necrosis observed in B37*Ht1* is considered a strong resistance reaction expressed by *Ht1*. Conversely, *Ht2* represented an unstable phenotype, which was confirmed by lower rates of mesophyll colonization and lower fungal DNA content. The necrosis observed in B37*Ht*2 was caused by fungal colonization. In this case, resistance is expressed by chlorosis or by smaller lesions and a low number of lesions. Instability of the *Ht2* resistance phenotype may be related to the influence of temperature and to the presence of the inhibitor gene *Sht1*. *Sht1* is epistatic to *Ht2* (Ceballos and Gracen, 1989). Therefore, resistance conferred by *Ht2* is considered oligogenic (Hooker, 1977). The *Ht3* phenotype can be easily identified by chlorotic spots. The *Ht3*-gene was introgressed from *Tripsacum floridanum* (Hooker, 1981), which is not an alternative host for *E. turcicum*. This implies, that the stability of the *Ht3* resistance phenotype might be related to a mechanism similar to non-host resistance. As an exception, the resistance conferred by *Htn1* is characterized as quantitative resistance (Hurni *et al.*, 2015).

Similar to B37*Htn1*, B37*Ht2* also presented a low average disease severity (17.4%). In the histological studies, XCE and MCE were even lower for B37*Ht2* than B37*Htn1* (Figure 5,

Supplementary figure 3). However, B37*Ht2* presented a similar fungal DNA content as the other lines. Differences in the average DNA content between the first and the second experiment can be correlated to differences in the level of disease severity (Figure 2G-H), as the race 23N isolate used in these experiments was able to overcome resistance provided by the *Ht2* gene. Even in the compatible interaction, the disease severity was not high which was in agreement with the low XPE, XCE, and XME observed in the histological studies. Therefore, the resistance mechanism underlying *Ht2* may be related to suppression of aggressiveness factors.

Exserohilum turcicum produces a non-host specific phytotoxin known as monocerin (Robeson and Strobel, 1982), which may be an aggressiveness factor. In addition to monocerin, a host-specific toxin, HT-toxin, has been described to inhibit chlorophyll formation, which might be the main cause of chlorosis and increase on lesion size (Bashan and Levy, 1992; Bashan *et al.*, 1995, Wang *et al.*, 2010; Li *et al.,* 2016). *Ht2* may either encode a mechanism of phytotoxin detoxification (Pedras *et al.*, 2001) and/or synthesis of phytoalexins (Lim *et al.*, 1970), such as a cyclic hydroxamic acid named DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) (Mace, 1973). Interestingly, some resistance genes appeared to be also effective in reducing disease severity and fungal colonization in the compatible interaction. This supports the hypothesis that qualitative resistance may affect infection also through an underlying quantitative resistance in the maize-*E. turcicum* pathosystem, as mechanisms of phytotoxin detoxification or the production of phytoalexins may be also related to quantitative resistance (Poland *et al.*, 2009).

From the genetic perspective, *E. turcicum* interacts with maize following the gene-for-gene concept (Mideros *et al.*, 2018). In such case, each *Ht*-gene should have a corresponding fungal avirulence gene. The first avirulence gene identified for *E. turcicum AVRHt1* corresponds to the *Ht1*-resistance gene (Mideros *et al.*, 2018). *AVRHt1* was expressed *in planta* by a race 23N isolate at 5 and 7 dpi (Hurni *et al.*, 2015), when xylem colonization started (Kotze *et al.*, 2019). Moreover, gene effector candidates encoded a hybrid polyketide synthase:nonribosomal peptide synthetase (PKS:NRPS) (Wu *et al.*, 2015), and virulence-associated peptidases leupeptin-inhibiting protein 1 fungalysin involved in the biosynthesis of secondary metabolites and cell wall degradation (Human *et al.*, 2020). The increase in XPE and XCE from 3 to 6 dpi indicates that between these time points, the pathogen is releasing virulence effectors. Transcriptional profiles showed that *Ecp6* and *SIX13-like* proteins, similar to the secreted xylem effectors of *Fusarium oxysporum*, were overexpressed at 5 and 7 dpi (Human *et al.*, 2020), which correlates to our findings in the histological studies and indicates that virulence effectors are being released at the time point before symptom expression (5-7 dpi).

The resistance phenotypes expressed by the *Ht* genes are diverse, as the *Ht1* gene expressed necrosis and chlorosis, *Ht2* was characterized by chlorosis and small lesions, *Ht3* showed chlorotic spots and *Htn1* conferred no lesions or wilt-type lesions. These lesions types reflect pathogen colonization, as plants displaying strong necrosis had the complete mesophyll colonized; instead of plants expressing chlorosis, where the xylem and mesophyll were weakly or not colonized. Besides differences on the resistance phenotype, the fungal DNA content was low in the compatible interaction. Indeed, a low fungal DNA content in plants carrying the *Ht* genes even in the compatible interaction shows that these genes have quantitative effect. In fact, *Htn1* was denominated as a source of quantitative resistance. Therefore, *Ht* genes may be associated with or confer additional quantitative resistance.

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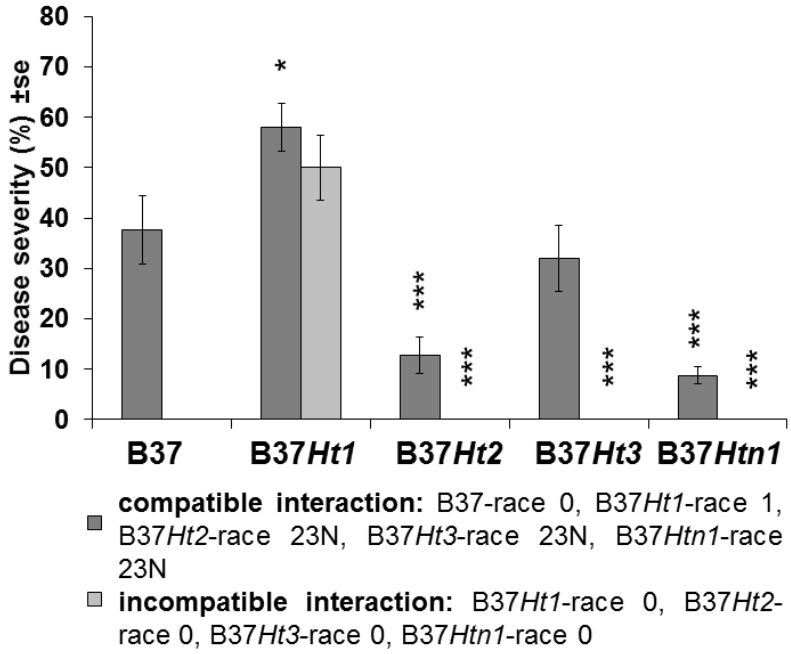
Supplementary Material

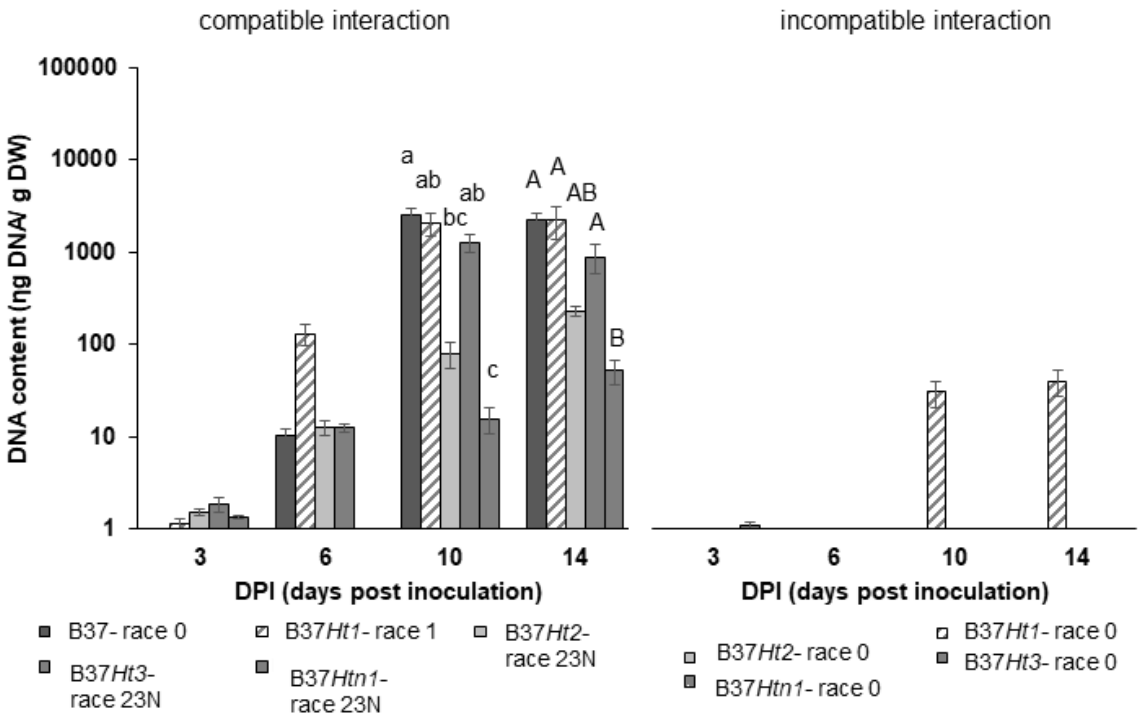
Supplementary table 1. Results from race phenotyping of plants used for *Exserohilum turcicum* DNA quantification and histological studies.

Isolate	Race	B37*Ht1*	B37*Ht2*	B37*Ht3*	B37*Htn1*
172-4	0	A	A	A	A
138-1	1	V	A	A	A
123-3	23N	A	V	V	V

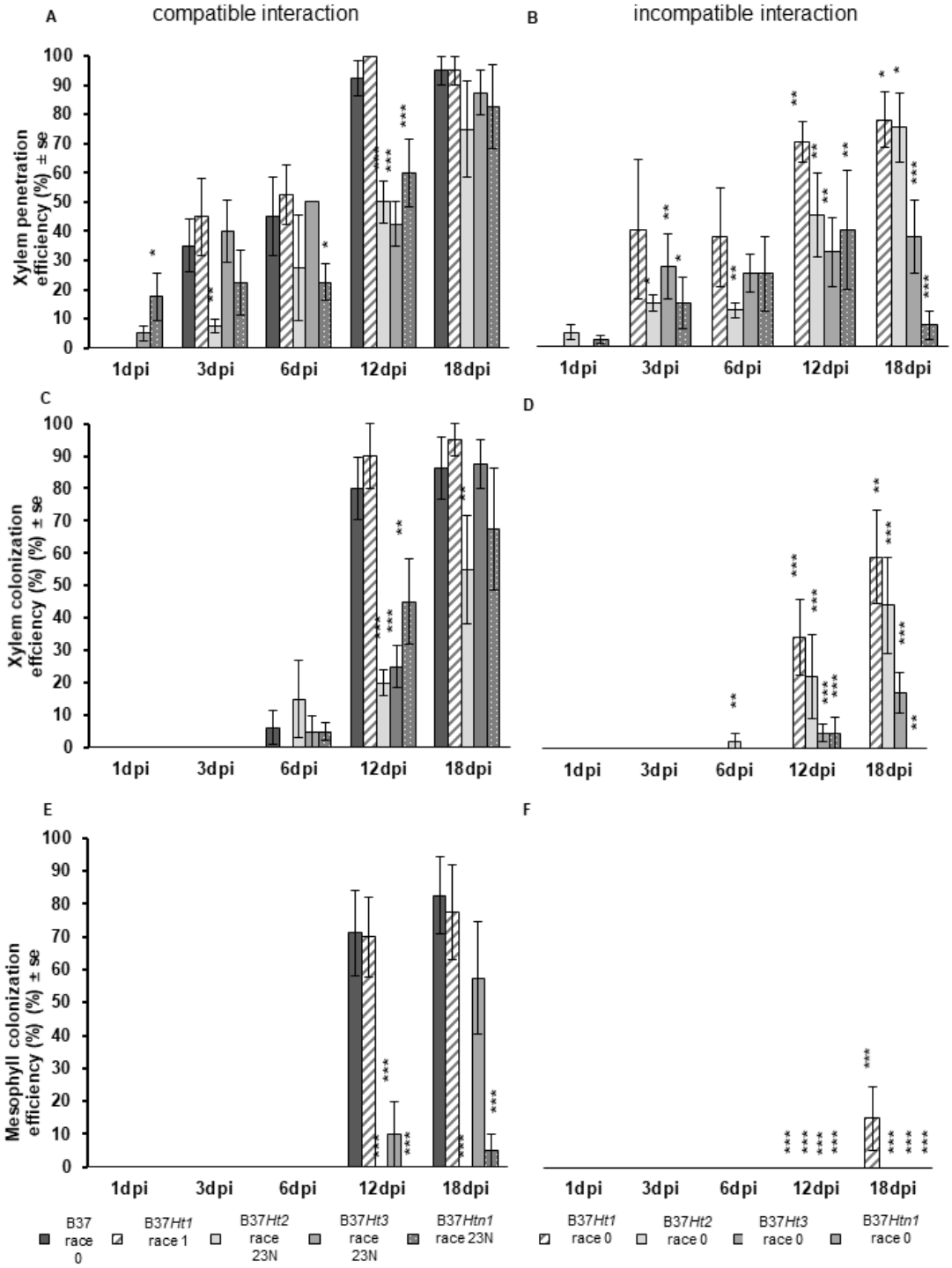
A – avirulent and V - virulent.



Supplementary figure 1. Disease severity and standard error for compatible and incompatible interactions between *Exserohilum turcicum* and the maize lines B37 without *Ht*-resistance genes and those with resistance genes *Ht1, Ht2, Ht3,* and *Htn1*. Disease severity was evaluated 14 days post inoculation (dpi). The graph presents data from the second replication. Data from each line with *Ht*-resistance genes was compared with B37 by Mann-Whitney-U test (* p-value ≤ 0.05, ** p-value ≤ 0.01, *** p-value ≤ 0.001).



Supplementary figure 2. DNA-contents with standard errors for compatible and incompatible interactions between *Exserohilum turcicum* and the maize lines B37 without *Ht*-resistance genes and those with resistance genes *Ht1*, *Ht2*, *Ht3,* and *Htn1*. Samples for qPCR-analysis were collected 0, 3, 6, 10 and 14 days post inoculation (dpi). The DNA content is presented in ηg DNA / g leaf dry weight. Only data from the second replication experiment are shown in the graph. Lowercase letters indicate significant differences between treatments at 10 dpi. Uppercase letters indicate significant differences between treatments at 14 dpi. Means sharing the same letter were not significantly different following Tukey-adjusted comparisons for data with a log-transformation (p-value ≤ 0.05).



Supplementary figure 3. Data from the second repetition for the effect of resistance genes *Ht1*, *Ht2, Ht3,* and *Htn1* in the maize line B37 on xylem penetration efficiency (XPE), xylem colonization efficiency (XCE), and mesophyll colonization efficiency (MCE) of *Exserohilum turcicum* compared between compatible (A,C,E) and incompatible (B,D,F) interactions. Samples were collected 1, 3, 6, 12, and 18 days after inoculation (dpi) with one isolate for each interaction. Data is from 10 penetration sites in four leaf segments (n=40). Data from each line with *Ht* resistance genes was compared with B37 by Chi-square test (* *p*-value ≤ 0.05, ** *p*-value ≤ 0.01, *** *p*-value ≤ 0.001). Bars indicate the standard error.

Chapter 4. Physiological and biochemical responses of maize lines carrying *Ht*-resistance genes against *Exserohilum turcicum*

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Abstract

Northern corn leaf blight (NCLB) is an important maize disease of worldwide importance, that is caused by the ascomycete *Exserohilum turcicum*. One of the main methods applied for NCLB control is the cultivation of resistant hybrids. Resistance derives from qualitative resistance genes in maize lines, the so called *Ht* genes. Maize lines carrying *Ht1* display necrotic lesions with chlorosis, *Ht2* displays chlorosis and small lesions, *Ht3* displays chlorotic spots and *Htn1* may not show any lesions or display wilt-type lesions. The objective of this study was to phenotypically characterize the differential resistance responses conferred by *Ht1*, *Ht2*, *Ht3* and *Htn1* and to evaluate effects on biomass yield under controlled conditions. In addition, physiological, biochemical and epidemiological parameters were studied in the differential set of line B37 carrying individual *Ht* resistance genes and for a commercial hybrid, which carries *Ht1*. Plants were inoculated with a race 0 isolate of *E. turcicum* conferring compatible interaction with B37 and incompatible interaction with plants carrying an *Ht* resistance gene. Five days post inoculation (dpi), the resistant lines displayed a reduction in CO_2 assimilation of 66 to 84% compared to healthy plants. At 14 dpi, line B37*Ht1* showed a decrease in CO_2 assimilation of 81%, similar to B37. Under controlled conditions and low disease severity (< 15% diseased leaf area), no reduction in dry weight of the above-ground biomass was detected for any of the lines (including the compatible interaction with B37) at 28 dpi. The healthy tissue seems to compensate for photosynthetic costs associated with the activation of resistance mechanisms in the incompatible interaction, as observed for B37*Ht2*, B37*Ht3,* B37*Htn1* and Scorpion. The extensive chlorosis combined with necrosis expressed in B37*Ht1* explains the low photosynthetic activity demonstrated in this line. At 6 dpi, low H_2O_2 detection and high peroxidase activity were observed in B37*Ht1*, in contrast to B37, indicating that this resistant line has different responses to pathogen infection when compared to the susceptible line. In addition, resistance in B37*Ht1,* B37*Ht2* and B37*Htn1* lead to a sporulation rate decreased by ~90%, whereas in B37*Ht2* this effect was associated with a

lower number of lesions. Our study confirms that resistance mechanisms of different *Ht* genes are distinct from each other resulting in different physiological responses of the plant. It is therefore worth conducting further studies to elucidate the defence mechanisms conferred by *Ht* genes.

Keywords: chlorophyll fluorescence, chlorophyll degradation, epidemiological parameters, gas exchange, northern corn leaf blight, peroxidase activity, plant-pathogen interactions, R genes*, Setosphaeria turcica*

Introduction

Northern corn leaf blight (NCLB), caused by the ascomycete *Exserohilum turcicum* (teleomorph *Setosphaeria turcica*), is one of the main foliar diseases in maize (*Zea mays*), (Galiano-Carneiro and Miedaner, 2017) and spread across maize-producing regions worldwide (CABI, 2019). The disease can be controlled by fungicide application or cultivation of resistant cultivars (Hooda *et al.*, 2017). Yield losses caused by *E. turcicum* in maize vary greatly according to the host developmental stage at which the infection occurs, the disease control strategies applied and the environmental conditions (Pataky *et al.*, 1988; Adipala *et al.*, 1993). When infection occurs before silking (flowering stage) and the cob leaf is affected, high yield losses caused are observed (Fajemisin and Hooker, 1974; Raymundo and Hooker, 1982; Welz, 1998; Ding *et al.*, 2015; Galiano-Carneiro and Miedaner, 2017). *E. turcicum* can cause yield reductions in resistant cultivars and susceptible cultivars of up to 17% and 63%, respectively (Raymundo and Hooker, 1982). In addition, *E. turcicum* causes higher yield losses in hybrids carrying qualitative resistance than in hybrids carrying quantitative resistance (Ullstrup, 1970). Leaf infection caused by *E. turcicum* is favored by moderate temperatures between 15 and 25°C, and frequent dew periods of at least 4 h and 90–100% relative humidity (Levy and Cohen, 1983b; Bentolila *et al.*, 1991; Ogliari *et al.*, 2005; Galiano-Carneiro and Miedaner, 2017). Under these conditions, maize yield losses caused by *E. turcicum* of up to 70% have been reported (Ullstrup and Miles, 1957).

Infection by *E. tucicum* in maize leaves begins with the pathogen conidia landing on the leaf surface and subsequent germination. Appressorium formation can usually be observed at the end of the germ tube 3 hours after conidia have attached to the leaf in the presence of high relative humidity (>95% RH) (Levy and Cohen, 1983a). Due to pressure exerted by the appressorium and secretion of lytic enzymes, the pathogen is able to penetrate directly through the epidermis (Knox-Davies, 1974; Kotze *et al.*, 2019). In the mesophyll, the hypha invaginates the membrane and forms an intracellular vesicle (Hilu and Hooker, 1964). This fungal structure is responsible for nutrient assimilation in the initial phases of infection (Hilu

and Hooker, 1964; Knox-Davies, 1974).Thus, the adjacent cells (vascular bundle sheath cells) are colonized until the hypha reaches the xylem vessel (Muiru *et al.*, 2008; Kotze *et al.*, 2019) and secretes monocerin (Robeson and Strobel, 1982; Cuq *et al.*, 1993) and HT-toxin (from *Helminthosporium turcicum*) (Bashan *et al.*, 1995; Li *et al.*, 2016). Monocerin is a non host-specific toxin that causes necrosis in maize, Johnson grass and cucumber, and is produced during compatible and incompatible interaction. Monocerin induces mortality of cells and protoplasts (Robeson and Strobel, 1982; Cuq *et al.*, 1993). The HT-toxin is host-specific and known to inhibit chlorophyll formation (chlorosis), to disturb the permeability of cell membrane, to increase lesion size and to overexpress a QM-like protein, which is related to stress response pathways (Bashan and Levy, 1992; Bashan *et al.*, 1995; Dong and Li, 1996; Dong *et al.*, 2001; Wang *et al.*, 2010).

Once inside the xylem vessel, hyphae grow and the pathogen starts colonizing the mesophyll. At this stage, the pathogen forms reproductive structures, which are important for disease spread over the field and the start of a new disease cycle (Kotze *et al.*, 2019). The incubation period of NCLB is 12–14 days and typical NCLB symptoms are long (up to 30 cm) elliptical grey lesions, which become necrotic in later disease stages when these lesions can coalesce and the entire leaf becomes blighted. Under high relative humidity, necrotic lesions sporulate and conidia are dispersed by rain and wind (Hooda *et al.*, 2017). *E. turcicum* survives in maize debris and can form chlamydospores as survival structures (Boosalis *et al.*, 1967).

Several genes that confer resistance in maize to *E. turcicum* are known (Hooker, 1963; Gevers, 1975; Hooker, 1977, 1981) and these genes have been extensively used by maize breeding programs worldwide. In general, maize lines carrying the resistant gene *Ht1* show necrotic lesions with chlorosis, lines carrying the *Ht2* gene display chlorosis and small lesions, lines carrying the *Ht3* have only chlorotic spots, and lines with *Htn1* show no lesions or wilt-type lesions when inoculated with *E. turcicum* (Hooker, 1963; Gevers, 1975; Hooker, 1981; Navarro *et al.*, 2020). In addition, molecular studies demonstrate that *Htn1* encodes wall associated receptor-like kinases (RLKs) and, is therefore able to recognize pathogen invasion and cell wall disruption (Hurni *et al.*, 2015).

Plant pathogen invasion is recognized by the plant immune system in incompatible interactions (Jones and Dangl, 2006). The recognition of microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) by transmembrane pattern recognition receptors (PRR proteins) results in accumulation of reactive oxygen species (ROS) (Malinovsky *et al.*, 2014), which will reprogram transcriptomes (Kachroo *et al.*, 2017). The accumulation of toxic substances, such as hydroxide peroxide (H_2O_2) and superoxide (O_2^-) leads to a

hypersensitive response (HR). Under higher concentrations of H_2O_2 and O_2^- in the host tissue, ROS may provoke programmed cell death (PCD) and hinder pathogen colonization in the infected tissue (Apel and Hirt, 2004). The imbalance between ROS production and ROS detoxification may cause PCD, and PCD is only effective if ROS scavenging enzymes, such as peroxidase, superoxide dismutase, catalase and glycolate oxidase, are suppressed in the apoplast (Apel and Hirt, 2004). In susceptible maize plants inoculated with *E. turcicum*, the content of H_2O_2 was higher at 15 and 20 dpi (Silveira *et al.*, 2019). In addition, the peroxidase activity was higher for resistant plants carrying the *Htn1* gene at 3 and 6 dpi (Shimoni *et al.*, 1991). Moreover, an increase in gene expression and activity of superoxide dismutase, catalase, ascorbate peroxidase, peroxidase, glutathione reductase, glutathione-S-transferase, transferase and glutathione peroxidase, enzymes related to antioxidant metabolism, was observed in susceptible plants (Shi *et al.*, 2018; Silveira *et al.*, 2019).

Pathogen invasion can lead to changes in several physiological processes in plants, such as photosynthesis, sugar translocation, and accumulation of carbohydrates (Berger *et al.*, 2007). Among these processes, photosynthesis is one of the first plant physiological processes affected by pathogen infection. The necrosis symptoms on leaves caused by pathogen colonization or strong plant defense responses decreases the radiation use efficiency on leaves and reduce the leaf net photosynthetic rate (Boote *et al.*, 1983). However, pathogens can cause disturbances in plant leaves that go beyond the necrotic area and affect the green tissue surrounding the lesions (Bastiaans, 1991). In these areas, the diffusive pathways of CO_2 are affected by stomatal closure or by reduction of CO_2 diffusion in plant cell mesophyll (Shtienberg, 1992; Bassanezi *et al.*, 2001; Nogueira Júnior *et al.*, 2017). Water vapor exchange is also affected since the stomatal processes are disturbed. The production of pathogen toxins impairs photochemical and biochemical processes in photosynthesis of green leaves by reducing the efficiency of the photosynthetic pigments or reducing the activity of photosynthesis enzymes such as Rubisco (Bassanezi *et al.*, 2001). However, the effect of pathogens on the photosynthesis of green areas surrounding lesions is not always negative. Some pathogens, such as rusts, can modify the metabolism of regions near pustules and increase CO_2 assimilation in so-called “green islands” (Walters *et al.*, 2008). Fungal diseases change the movement of assimilates according to the trophic interactions between pathogens and plants. In the biotrophic phase, fungi manipulate plant carbohydrate metabolism, redirecting plant sugars for their own needs, by inducing a physiological sink for assimilates (Zadoks and Schein, 1979). In the necrotrophic phase, pathogens infect living tissues, but mainly derive their nutrients from tissue killed, usually by toxins, in the course of colonization (Whipps and Lewis, 1981). Fungi of both trophic patterns can cause a decrease in the export of photoassimilates from

infected leaves, but biotrophic/hemibiotrophic pathogens generally increase the import of photosynthetic products to the infected tissues (Whipps and Lewis, 1981; Owera *et al.*, 1983; Nogueira Júnior *et al.*, 2017). The sum of the effects of pathogens on light interception, CO_2 diffusion and assimilation, carbohydrate metabolism and transport will eventually affect the plant growth and yield build-up (Boote *et al.*, 1983). Impairments on photosynthesis were observed in susceptible maize plants infected by *E. turcicum.* Photosynthetic efficiency is reduced in the course of disease development (Silveira *et al.*, 2019). The reduction is not restricted to the necrotic leaf tissue in lesions, but also in leaf tissue adjacent to the lesions. *E. turcicum* also affects the translocation of photosynthates causing the import of photoassimilates from healthy leaf tissue into lesions. This further reduces the pool of photosynthates available for dry matter increment (Levy and Leonard, 1990).

Damage to leaf photosynthesis during the first stages of infection is also observed in incompatible interactions and may be considered as metabolic costs of resistance (Berger *et al.*, 2007). In the incompatible interaction between tobacco and *Phytophthora nicotianae,* plant defense mechanisms against pathogens induce changes in carbohydrate metabolism, such as decrease on sucrose efflux. This shift in plant metabolism is related to increases in apoplastic invertase activity and an early blockage of intercellular sugar transport by callose deposition (Scharte *et al.*, 2005). Grapevine cultivars resistant to *Plasmopara viticola* showed high reductions in the photosynthetic rate and stomatal conductance a few hours after the pathogen inoculation. Damage to photosynthesis was associated with plant responses to pathogen infection such as the oxidative burst (Nogueira Júnior *et al.*, 2020). A similar pattern has been observed in the incompatible interaction between barley *and Erysiphe graminis* f. sp. *hordei*, where pathogen recognition occurs during haustoria development in host cells. After recognition, several histochemical and biochemical alterations are induced in hosts and those responses may cause damage to photosynthesis of resistant cultivars faster than in susceptible cultivars (Smedegaard-Petersen and Tolstrup, 1985).

The resistance expressed by *Ht* genes has been not well described at physiological, biochemical and epidemiological levels. Distinct resistance reactions on the maize line B37 may cause different effects on plant host physiology. Therefore, the main objective of this study was to compare physiological and biochemical responses in the maize line B37 carrying *Ht1*, *Ht2*, *Ht3* and *Htn1* resistance genes, in order to better understand resistance responses conferred by these genes. For this purpose, experiments were conducted under controlled conditions, and gas exchange, chlorophyll fluorescence and peroxidase activity were measured in non-inoculated and *E. turcicum*-challenged plants. In addition, the effect

of resistance genes on epidemiological components, such as the number of lesions, lesion length and sporulation, was investigated. Moreover, plant biomass was assessed to elucidate whether the respective plant-pathogen interaction not only influences plant physiology directly but also the attainable biomass yield. To further ensure the general validity of the present study, a commercially available maize hybrid, “ES Scorpion”, carrying *Ht1* resistance was included in the studies.

Material and Methods

Plant material and inoculation

Maize plants from the differential set of the line B37 without a qualitative resistance gene and with the resistance genes *Ht1*, *Ht2*, *Ht3*, *Htn1*, and the hybrid cultivar Scorpion, which carries the *Ht1* gene (see supplementary material), were cultivated in the greenhouse at 24 ± 3°C, 70% air humidity and a day/night light regime of 14/10 h. Two seeds per pot (11 cm x 11 cm x 10 cm) were sown in a mixture of substrate, compost and sand (3:3:1). Seeds were provided by KWS Saat SE (Einbeck, Germany). The *E. turcicum* isolates used for inoculation are from the collection of the Institute of Plant Pathology and Crop Protection of the University of Göttingen. All isolates had been race determined in previous works (Hanekamp, 2016; Navarro *et al.*, 2021) and conidia were stored in fresh glycerin (25%) cultures. Glycerin cultures were preserved for up to two years until they were transferred to Petri dishes containing V8 medium (75 ml V8 vegetable juice, 1.5 g $CaCO_3$). The plates were kept at 24°C in the dark for at least 21 days. Conidia were harvested by washing the plates and inoculum suspensions were prepared in concentrations of 750 to 3,000 spores ml^{-1}, according to the experiment. Maize plants were inoculated when the fifth and sixth leaves were unfolded, around 30 days after sowing, using a sprayer. Each plant received around 7 ml of the conidia suspension until the point of run-off was reached, and control plants were sprayed with water. After inoculation, all plants were maintained in high-humidity conditions (> 95°C) for 24 hours. The lines B37*Ht1*, B37*Ht2*, B37*Ht3* and B37*Htn1* were inoculated with a race 0 isolate, which is avirulent on these resistance lines, and leads to an incompatible interaction. The same isolate race 0 was inoculated in B37 without resistance genes, resulting in a compatible interaction. The hybrid Scorpion was inoculated with both an avirulent (race 0) and virulent (race 1) isolate. This procedure was applied in all experiments (gas exchange, biochemical responses, epidemiology and biomass).

Leaf gas exchange and chlorophyll fluorescence

Three experiments were performed to assess the effect of resistance responses conferred by *Ht* genes on *E. turcicum* on gas exchange and chlorophyll fluorescence of maize plants

from the differential set of B37 and the maize hybrid Scorpion. In Experiment 1, gas exchange variables were measured in three healthy and three inoculated plants (n = 3 biological replicates) from each line and evaluation date. The inoculum concentration used in the gas exchange experiment was set to 1,500 spores ml^{-1}. After inoculation, plants were maintained in a climate chamber under controlled conditions. The day/night regime was 14 h light/10 h dark, a light intensity of 120 ± 10 µmol m^{-2} s^{-1}, a temperature of 25/20°C day/night and air humidity of 70%. Gas exchange measurements were performed at 5 and 14 days post inoculation (dpi). Measurements of leaf CO_2 assimilation (A), stomatal conductance (gs), transpiration (E) and intercellular CO_2 concentration (Ci) were performed using a portable infrared gas analyzer (GFS-3000, Heinz Walz GmbH, Germany) equipped with a fluorometer (3010-S, Heinz Walz GmbH, Germany). The cuvette size was 4 cm^2. The measurements were performed with an air CO_2 concentration (Ca) of 400 ppm CO_2, 60% relative humidity, and photosynthetic active radiation (PAR) of 1000 µmol m^{-2} s^{-1}. Measurements were performed between 9:00 h and 16:00 h. The instantaneous carboxylation efficiency (k) was calculated as A/Ci (Machado *et al.*, 2005). The percentage of disease severity was quantified in the area covered by the portable infrared gas analyzer chamber. The diseased area was estimated by the software Assess 2.0 (Lakhdar Lamari, 2008, APS, USA). The relative values of A, gs, E and k, were calculated by considering the ratio of the variable values collected from inoculated plants (Yx) and to the mean values of healthy plants (Yo), in each B37 line, in order to compare the effect of *E. turcicum* between the lines, obtaining the following variables: relative photosynthesis (Px/Po), relative transpiration (Ex/Eo), relative stomatal conductance (gsx/gso) and relative instantaneous carboxylation efficiency (kx/ko). The experiment was replicated once.

In Experiment 2, chlorophyll fluorescence variables were measured in three healthy and three inoculated plants (n = 3 biological replicates) from each line and evaluation date. Chlorophyll fluorescence measurements were performed at 5 and 14 days post inoculation (dpi) using a fluorimeter (FluorCam 2.0, Photon Systems Instruments, Czech Republic) and applying the “quenching” protocol. The quantum yield of photosystem II (QY = Fv / Fm), where Fv is the fluorescence increment due to the transition from a dark-adapted state with all-open reaction centers to an all-closed state during a saturating flash of light, and Fm is the maximum fluorescence in the dark-adapted state, and the non-photochemical quenching relaxing in the dark (nPQ), which is the conversion of excitation energy into thermal energy, were measured on the symptomatic area in inoculated plants. Plants were pre-adapted to dark conditions for at least 2 h before realizing the chlorophyll fluorescence measurements. The relative values of QY and nPQ were calculated by considering the ratio of the variable values collected from inoculated plants (Yx) and to the mean values of

healthy plants (Yo), in each B37 line, obtaining the following variables: relative quantum yield (QYx/QYo) and relative non-photochemical quenching (nPQx/nPQo). The experiment was replicated once.

An additional experiment (Experiment 3) was performed with the commercial maize hybrid Scorpion. Plants were inoculated with the isolate race 0 (incompatible interaction) and with the isolate race 1 (compatible interaction). Plants sprayed with water served as control plants (n = 3 biological replicates). The inoculum concentration was 1,500 spores ml^{-1}. After inoculation, the plants were maintained in a climate chamber under controlled conditions as described above. Gas exchange measurements were performed at 5 and 14 dpi and the variables A, gs, E, Ci and k were obtained as in the experiment with the differential set B37.

Peroxide (H_2O_2) detection and peroxidase activity (POX)

One experiment was performed to investigate the histochemical detection of peroxide (H_2O_2) and superoxide (O_2^-), using light microscopy, and a second experiment was conducted to measure the biochemical peroxidase activity (POX) in maize plants from the differential set B37 inoculated with *E. turcicum.* In the experiment conducted to detect H_2O_2 and O_2^-, three healthy and three inoculated plants (n = 3 biological replicates) from each line were evaluated. The plants were inoculated with 3,000 spores ml^{-1} and samples were collected at 1, 3, and 6 dpi. Two leaf segments of 6 cm^2 per plant were cut from the fifth unfolded leaf. One segment was placed in a solution of 10 mM phosphate buffer (pH 3.8 – adjusted with HCl) and 0.1% DAB (3,3-diaminobenzidin) and the second leaf segment was placed in a solution of 10 mM phosphate buffer (pH 7.8) and 0.01% NBT (nitroblue tetrazolium). After one hour in the dark, leaf segments were washed with demineralized water and maintained under direct light for one hour. In the next step, the leaf segments were transferred into a destaining solution of 0.15% trichloroacetic acid (TCA) and ethanol:chloroform (4:1). Microscope analysis was realized using 50% glycerin as the mounting media. A positive peroxide localization was considered when the penetration site was a dark showing brownish color (Figure 1) and a positive superoxide localization was considered when it showed a blue color (Hückelhoven *et al.*, 2000). The percentage of H_2O_2 and O_2^- detection was calculated by considering the ratio of the number of stained penetration sites to the number of total penetration sites visualized on each leaf segment (biological replicate). The experiment was replicated once.

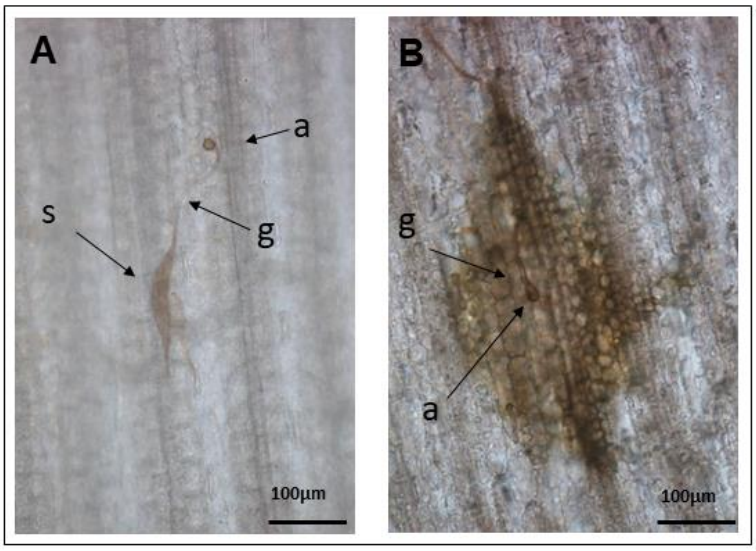

Figure 1. Peroxide detection (H_2O_2) by histochemical staining with DAB (3,3-diaminobenzidin). The infection was considered when the spore (s) presented a germinate tube (g) and an appressorium (a) (A). Peroxide detection was considered when a brownish color was observed surrounding the appressorium (B).

A second experiment was performed to measure peroxidase (POX) activity in maize plants from the differential B37 set inoculated with *E. turcicum.* Three healthy and three inoculated plants (n = 3 biological replicates) from each line were evaluated. The plant were inoculated with 3,000 spores ml^{-1} and the fourth and fifth leaf of each plant were harvested and then pooled into one biological replicate. Three plants were harvested per treatment (n = 3 biological replicates), making a total of 90 harvested plants per experiment. The samples were collected at 1, 3, and 6 dpi. Leaf segments were harvested and directly frozen in liquid nitrogen. The samples were ground with a mortar and pestle and then stored at -80°C. The extract was prepared with 50 mg of leaf sample in addition to 500 µl of cold 15 mM sodium phosphate buffer (pH 6.0). After centrifugation (10000 *g*, 10 min, 4°C), 100 µl of the supernatant was added to a cuvette and mixed with 1500 µl of assay mix. The assay mix was composed of 15 nM sodium phosphate buffer, pH 6.0, 0.0066% of hydrogen peroxide (1100 µl l^{-1} of 30% H_2O_2) and 0.0066% (660 µl l^{-1}) of O-methoxyphenol (guaiacol) (Shimoni *et al.*, 1991). The assay mix was prepared every two hours. The oxidative reaction between guaiacol and H_2O_2 was catalyzed by peroxidase and produced the brownish tetraguaiacol (Schopfer, 1989). The increase in absorbance was recorded at 470 nm for 4 min (2 measurements s^{-1}) using a spectrophotometer (Specord 40 – Analitik Jena, Germany).
The protein content was measured using the Bradford assay (Bradford, 1976). One aliquot of 50 µl from sample extract was pipetted onto a 96-well plate and 200 µl of Bradford solution was added (Roti® -Quant solution – Carl ROTH®, Karlsruhe, Germany). The

Bradford solution was composed of 0.01% (w/v) Coomassie Brilliant Blue G-250 dissolved in 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. The Coomassie Brilliant Blue dye bonded to the protein and the protein-dye complex was dispersed in the solution. After 15 min of reaction, the absorbance was measured at 595 nm by a photometer (μQuant BioTek Instruments, INC). The standard curve was prepared with bovine serum albumin in concentrations of 10, 20, 40, 60, 80 and 100 μg mL^{-1}. The protein content was measured in mg protein ml^{-1}.

The enzyme activity was calculated by the Beer-Lambert law: $Abs_\lambda = \varepsilon_\lambda c\, l$; where Abs_λ is the absorbance or optical density, ε_λ is the extinction coefficient (for tetraguaiacol: $\varepsilon_{\lambda=470nm} = 2.66\ 10^7\ cm^2\ mol^{-1}$) (Chance and Mahehly, 1955), c is the concentration of the reaction product in the solution, in this case the enzyme activity (mol ml^{-1} min^{-1} – expressed by nKatal ml^{-1}), and *l* is the optical path length (calculations can be followed in Appendix 2). The enzyme activity was divided by the protein content and expressed in nkat*mg protein fresh $weight^{-1}$ (Schaffrath *et al.*, 1995). The experiment was replicated once.

Epidemiological components

Epidemiological components in maize plants from the differential set of the line B37 inoculated with *E. turcicum* were measured in two experiments. One experiment was conducted to estimate pathogen sporulation and the other to estimate the number of lesions and lesion growth. In order to reduce the effect of pathogen aggressiveness, three isolates were inoculated per line. Eight plants were sampled per treatment (line x isolate), giving 24 sampled plants per line (n = 8 biological replicates). The inoculum concentration was set to 750 spores ml^{-1} for the experiment to evaluate the number of lesions and lesion length, and to 1,500 spores ml^{-1} for the experiment to determine sporulation. The sporulation experiment was repeated once. The number of lesions was counted on the third, fourth, fifth and sixth leaves at 13 dpi. The length of three lesions per leaf was measured in the fourth and fifth leaves at 15 dpi. Leaf pieces of 6 cm^2 (3 cm x 2 cm) from the fifth leaf of the eight inoculated plants were sampled at 14 dpi to estimate sporulation. The transition between green and chlorotic/necrotic areas was chosen as the sampling area. Leaf samples were placed in a humid chamber, i. e. plastic boxes containing moistened filter paper to maintain high humidity and stimulate conidia production. Pictures of the sampled area were used for quantification of the diseased area. Leaf pieces were measured using the software Image J1.52a (Wayne, Rasband, National Institute of Health, USA) and the percentage of diseased area was estimated using the software Assess 2.0 (Lakhdar Lamari, 2008, APS, USA). After three days under high-humidity conditions, each leaf piece was placed in a

Falcon tube containing 4 ml of distilled water and the surfactant Silwet Gold® at 125 ppm. The Falcon tubes were frozen at -20°C for further procedures. Sporulation was measured after mixing the sample and placing one aliquot of 50 µl from the spore suspension in a Fuchs-Rosenthal chamber. The spore concentration was calculated by the average of the three aliquots and divided by the diseased area.

Leaf area and plant biomass estimation

Two experiments were performed, one with the differential set of the line B37 and the other with the commercial maize hybrid Scorpion, to estimate leaf area and plant biomass of healthy and *E. turcicum* inoculated plants at 14 and 28 dpi. In the experiment using the differential set of the line B37, two plants were pooled in one biological replicate, making a total of four biological replicates per line, treatment and time (n = 4 biological replicates). In total, 160 plants were sampled per experiment. The experiment was replicated once. The inoculum concentration was set to 1,500 spores ml^{-1}. After inoculation, plants were maintained in the greenhouse under conditions of 16 h light/8 h dark, a light intensity of 600 ± 200 µmol m^{-2} s^{-1} and a temperature of 25±5°C. Before sampling, the disease severity of all leaves was evaluated based on a diagrammatic scale (Pataky, 1992). The disease severity for each plant was calculated by the average of all leaves. The leaf area (LA) was measured using an area meter (LI3100C, LI-COR Biosciences, US). The dry weights of the aerial (Da) and root (Dr) biomass were measured. The relative Da, Dr, and LA were calculated by the ratio of the variable values collected from inoculated plants (Yx) and to the mean values of healthy plants (Yo), obtaining the following parameters: relative aerial biomass (Dax/Dro), relative root biomass (Drx/Dro) and relative leaf area (LAx/LAo).

In the experiment using the commercial maize hybrid Scorpion, eight plants were inoculated with the isolate race 0 (incompatible interaction), eight plants were inoculated with the isolate race 1 (compatible interaction) and eight plants were sprayed with water to serve as control plants. Two plants were pooled in one biological replicate, resulting in four biological replicates per line, treatment and time (n = 4 biological replicates). The inoculum concentration was 1,500 spores ml^{-1}. After inoculation, plants were maintained in the greenhouse under conditions of 16 h light/8 h dark, a light intensity of 600 ± 200 µmol m^{-2} s^{-1}, and a temperature of 25±5°C. Before sampling, the disease severity of all leaves was evaluated based on a diagrammatic scale (Pataky, 1992). The disease severity for each plant was calculated by the average of all leaves. The leaf area (LA) was measured using area meter (LI3100C, LI-COR Biosciences, US). The dry weights from the aerial (Da) and root (Dr) biomass were measured as in the experiment with the differential set of the line B37.

Data analysis

Data analysis was performed using R (version 3.6.0) (R core team 2019). Mixed model analysis was performed using the package lmer. Data from the relative values measured by gas exchange (Px/Po, Ex/Eo, gsx/gso, kx/ko) for each time point (5 and 14 dpi) were fitted to a mixed model with maize line as the fixed factor and experiment replication as the random effect. Data regarding disease severity at 14 dpi from both experimental replicates had homogeneity of variance applying the Bartlett's test and therefore were combined in the analysis. Maize lines were compared by analysis of variance (ANOVA) and by multiple comparison applying Tukey's test (p-value ≤ 0.05). For the maize hybrid Scorpion, the absolute values A, E, gs, and k were compared between treatments (healthy plants, compatible interaction, and incompatible interaction) by analysis of variance (ANOVA) and by multiple comparison applying Tukey's test (p-value ≤ 0.05).

Data on the percentage of penetration sites with H_2O_2 localization and POX activity were compared between lines for each time point (1, 3 and 6 dpi). Data of the percentage on penetration sites with H_2O_2 detection were fitted to a mixed model with line as the fixed factor and experiment replication as the random effect. An analysis of variance (ANOVA) was performed and lines were compared by multiple comparison applying Tukey's test (p-value ≤ 0.05).

Data on the number of lesions, lesion length and sporulation from all three inoculated isolates were combined for the analysis to verify the effect of the line on the epidemiological components. The number of lesions and lesion length were analyzed using the non-parametric Kruskal-Wallis test and Dunn's test for multiple comparisons (p-value ≤ 0.05). Data on sporulation were Box-Cox transformed and fitted to a linear mixed model, with line as fixed factor and experiment replications as random effect compared by multiple comparison using Tukey's test (p-value ≤ 0.05).

Data from the relative values of leaf area, plant biomass (Dax/Dao, Drx/Dro, Lax/LAo) and disease severity for each time point (14 and 28 dpi) were fitted to a mixed model with line as fixed factor and experiment replication as random effect. Maize lines were compared by analysis of variance (ANOVA) and by multiple comparison applying Tukey's test (p-value ≤ 0.05). For the maize hybrid Scorpion, absolute values of dry weight from the aerial (Da) and leaf area (LA) and the disease severity were compared between treatments by analysis of variance (ANOVA) and by multiple comparison applying Tukey's test (p-value ≤ 0.05). Experiments with the hybrid Scorpion were conducted once.

Results

Leaf photosynthesis impairments and chlorophyll fluorescence

In general, the lines B37, B37*Ht1*, B37*Ht2*, B37*Ht3* and B37*Htn1* showed distinct symptoms for each incompatible interaction (Figure 2). Maize lines carrying the *Ht1* gene showed strong necrotic lesions surrounded by chlorosis, *Ht2* showed chlorosis and small lesions, *Ht3* showed chlorotic spots and *Htn1* showed no lesions or wilt-type lesions in some plants. Disease severity in the measured area was higher for B37 and B37*Ht1* with an average of 33% and 24%, respectively (Figure 3). For the lines B37*Ht2* and B37*Ht3*, the disease severity was lower than 1%. The line B37*Htn1* showed an average of 3% of disease severity in the leaf area were gas exchange was measured. Differences in values of CO_2 assimilation (A) were also observed in healthy plants, and the lines B37*Ht3* and B37*Htn1* had the lowest values of A on both evaluated days (5 and 14 dpi) in both experiment replicates, with average values of A of 10.4 and 11.2 µmol CO_2 m^{-2} s^{-1}, respectively. Healthy plants from B37 and B37*Ht2* lines showed the highest A with average values of 16.4 and 17.2 µmol CO_2 m^{-2} s^{-1}, respectively. The average value of A for the healthy plants of the line B37*Ht1* was 12.7 µmol CO_2 m^{-2} s^{-1}, demonstrating no significant differences from the other lines (Supplementary figure 1).

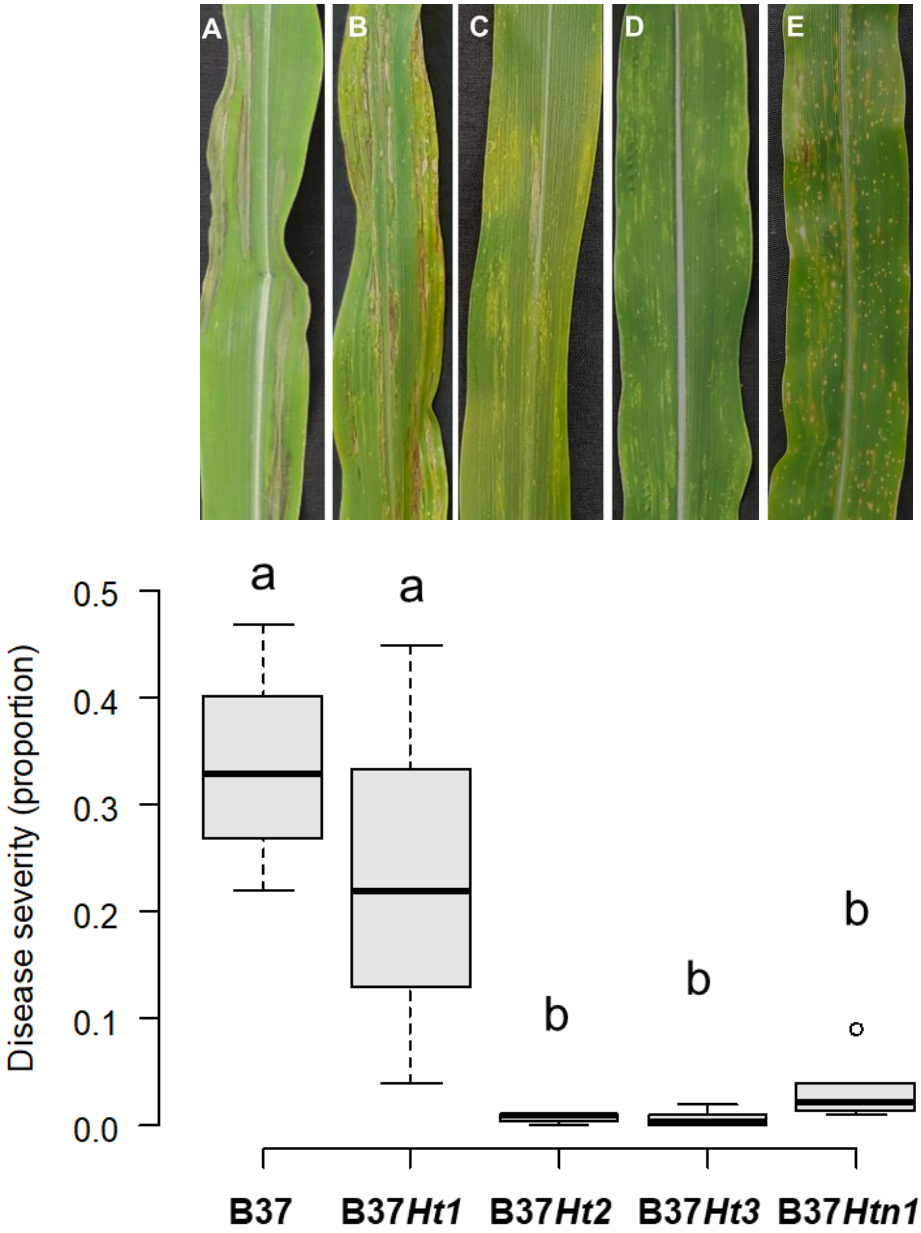

Figure 2. Maize lines B37 (A), B37*Ht1* (B), B37*Ht2* (C), B37*Ht3* (D), and B37*Htn1* (E) inoculated with *Exserohilum turcicum* at 14 days post inoculation. All plants were inoculated with a race 0, which confers a compatible interaction with the line B37 and an incompatible interaction with B37*Ht1*, B37*Ht2*, B37*Ht3* and B37*Htn1*. Disease severity of the leaf area used of gas exchange measurements measured at 14 days post inoculation (F). The lower quantile represents 0.25 and the upper quantile 0.75 of the data range. Data from both experiments are combined (n = 6 biological replicates). Samples were compared by analysis of variance (ANOVA) followed by Tukey's test (*p*-value ≤ 0.05) as a multiple comparisons test. Lines that differ between letters are not statistically significant using Tukey's test (*p*-value ≤ 0.05). Dots above the boxes are outliers calculated by the package ggplot2 in R.

In addition, differences in relative net photosynthetic rate (Px/Po), transpiration (Ex/Eo), stomatal conductance (gsx/gso), and carboxylation efficiency (kx/ko) between lines were observed (Figure 4). At 5 dpi, the average reduction of A values in inoculated plants of the compatible interaction (susceptible) of B37 was 38%, whereas reductions of 66 to 71% were recorded in inoculated plants bearing *Ht* genes (incompatible interactions). At 14 dpi, when fungal colonization is typically established, the values of A in the susceptible line were reduced by 84% in the inoculated plants compared to the healthy plants of B37. Inoculated plants from the resistant line B37*Ht1* demonstrated a similar reduction when compared to the susceptible line with an A value of 81% lower than the healthy plants. The line B37*Htn1* showed a 57% reduction in A, again similar to the susceptible line and to B37*Ht1* and B37*Ht2*. The net photosynthetic rate of B37*Ht2* (27% reduction) was similar to the values obtained in the line B37*Ht3* (+7%), where no reduction of A was verified in inoculated plants.

At 5 dpi, the average values of Ex/Eo were not significantly different between lines, varying from 38% to 56% reduction. At 14 dpi, B37*Ht3* demonstrated a 32% higher E than healthy plants, whereas the other lines exhibited reductions ranging from 25 to 61%. (Figure 4). The average values of gsx/gso were reduced by 40% to 58%, although they displayed no differences between the lines at 5 dpi. When symptoms were already established (at 14 dpi), B37*Ht3* displayed values of gsx/gso 32% higher than healthy plants. The average values of B37, B37*Ht1*, B37*Ht2* and B37*Htn1* were reduced by 25% to 60%.

In the early stage of colonization (5 dpi), the kx/ko observed in inoculated plants was 0.33, 0.29 and 0.32 for the lines carrying *Ht1, Ht3* and *Htn1*, respectively. These values are 45%, 54% and 49% lower than those displayed by the inoculated susceptible line B37, which showed a kx/ko of 0.62 (Figure 3). At 14 dpi, the average values of kx/ko were reduced to 89 and 85% for B37 and B37*Ht1* respectively. The line B37*Htn1* demonstrated a 58% reduction of k in inoculated plants compared to healthy plants from the same line. Inoculated plants from the lines B37*Ht2* and B37*Ht3* displayed the highest values of k. Average values of k were reduced to 29% and 19%, respectively (Figure 4).

Chlorophyll fluorescence measurements showed chlorophyll degradation in the necrotic areas and small damage in the area surrounding the necrosis and yellow sites (data not shown). At 5 dpi, the QY of inoculated plants was close to 1, and therefore similar to healthy plants. However, at 14 dpi, the line B37*Ht1* showed the lowest value of QY compared to the other lines (Figure 5). At 5 dpi, the highest values of nPQx/nPQo were observed for the resistant lines, between -28% and +28% when compared to healthy plants. Inoculated plants of the susceptible line B37 showed nPQ values 46% lower than healthy plants. At 14 dpi, B37 showed the highest value of nPQx/nPQo (+89%), followed by B47*Htn1* (+56%).

Values for B37*Ht1*, B37*Ht2* and B37*Ht3* varied from -7% to +16%. However, the maize lines were not statistically different for nPQx/nPQo at 14 dpi.

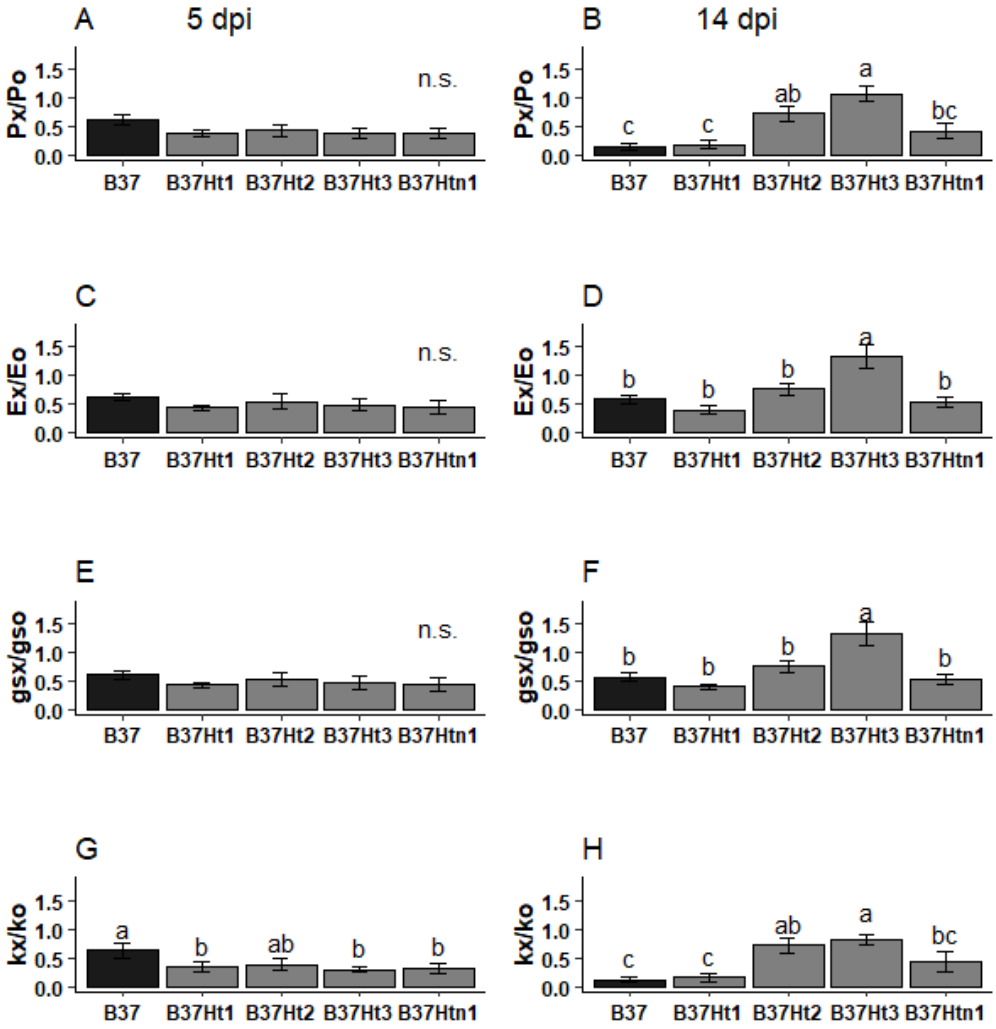

Figure 4. Relative leaf CO_2 assimilation [Px/Po] (A,B), relative transpiration [Ex/Eo] (C,D), relative stomatal conductance [gsx/gso] (E,F), and relative instantaneous carboxylation efficiency [kx/ko] (G,H) in inoculated plants of the line B37 without resistance genes and with resistance genes *Ht1*, *Ht2*, *Ht3* and *Htn1.* Px/Po, Ex/Eo, gsx/gso and kx/ko were calculated by considering the ratio of the variable values collected from inoculated plants to the mean values of healthy plants in each B37 line. Disease plants were inoculated with one race 0 isolate, which presented compatible interaction with the line (B37) without a resistance gene, represented by the black bar, and incompatible interaction with all resistance genes, represented by gray bars. Gas exchange measurements were realized at 5 (A,C,E,G) and 14 days post inoculation (dpi) (B,D,F,H). Data were fitted to a linear mixed model applying experiment replications as random effect. Data were analyzed by multiple comparison applying Tukey's test. Means sharing the same letter are non-significantly different, n.s. means not significant differences between lines (p-value ≤ 0.05). Bars indicate standard error (n = 3 biological replicate).

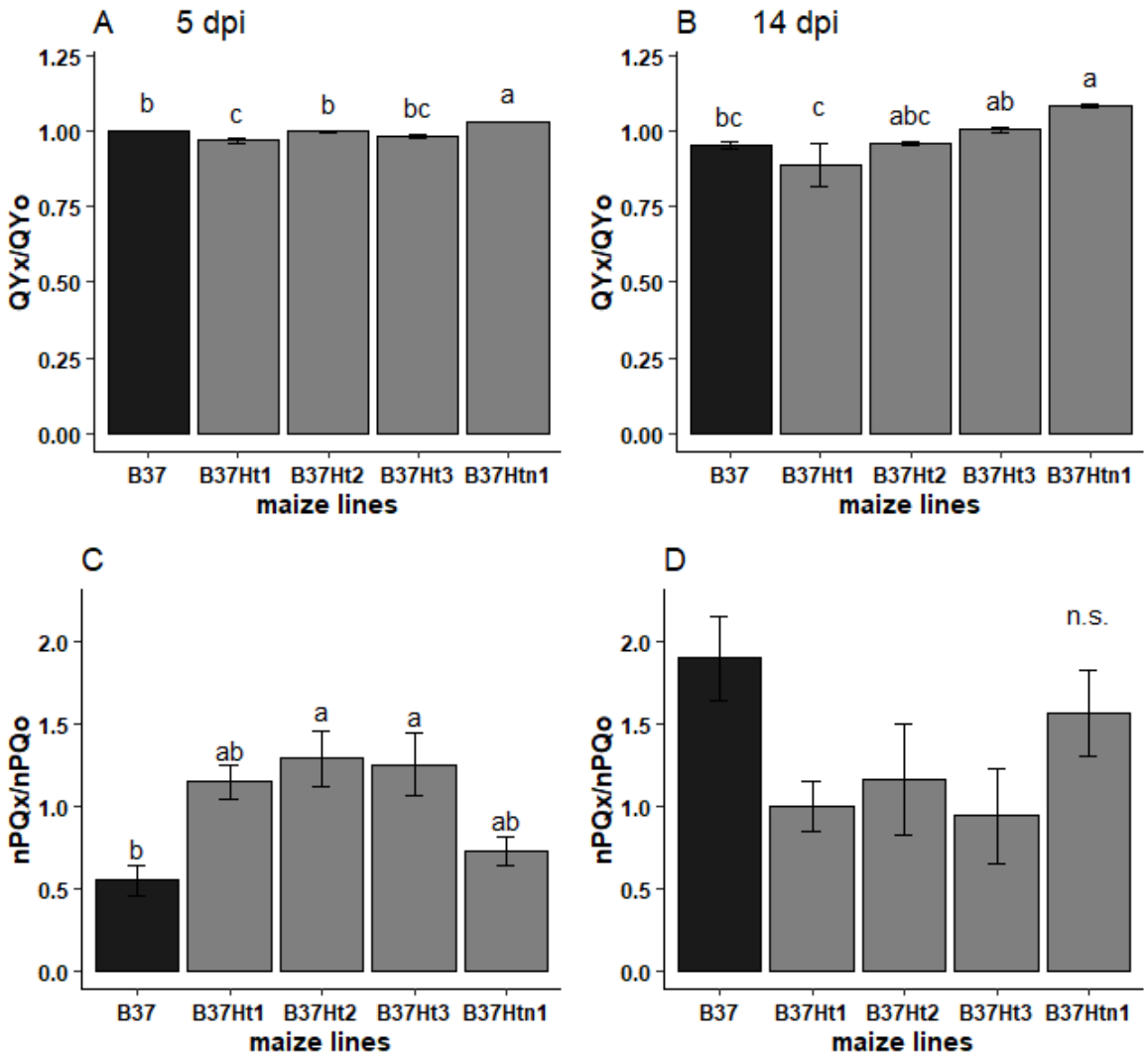

Figure 5. Relative quantum yield of photosystem II [QYx/QYo] (A,B), and the non-photochemical quenching [nPQx/ nPQo] (C,D) of inoculated plants of the line B37 without resistance genes and with resistance genes *Ht1*, *Ht2*, *Ht3,* and *Htn1.* Disease plants were inoculated with one race 0 isolate, which presented compatible interaction with the line (B37) without a resistance gene, represented by the black bar, and incompatible interaction with all resistance genes, represented by gray bars. Chlorophyll fluorescence measurements were realized at 5 (A,C) and 14 days post inoculation (dpi) (B,D). Data were fitted to a linear mixed model applying experiment replications as random effect. Data were analyzed by multiple comparison applying Tukey's test. Means sharing the same letter are not significantly different, n.s. means non-significant differences between lines (*p*-value ≤ 0.05). Bars indicate standard error (n = 3 biological replicates).

Plants from the cultivar Scorpion displaying compatible interaction showed higher variation in disease severity, with average values of 36% (Figure 6). The resistance reaction (incompatible interaction) shown by the hybrid Scorpion was characterized by necrosis surrounded by chlorosis, and the disease severity recorded was 14%. Absolute values of A, E, gs and k for the hybrid Scorpion showed a reduction in the incompatible interaction only at 5 dpi, while for the compatible interaction, reductions were observed at 5 and 14 dpi (Figure 7). At 5 dpi, the incompatible interaction demonstrated a reduction in A of 45% compared to the control, whereas a reduction of 58% was conferred by the compatible interaction. At 14 dpi, the A values for plants showing the incompatible interaction (21 µmol CO_2 m^{-2} s^{-1}) showed no significant differences when compared to healthy plants (17 µmol CO_2 m^{-2} s^{-1}). However, in the compatible interaction, A values were reduced by 45%. At 5 dpi, the E values were reduced by 46% in the incompatible interaction. In contrast, a reduction of only 28% for E values was measured for the compatible interaction. However, in the late stages, at 14 dpi, the compatible interaction (1.47 mmol m^{-2} s^{-1}) showed lower values of E when compared to the incompatible interaction (1.73 mmol m^{-2} s^{-1}). At 5 dpi, the gs values were reduced by 29% for the compatible interaction and by 47% for the incompatible interaction. At 14 dpi, a reduction of 23% of reduction was observed for the compatible interaction, whereas for the incompatible interaction the gs was reduced by 10%. At 5 dpi, the k was reduced by 60% and by 40% for incompatible and compatible interactions, respectively. At 14 dpi, the incompatible interaction showed no significant differences in the values of k compared with control plants. However, the average value of k in the compatible interaction was reduced to 29% (Figure 7).

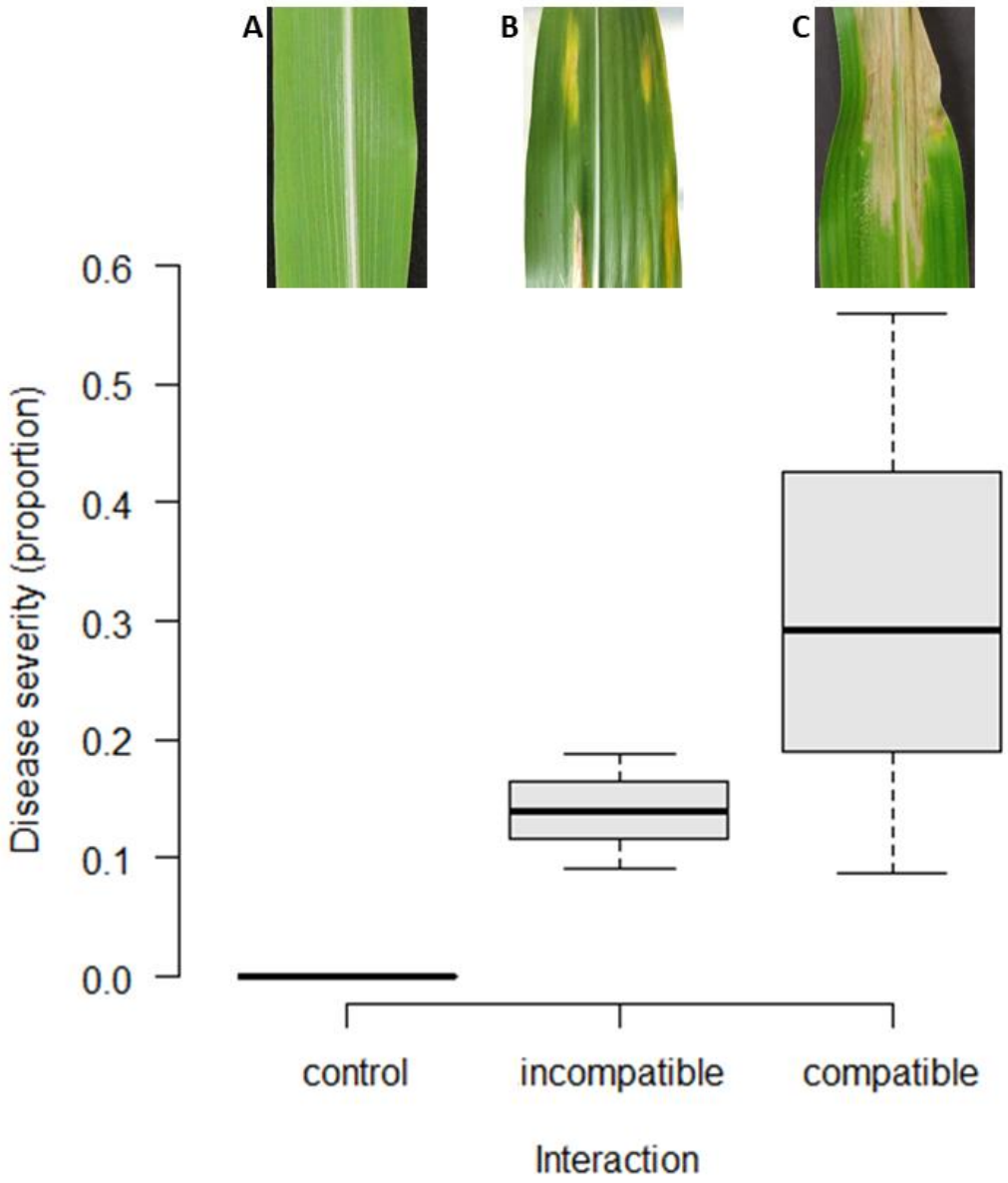

Figure 6. Disease severity of the maize hybrid Scorpion on a healthy plant (A), with a resistant reaction (incompatible interaction) displaying necrosis and chlorosis (B) and with necrosis, indicating susceptibility (compatible interaction) (C). The boxplot distribution represents the mean disease severity of the area in which gas exchange was measured in control plants (healthy plants), incompatible interaction (inoculated with isolate race 0) and compatible interactions (inoculated with isolate race 1). The lower quantile represents 0.25 and upper quantile 0.75 from the samples (n = 3 biological replicates).

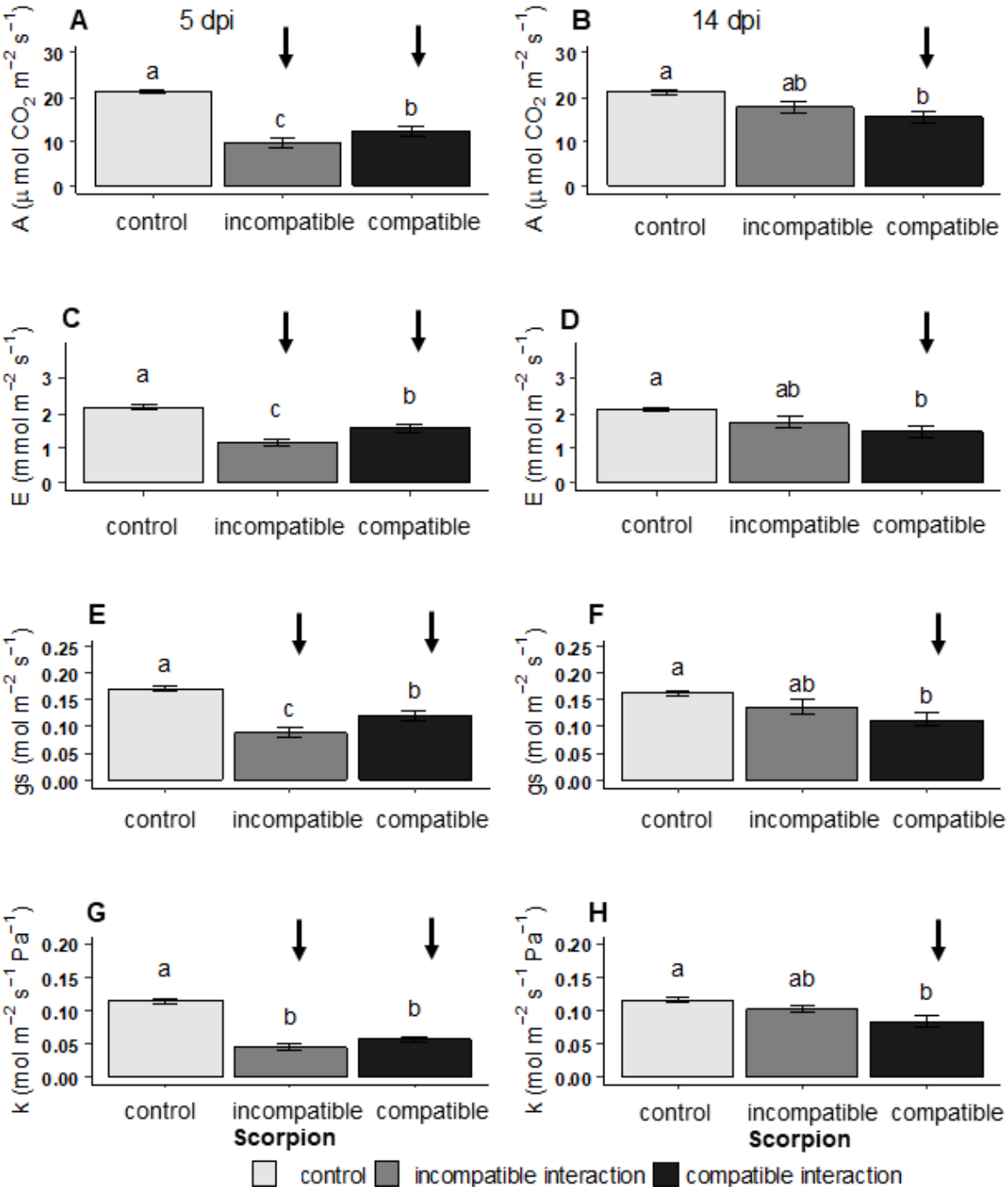

Figure 7. Leaf CO_2 assimilation [A] (A,B), transpiration [E] (C,D), stomatal conductance [gs] (E,F) and instantaneous carboxylation efficiency [k] (G,H) in healthy plants (control), and *E. turcicum* inoculated maize plants of the hybrid cultivar Scorpion, which carry the *Ht1* gene. Diseased plants were inoculated with race 0 (avirulent; incompatible interaction), and race 1 (virulent, compatible interaction). Gas exchange measurements were realized at 5 (A,C,E,G) and 14 days post inoculation (dpi) (B,D,F,H). Means sharing same letter are not significantly different according to Tukey-adjusted comparisons (p-value 0≤ 0.05). Bars indicate the standard error (n = 3 plants). Arrows indicate significant reduction when compared to control plants.

Peroxide detection and increase in peroxidase activity

In general, the percentage of penetration sites with peroxide (H_2O_2) detection and peroxidase activity (POX) increased over time (Figure 8). At 1 dpi, no significant differences in peroxide detection and peroxidase activity were observed between the lines. At 3 dpi, the line B37 exhibited a high percentage of penetration sites displaying peroxide positive reactions, whereas the POX was low. The highest percentages of penetration sites showing peroxide detection at 3 dpi were observed for B37 and B37*Htn1.* For POX, B37*Ht3* demonstrated higher activity than B37 and B37*Ht1*. At 6dpi, B37*Ht2* and B37*Htn1* differed from B37*Ht1* in the percentage of H_2O_2 localization, whereas for POX B37*Htn1* differed from B37. In addition, B37*Htn1* showed high values of both variables at 6 dpi. No DAB staining was observed in control plants and no superoxide (O_2^-) could be detected using NBT stain.

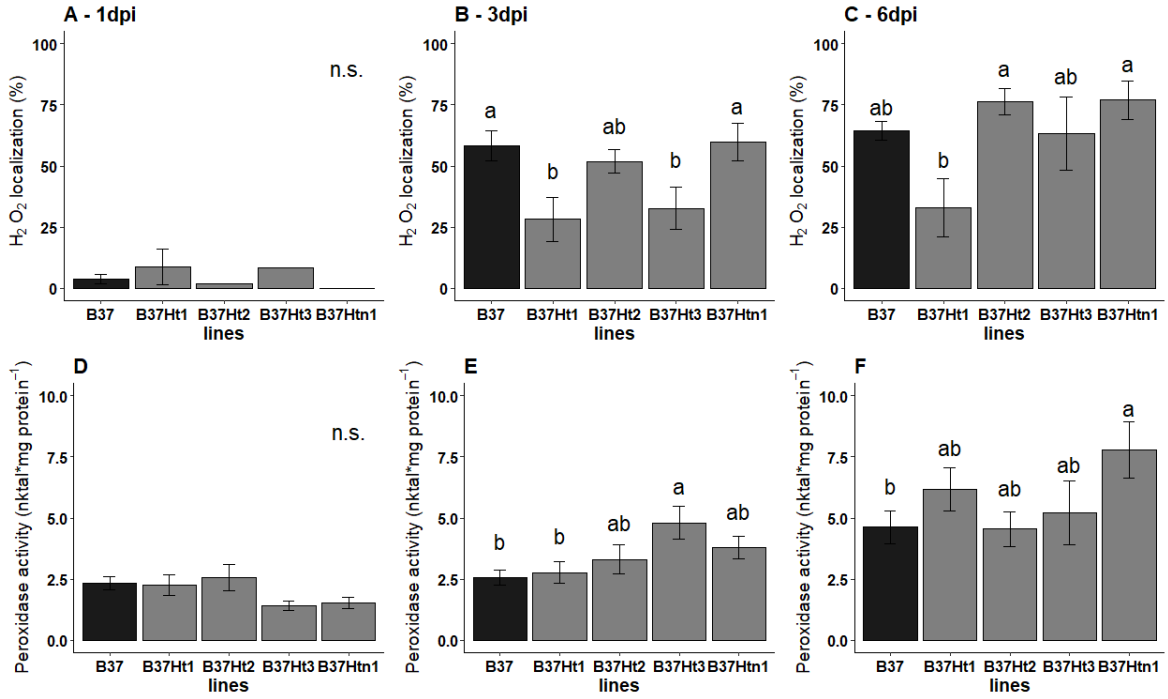

Figure 8. Percentage of penetration sites showing peroxide (H_2O_2) positive reactions by histochemical reaction with DAB (3,3-diaminobenzidin) (A-C) and peroxidase activity (nktal*mg protein^{-1}) (D-F) on the maize line B37 without resistance genes and with resistance genes *Ht1*, *Ht2*, *Ht3,* and *Htn1* inoculated with one race 0 isolate. Leaf samples were collected from three plants per treatment at 1, 3, and 6 days post inoculation (dpi). Data were fitted to a linear mixed model applying experiment replications as random effect. Data were analysed by multiple comparison applying Tukey's test. Means sharing the same letter are non-significantly different, n.s. means not significant differences between lines (*p*-value ≤ 0.05). Bars indicate standard error (n = 3 biological replicates).

Reduction in number of lesions per leaf, lesion length, and sporulation

The number of lesions per leaf was higher for the incompatible interaction with B37 followed by B37*Ht1* due to strong chlorosis and necrosis (Figure 9). B37*Ht2* displayed some lesions, whereas B37*Ht3* and B37*Htn1* showed only one small lesion in one or a maximum of two leaves. The largest lesions were observed for B37, followed by B37*Ht1* and B37*Ht2*. B37 and B37Ht2 showed the highest sporulation of *E. turcicum* per disease area. B37*Ht3* and B37*Htn1* showed high variation due to the few samples displaying lesions. In B37*Htn1* only two lesions were observed in all plants. In B37*Ht1*, B37*Ht3* and B37*Htn1*, observed symptoms were always related to chlorosis in the incompatible interaction.

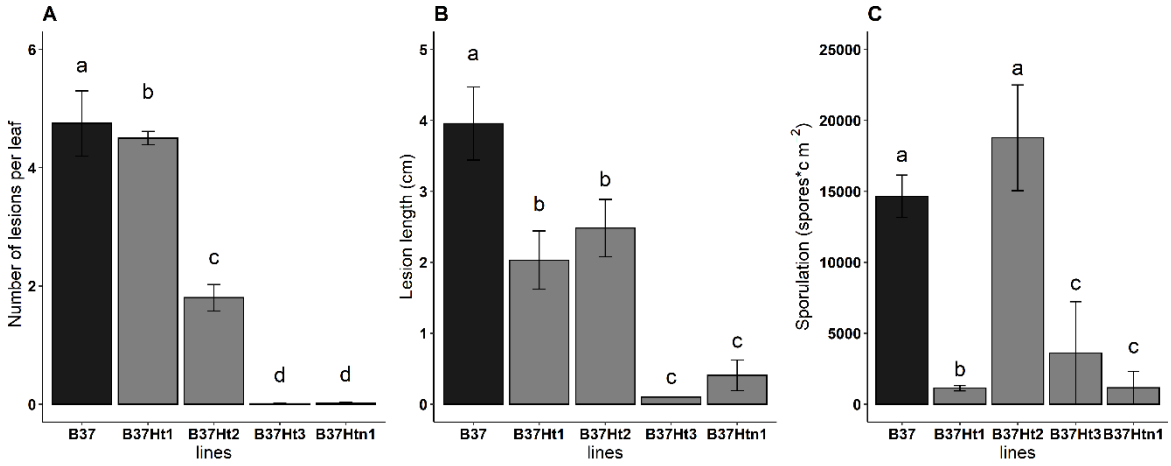

Figure 9. Number of lesions per leaf, lesion length and sporulation (spores cm^{-2} of diseased area) by *Exserohilum turcicum* infection at 14 days post inoculation on B37, B37*Ht1*, B37*Ht2*, B37*Ht3* and B37*Htn1*. All lines were inoculated with three race 0 isolates conferring compatible interaction with B37 and incompatible interaction with the other lines. Bars indicate the standard error. Data on the number of lesions and lesion length were analyzed the using non-parametric Kruskal-Wallis test. Means sharing same letter are not significantly different for Dunn's multiple comparisons test (p -value ≤ 0.05). Data on the sporulation were Box-Cox transformed and fitted to the linear mixed model, with replications as random effect. Means sharing same letter are not significantly different for Tukey-adjusted comparisons (p-value ≤ 0.05).

Leaf area and plant biomass estimation

No reduction in shoot (Figure 10) or root (data not shown) biomass was observed for inoculated plants at 14 dpi (data not shown) or at 28 dpi. However, a slight decrease in leaf area was observed for the susceptible line, being only significantly different for B37*Ht2*. In general, values of disease severity were lower than 10% in the biomass experiments. The exception was B37*Ht1,* which presented the highest values of disease severity (average of

11.72%). For the hybrid Scorpion, there were no differences in shoot biomass or leaf area between treatments. In addition, no differences in disease severity were observed between compatible and incompatible interactions (Figure 11).

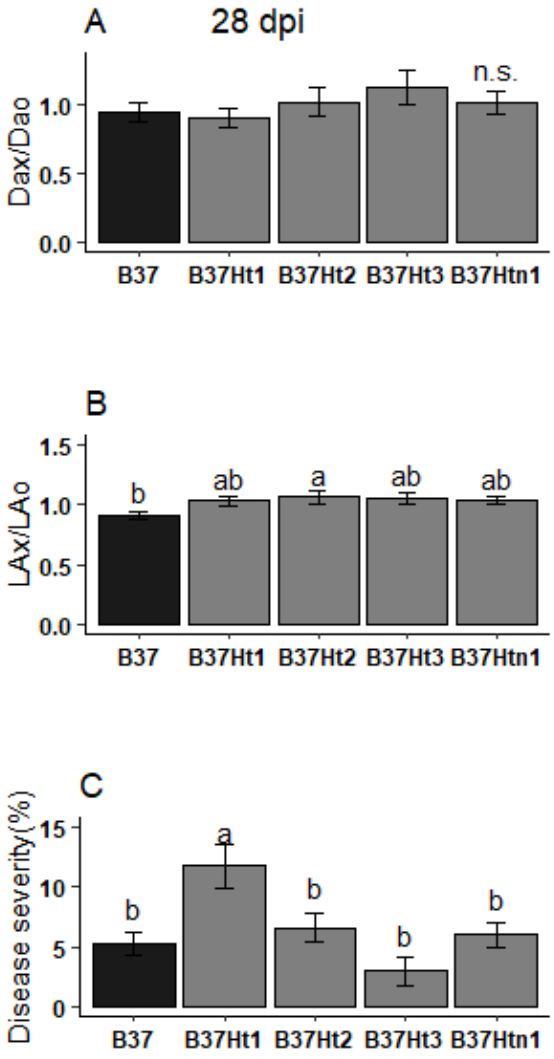

Figure 10. Relative ratio of dry weight of the shoot biomass (stem and leaves) [Dax/Dao] (A), leaf area [LAx/LAo] (B) and disease severity (C) between inoculated plants (Yx) and healthy plants (Yo) of the maize line B37 without resistance genes and with resistance genes *Ht1*, *Ht2*, *Ht3,* and *Htn1* from plant harvest at 28 days post inoculation (dpi). Diseased plants were inoculated with one race 0 isolate, which represented a compatible interaction, with the line (B37) without a resistance gene represented by the black bar, and incompatible interaction with all resistance genes, represented by gray bars. Data were fitted to a linear mixed model applying experiment replications as random effect. Data were analyzed by multiple comparison applying Tukey's test. Means sharing the same letter are not significantly different, n.s. means non-significant differences between lines (p-value ≤ 0.05). Bars indicate standard error (n = 4 biological replicates).

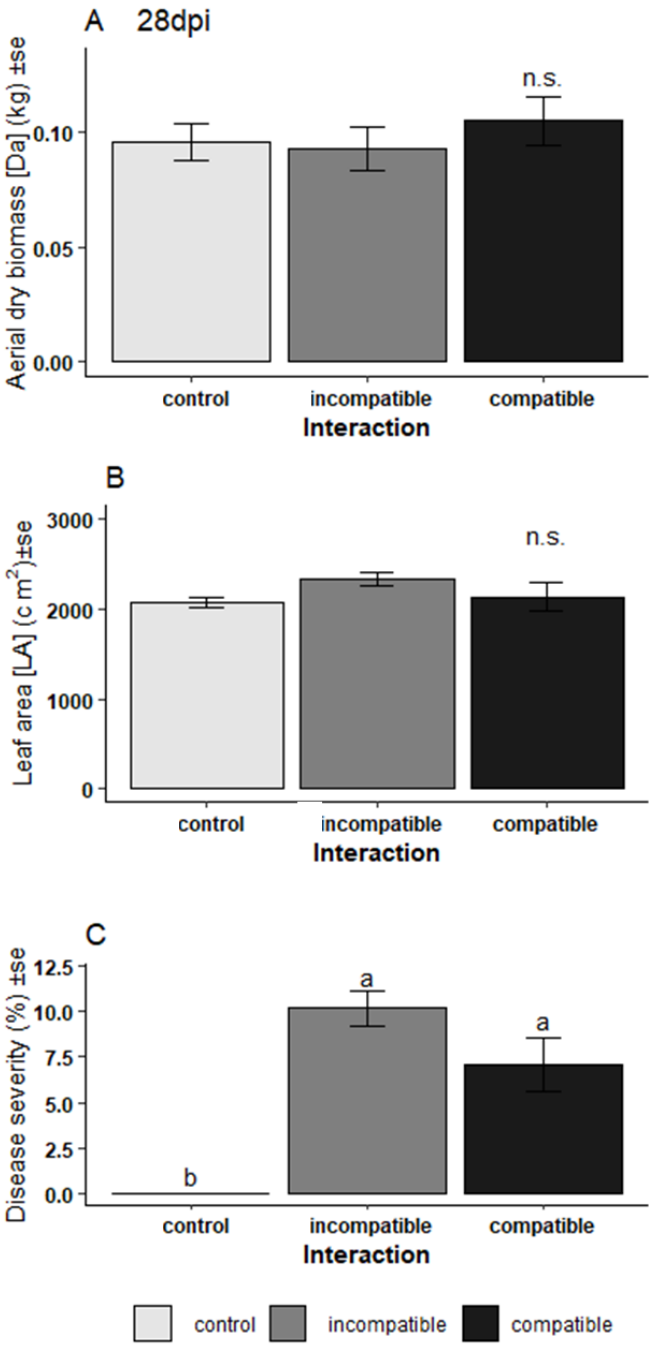

Figure 11. Dry weight of the shoot biomass (stem and leaves) [Da] (A), leaf area [LA] (B), and disease severity (C) of inoculated maize plants of the hybrid cultivar Scorpion, which bears the *Ht1* gene, harvested at 28 days post inoculation (dpi). Diseased plants were inoculated with one race 0 isolate, which was avirulent to Scorpion (incompatible interaction), and one race 1 isolate, which was virulent to Scorpion (compatible interaction). Data were fitted to a linear mixed model applying experiment replications as random effect. Data were analyzed by multiple comparison applying Tukey's test. Means sharing the same letter are not significantly different, n.s. means non-significant differences between lines (*p*-value ≤ 0.05). Bars indicate standard error (n = 4 biological replicates).

Discussion

The resistance responses of *Ht* genes against *E. turcicum* distinctly affected gas exchange, chlorophyll fluorescence, and oxidative burst in maize, as demonstrated by the experiments conducted in this study. The incompatible interaction of plants carrying the *Ht1* gene and inoculated with *E. turcicum* caused reductions in physiological activities similar to the compatible interaction. The visual severity of the resistance response in a plant bearing *Ht1* is similar to, or even higher than the disease severity caused by the pathogen in a compatible host (Figures 3 and 5). Conversely, the resistance response to *E. turcicum* expressed by *Ht3* did not affect most of the gas exchange and chlorophyll fluorescence variables, as no damaged leaf tissue could be observed in the incompatible interaction (Figure 4).

The lines carrying *Ht* genes decreased the carboxylation efficiency a few days after infection by *E. turcicum* and no effect was observed in water vapor diffusion variables (transpiration and stomatal conductance) after 5 dpi. Reductions in carboxylation efficiency caused by pathogens are usually related to biochemical limitations in the photosynthetic process (Navarro *et al.*, 2019). However, decreased carboxylation efficiency was not observed for the susceptible line B37 (compatible interaction) with *E. turcicum* at 5 dpi. At 14 dpi, the resistant line B37*Ht3* recovered in its photosynthetic activity. In contrast, the resistant line B37*Ht1* demonstrated drastic reductions in several gas exchange variables and in carboxylation efficiency, similarly to the compatible interaction of line B37.

In plant-pathogen compatible interactions the photosynthetic rate usually remains unaltered during the first stages of the interaction, as observed for the compatible interaction on B37 (susceptible line). In other pathosystems, photosynthesis is not reduced in the compatible interaction with biotrophs up to 6 dpi, as observed for powdery mildew (*Blumeria graminis*) on barley (Swarbrick *et al.*, 2006) and downy mildew (*Plasmopara viticola*) on grapevine (Nogueira Júnior *et al.*, 2020). However, photosynthesis of susceptible plants is usually progressively reduced up to 60% compared with healthy leaves after 7 dpi (Swarbrick *et al.*, 2006; Nascimento *et al.*, 2019; Nogueira Júnior *et al.*, 2020), similar to B37 and B37*Ht1* in the present work. After 5 dpi, a reduction in carboxylation efficiency was observed for maize in the incompatible interaction with *E. turcicum*, as demonstrated for incompatible interactions between *Blumeria graminis* f. sp. *hordei* and barley (Swarbrick *et al.*, 2006), and *Plasmopara viticola* and grapevine (Nogueira Júnior *et al.*, 2020).

Commonly, histochemical and biochemical alterations expressed during plant defense responses against pathogen infection in the incompatible interactions enhance carbohydrate hydrolysis and represses photosynthesis-associated proteins. These

impairments in the photosynthesis might be reversible, as for *Austropuccinia psidii* in Eucalyptus (Alves *et al.*, 2011), or not, as for *Plasmopara viticola* in resistant grapevine and *E. turcicum* and the maize line B37*Ht1,* according to the type of resistance response expressed by the resistance genes.

Resistance mechanisms of maize against *E. turcicum* can be related to morphological or biochemical alterations. Morphological alterations associated with resistance are thicker vessel walls (Hilu and Hooker, 1964), which may reduce hyphal penetration into xylem vessels (Navarro *et al.*, 2020). Necrosis was verified when the pathogen reached the xylem vessel (Kotze *et al.*, 2019) and necrotic symptoms are associated with cell death, which is provoked by the release of phytotoxins (Kotze *et al.*, 2019). In addition to the morphological alterations, the release of antibiotic substances can be involved in maize defense. A cyclic hydroxamic acid called DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) inhibited *E. turcicum* spore germination, decreasing the number of lesions in higher concentrations (Couture *et al.*, 1971). DIMBOA was found in maize and in many plants from the family Poaceae (Niemeyer, 1988) and also inhibited insect pests such as the European corn borer, *Ostrinia nibilalis* (Frey *et al.*, 1997), and beet armyworm, *Spodopotera exigua* (Rostás, 2007). The release of DIMBOA occurred following an increase in *β*-glucosidase activity, however, *β*-glucosidase was localized close to the small vascular bundles in *E. turcicum* inoculated and non-inoculated leaves (Mace, 1973). Besides DIMBOA, the production of phenolic phytoalexins has been shown to be induced by isolates that are pathogenic on maize (Lim *et al.*, 1970; Obi *et al.*, 1980). Phytoalexins have been shown to correlate with chlorosis expressed in the resistant phenotype (Calub *et al.*, 1973; Lim *et al.*, 1968; Obi *et al.*, 1980).The increase in phytoalexin production was related to more aggressive isolates (Lim *et al.*, 1970) and to homozygous plants (*Ht*/*Ht*) for the resistance gene (Calub *et al.*, 1973).

In addition to phytoalexins, other substances such as compounds involved in the oxidative burst and hormones may play a role in resistance. ROS can act in signaling in the first stages of infection. However, the percentage of penetration sites, at 1 dpi, where H_2O_2 and O_2^- could be detected was small or missing, respectively (Figure 8). These molecules were probably produced in low quantities, undetectable by the applied methods. Nonetheless, ROS are also known to act on defense signaling by salicylic acid (SA) (Lamb and Dixon, 1997). which was also reported from the maize - *E. turcicum* pathosystem (Wu *et al.*, 2015). In the present study, H_2O_2 was detected in more than 50% of the penetration sites at 3 and 6 dpi, corroborating the high H_2O_2 production in susceptible plants at 15 dpi (Silveira *et al.*, 2019). H_2O_2 accumulation in the late infection stages may be related to vascular colonization, as after 6 dpi *E. turcicum* is able to penetrate into the xylem (Navarro *et al.*,

2020). Wheat infected with the hemibiotroph *Zymoseptoria tritici* (teleomorph: *Mycosphaerella graminicola*) has shown high H_2O_2 accumulation during pathogen reproduction, at the necrotrophic stage (Shetty *et al.*, 2007). In our experiments, the peroxidase activity was higher in inoculated plants at 6 dpi (Figure 8). The highest values of POX activity were observed for B37*Htn1*, differing from the susceptible line B37. Similar results for POX activity were obtained for the line B73 (Shimoni *et al.*, 1991). The lowest percentage of penetration sites with H_2O_2 detection was observed in the line B37*Ht1*. However, the low detection of H_2O_2 might be related to cell death, as high disease severity was detected for this line (Figures 2 and 3). The resistance phenotype conferred by *Htn1* described in the literature was not related to chlorosis but to an extended latent period and to quantitative resistance (Hurni *et al.*, 2015). After six to eight weeks, healthy plants were showing yellow spots (Navarro *et al.*, 2020). These symptoms were observed in the lower leaves and were described as wilt-type lesions (Gevers, 1975). Wilt-type lesions, also called "lesion-mimics", might have an inappropriate resistance mechanism like hypersensitive response (HR), which demands energy (Balint-Kurti, 2019), corroborating the low values of CO_2 assimilation in healthy plants (Supplementary figure 1). Wilt-type lesions could explain the higher percentages of H_2O_2 detection and high peroxidase activity in plants carrying the *Htn1* gene.

A proteomic study realized at 3 dpi with the incompatible interaction between the line A619*Ht2* and a race 13 isolate identified up-regulation of 50 proteins with multiple functions. Proteins related to energy metabolism, such as proteins related to ATP synthesis, represented 46% of the up-regulated proteins. ATP is important for phytotoxin detoxification and cell apoptosis (Zhang *et al.*, 2014). The apoptosis leads to the detachment of the plasma membrane from the cell wall, which preserves its integrity and permits *de novo* synthesis of proteins (Veloso and van Kan, 2018), and consequently avoids the use of nutrients by pathogens during the necrotrophic phase and during the biotrophic phase by PCD. The proteins related to defense against pathogens represented 18% of up-regulated proteins, and were identified as *β*-glucosidase, superoxide dismutase, and polyamine oxidase. Enzymes that act on the oxidative burst are related to the accumulation of ROS (Zhang *et al.*, 2014) and disease signaling (Apel and Hirt, 2004). Our results obtained in the gas exchange and chlorophyll fluorescence experiments confirmed that photosynthesis was affected in the initial stages of infection in the incompatible interactions. These data correlate with the decrease in Rubisco activity observed in the proteomic study (Zhang *et al.*, 2014). The up-regulation of genes from the family of laccase-like multicopper oxidases (LMCO), which are released by the pathogen during early stages of infection, indicates that those genes play an important role in pathogenesis. LMCO genes increase laccase activity,

which is important for lignin degradation, detoxification of phenolic substances and lignocellulose degradation (Liu *et al.*, 2019).

The resistance response of *Ht1* displays an extensive chlorosis combined with necrosis (Figures 2 and 6) which explains the low photosynthetic activity, as observed for chlorophyll fluorescence, which causes impairments in the photosynthetic process at 14 dpi (Figures 4, 5 and 6). Chlorosis can be associated with loss of cell membrane integrity and accumulation of water in the apoplast of the infected tissue (Lindenthal *et al.*, 2005). Chlorosis is caused by reduction in chlorophyll fluorescence and is usually associated with damage to chloroplasts (Buchanan *et al.*, 1981). Fungal infection can cause reduction in photophosphorylation due to decrease in the rate of non-cyclic electron transport, which has water as an electron donor (Buchanan *et al.*, 1981). Host resistance responses might modify guard cell regulation of the stomatal apertures (Grimmer *et al.*, 2012). Consequently, water loss promotes cell death and desiccation of healthy tissue (Lindenthal *et al.*, 2005), leading to necrosis. At 5 dpi, a reduction in instantaneous carboxylation efficiency (k) indicates that the resistance genes have an earlier response to fungal infection, whereas for the susceptible line k is not reduced. The increase in nPQ indicates that energy is being transferred to other non-photosynthetic processes. Some resistance mechanisms might be involved in electron transport by changing the cell membrane and through chlorophyll disintegration (Lindenthal *et al.*, 2005). At 14 dpi, the healthy tissue seems to compensate for photosynthetic costs associated with the activation of resistance mechanisms in the incompatible interaction, as observed for Scorpion (Figure 9).

The expression of resistance mechanisms conferred by *Ht* genes affected some epidemiological components, such as sporulation (Abadi *et al.*, 1989). The suppression of sporulation reduces the disease secondary cycle and may affect the apparent infection rate (*r*) (Parlevliet, 1979). Instead of suppression of sporulation, the *Ht2* gene reduced the number of lesions and lesion size (Figure 9). Even in the compatible interaction, the line B37*Htn1* was able to decrease the sporulation by 64% compared to the line without resistance genes (Figure 9). A delay in disease development and a slow disease growth rate are important breeding goals (Sigulas *et al.*, 1988). Indeed, it is more laborious to measure lesion length than latent period and therefore the former is less suitable as screening parameter in breeding selection for resistance to *E. turcicum* (Carson, 2006). However, lesion expansion is an important epidemiological component for secondary infection (Berger *et al.*, 1997), especially for tropical pathosystems (Bergamin Filho and Amorim, 1996).

Previous studies with *Arabidopsis thaliana* carrying the resistance gene *RPM1*$^{+}$*,* which confers resistance against *Pseudomonas syringae* pv*. maculicola*, showed that plants were

smaller, had lower reproductive fitness and presented 9% fewer seeds than *rpm1* plants (Brown, 2003). In maize, plants carrying qualitative resistance presented a 8.45 to 13.2% yield reduction, when compared to susceptible plants (Ullstrup, 1970). However, in our experiments, we were not able to detect a reduction in the shoot biomass (Dax/Dao) at 28 dpi for the lines (Figure 10) or for the hybrid (Figure 11), most likely due to the low level of disease severity (Figures 3 and 7). Thus, low disease severity does not necessarily suggest that the inoculation procedure was unsatisfactory but rather that maize growth is fast, especially during stem elongation and formation of the upper leaves. The experiments regarding biomass production presented in this work were conducted in the greenhouse under controlled conditions. However, field experiments should be conducted to investigate the effect of resistance genes on plant biomass (silage maize) and/or grain yield (grain maize) in practical conditions. Commonly, a reduction in energy production might affect yield formation. The presence of the *Ht1* resistance gene demands energy from the host (Ullstrup, 1970). By conducting a three-year field trial, Ullstrup (1970) was able to show that NCLB led to a yield reduction of about 40% when susceptible cultivars were infected. Qualitative resistance through the use of *Ht1*, on the other hand, led to a yield reduction of only 19%. Quantitative resistance however resulted in the lowest yield reduction of only 5%. Another study showed that hybrids carrying the *Ht1* gene had a 6 to 18% yield loss, which was less than those hybrids without resistance genes (6 to 51 % yield loss) (Pataky, 1994). Resistance costs were correlated with chlorotic-type lesions (Ullstrup, 1970; Pataky, 1994; Lipps *et al.*, 1997). In line with its extremely susceptible background, the *Ht1* gene expresses extensive chlorosis (Ullstrup, 1970).

A recent study has shown that *Ht2* and *Ht3* are identical and allelic to the *Htn1* gene. The allele *Ht2/Ht3* differ from *Htn1* by multiple amino acid polymorphisms that may affect an extracellular domain, consequently leading to changes in triggered resistance mechanisms (Yang *et al.*, 2021). Additionally, Yang *et al.* (2021) identified extremely high genetic variation between B37 and the line B37*Ht1*, B37*Ht2*, B37*Ht3* and B37*Hnt1 (*from 8 to 61%) that were supposed to be near isogenic lines. The same authors suppose that differences in the phenotype between *Ht2* and *Ht3* are due to distinct genetic background. In fact, the genetic background seems to have a much stronger effect on the phenotype, especially when accounting for resistance, as differences on the physiological responses to the pathogen infection and the pattern of epidemics could be clearly observed between the line B37*Ht2* and B37*Ht3* (which are actually the same allele). In the pathosystem *Magnaporthe oryzae* - rice, the genes *Pi35* and *Pish* are allelic and multiple functional polymorphisms increase resistance (Fukuoka *et al.*, 2014). Additionally, Fukuoka *et al.* (2015) identified that an amino acid residue of *Pi35* is associated with quantitative resistance.

It is known that *Ht* resistance genes can cause physiological and biochemical alterations, which may subsequently influence biomass production. Indeed, the *Ht* genes have reduced disease severity and have led to a delayed progress of infection in compatible interactions, which suggests that maize R genes against *E. turcicum* are associated with, or confer, additional quantitative resistance (Navarro *et al.*, 2020). Such increase in the level of resistance suggests that this might be a residual effect of "defeated" *Ht* genes, which confer additional quantitative resistance (Navarro *et al.*, 2020). The pathogen is in constant co-evolution with the host, which explains why the pathogen can overcome some resistance mechanisms (Brown, 2003). As hypothesized by Poland *et al.* (2009), some *Ht* genes may condition incomplete resistance, which can be a weaker form of an R-gene, leading to minor-gene-for-minor-gene interaction (Parlevliet and Zadoks, 1977). As observed on this study, the reduction on net photosynthetic rate ranged from 0% to 94%, which demonstrates that not only the resistance gene, but the genetic background is responsible for distinct phenotype reactions and consequently on photosynthetic processes. The advance of molecular methods may help to better understand coevolution between host and pathogen (Thrall *et al.*, 2012). Candidate effectors involved as avirulence factors in interactions with resistance response factors have previously been studied showing that these effector candidates of the *Ht1* gene are involved in the biosynthesis of secondary metabolites and cell wall degradation (Wu *et al.*, 2015; Human *et al.*, 2020). Nonetheless, more studies are needed to identify virulence factors involved in host-pathogen interactions and resistance mechanisms involved in resistance conferred by the *Ht* genes.

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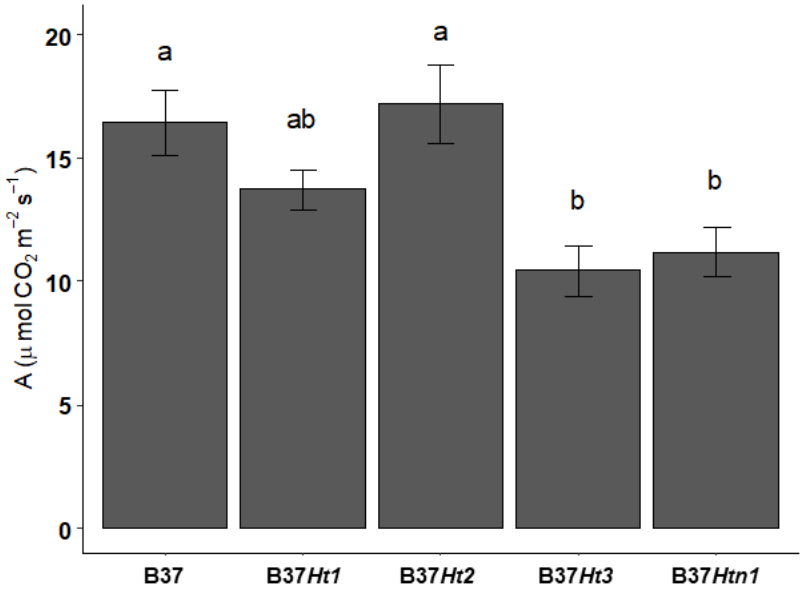
Supplementary Material

Appendix 1. The German commercial hybrid ES Scorpion was inoculated with six *E. turcicum* isolates of different races (race 0, 1,2,3, 3N and 23N). Inoculations were performed by spraying an average 7 ml of an inoculum suspension of 3000 spores ml^{-1} for each plant until the point of run-off. Four plants were inoculated per isolate. Disease phenotype (Bigirwa *et al.*, 1993; Hanekamp, 2016) and disease severity (Levy and Pataky, 1992) were evaluated 14 days post inoculation following diagrammatic scales. The disease phenotyping basically describes plants as susceptible when showing green-grey lesions, and as resistant when chlorotic lesions are visualized. Data of disease severity were compared between hybrids by analysis of variance (two-way ANOVA) and by multiple comparison applying Tukey test (p-value ≤ 0.05) using R software 3.6.0 (Core Team 2019). ES Scorpion showed similar phenotype and high disease severity, as observed for the line B37*Ht1.* Average values of disease severity were high displaying 62%.

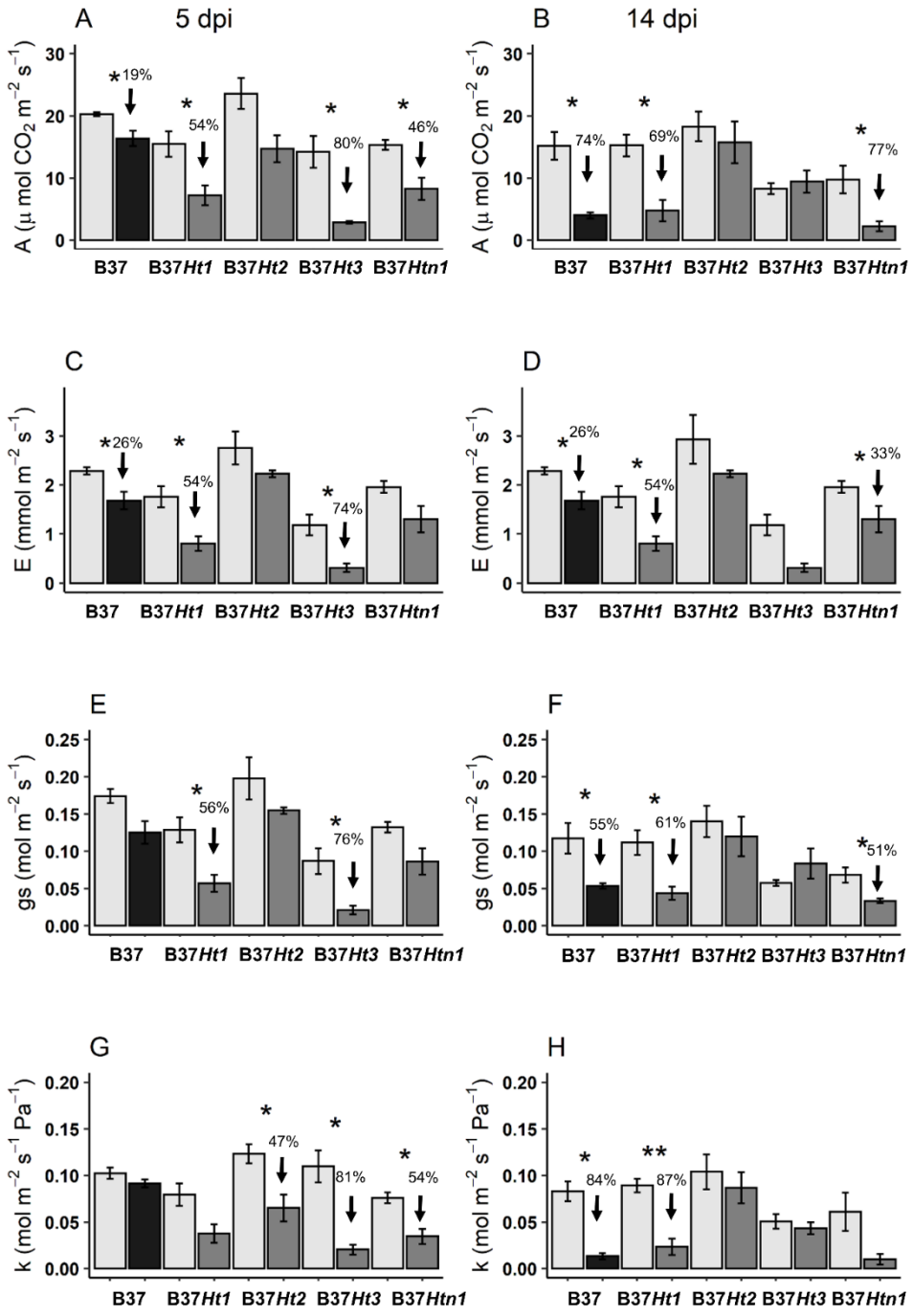
Supplementary table 1. Disease phenotype for 3 maize hybrids commercialized in Germany. Each hybrid was inoculated with *Exserohilum turcicum* isolates from 6 different races (race 0, 1, 2, 3, 3N and 23N). Disease phenotype was evaluated at 14 days post inoculation.

Isolates	Mize hybrid ES Scorpion
Race 0	R
Race 1	S
Race 2	R
Race 3	R
Race 3N	R
Race 23N	R

S – plants showing susceptible phenotype, R – plants showing resistant phenotype



Supplementary figure 1. Leaf CO_2 assimilation [A] for healthy plants (control) of the line B37 without resistance genes and with resistance genes *Ht1*, *Ht2*, *Ht3* and Htn1 for both experiments replicates. Means sharing same letter are not significantly different for Tukey-adjusted comparisons (p-value ≤ 0.05). Data from both replications are presented in the graphics. Bars indicate the standard error.



Supplementary figure 2. Leaf CO_2 assimilation [A] (A,B), stomatal conductance [gs] (C,D), transpiration [E] (E,F) and instantaneous carboxylation efficiency [k] (G,H) in healthy plants (control - represented by light grey bars) and inoculated plants with one race 0 isolate, which presented compatible interaction with the line B37 by the black bar, and incompatible interaction with all resistance genes, represented by gray bars. Leaf samples were measured at 5 (A,C,E), and 14 (B,D,F) days post inoculation (dpi). Bars indicate standard error (n = 4 biological replicate). Data were compared by anaysis of variance, and values of healthy plants were compared with inoculated plants by T-test (* *p*-value ≤ 0.05). Graphs show data of the first repetition.

Appendix 2. Calculation of the peroxidase activity

- **Absolute peroxidase activity**

1) A linear regression was plotted with data of absorbance per time and the slope was calculated (time unit in second).

2) Beer-Lambert Law: $\mathbf{Abs_\lambda = \varepsilon_\lambda c\ l}$

c = abs **/** ε l

c = slope * 60 **/** 2.66 10^7 cm^2 mol^{-1} * 1cm

c = slope * 22,55 *10^{-7} mol ml^{-1} min^{-1}

c = slope * 22,55 *10^{-7} * 10^6 µmol min^{-1} ml^{-1}

3) Transformation in nKatal

c = slope * 2,255 * 16.67 nkat ml^{-1}

4) Enzyme activity in the sample extract

c = slope * 37,6 / reaction volume

c = slope * 37,6 * volume sample extract / reaction volume → nkat in 1 ml sample extract

Beer-Lambert Law: $Abs_\lambda = \varepsilon_\lambda c\ l$

***Abs*$_\lambda$** → absorbance or optical density

ε_λ → extinction coefficient [for guaiacol and peroxidase: $\varepsilon_{\lambda=470nm}$ = 2.66 10^7 cm^2 mol^{-1}] (Chance and Mahehly, 1955)

c → concentration of the reaction product in the solution, in this case the enzyme activity (mol ml^{-1} min^{-1} – expressed by nKatal ml^{-1})

l → optical path length.

- **Protein content**

Calculation with the calibration curve.

Final unit is mg protein ml^{-1}

Enzyme units:

1 katal – kat = 1 mol s^{-1}

1U (enzyme unit) = µmol min^{-1} = 16.67 nkat

❖ **Peroxidase activity** (nktal*mg $protein^{-1}$) **= absolute peroxidase activity** (nkat) ***/*** **protein content** (mg protein ml^{-1})

Chapter 5. The efficacy of *Ht2*-resistance to *Exserohilum turcicum* in maize is not related to the pre-inoculation temperature

Abstract

Northern corn leaf blight (NLCB) is an important leaf disease caused by the ascomycete *Exserohilum turcicum*. NCLB control is based on host resistance, and more specifically on qualitative genes called *Ht* genes. The resistance phenotype conferred by to *E. turcicum* can differ depending on environmental conditions. In the literature, there are reports about the susceptibility of maize plants bearing the *Ht2* gene when maintained under low post-inoculation temperature (22/18°C) and low light intensity (324 - 162 µmol m^{-2} s^{-1}), however it is not clear which factor (host resistance or pathogen virulence) is mostly affected by temperature. Our objective was to assess the influence of pre-inoculation temperature on the efficacy of *Ht2*-resistance to *E. turcicum.* Maize plants from the line B37 with no resistance gene and for the line B37*Ht2* (with the *Ht2* gene) were pre-disposed to warm (30/25°C) and moderate (20/15°C) temperature regimes for 10 days. Spray inoculations were performed with three Brazilian and three German isolates. After inoculations, all plants were maintained under the same temperature regime (25/20°C). The disease severity and DNA content of *E. turcicum* were evaluated 21 days post inoculation (dpi). No significant differences in disease severity and *E. turcicum* DNA content were observed between plants maintained at different pre-inoculation temperatures. There was no influence of pre-inoculation temperature on the expression of resistance in the maize line bearing the *Ht2* gene. The resistance conferred by the *Ht2* was confirmed by quantifying low amounts of fungal DNA in the line B37*Ht2* at 21 dpi. Changes in resistance levels of maize plants bearing the *Ht2* gene reported in the literature for plants exposed to different post-inoculation temperatures might be related to the influence of temperature on pathogen aggressiveness factors. Further studies are necessary to determine how expression of the *Ht2* gene is up- and down-regulated, and consequently what pathways are being activated to trigger resistance.

Keywords: disease resistance, R gene, qPCR, *Setosphaeria turcica, Zea mays*

Introduction

Changes in environmental conditions, such as the increase in temperature can modify cropping systems (Velásquez *et al.*, 2018), which may lead to changes in disease epidemics. The dynamic of diseases can be affected by the amount of primary inoculum, the rate of disease progress, and the potential duration of the epidemic (Juroszek and Tiedemann, 2013). Environmental conditions are the main drivers of these variables, affecting pathogen aggressiveness and the efficacy of resistance genes (Agrios, 2005). Pathogen reproduction, survival, germination and the expression of virulence factors, such as virulence proteins or toxin production, may change under different weather conditions (Velásquez *et al.*, 2018). Conversely, host physiology is strongly affected by abiotic factors, such as heat and drought stress or low nutrients availability increasing plant susceptibility to pathogens (Coakley *et al.*, 1999).

The increase in temperature may inactivate or enhance the efficacy of resistance genes according to the pathosystem and resistance gene (Vanderplank, 1968; Coakley *et al.*, 1999; Onaga *et al.*, 2017). For instance, the resistance promoted by the *Pg3* and *Pg4* genes in oats against *Puccinia graminis* f. sp. *avenae* is inactivated with increase in temperature (Vanderplank, 1968; Coakley *et al.*, 1999). The same tendency was observed for the resistance genes *Lr20* and *Sr15* in wheat against *Puccinia recondita* and *Puccinia striiformis* f.sp. *tritici.* Wheat plants exposed to a high pre-inoculation temperature (30°C) were susceptible to leaf and stem rust (Ramage and Sutherland, 1995). Interestingly, *Triticum dicoccum* bearing the *Rmg7* gene, which confers resistance to *Magnaporthe oryzae* pathotype *Triticum,* lost its resistance under warm temperature conditions (25°C), compared to plants exposed to pre-inoculation temperatures of 20 and 22°C. In this case, only the virulent isolate was able to infect under 25°C (Tagle *et al.*, 2015). The resistance conferred by *Rmg7* was stable when plants were inoculated with an avirulent isolate carrying the *AvrRmg7* gene (Tagle *et al.*, 2015; Anh *et al.*, 2018). Conversely, high pre-inoculation temperatures increased the expression of the *Pi54* gene (R gene) against *Pyricularia oryzae* in japonica rice. The japonica background of rice cultivars increases the expression of resistance of *Pi54* gene (Onaga *et al.*, 2017). The increase in resistance in rice plants exposed to high pre-inoculation temperatures is due to an increase in callose deposition (Onaga *et al.*, 2017). An increase in resistance with the increase of temperature is also observed in wheat and barley varieties infected by *Puccinia striiformis* (Sharp, 1962). For instance, high temperatures increase cell wall lignification in grasses, which enhances resistance to fungal pathogens (Coakley *et al.*, 1999). Moreover, temperature and light intensity may affect the resistance function of some *Ht* genes for the pathosystem maize-*Exserohilum turcicum* (Thakur *et al.*, 1989a; Thakur *et al.*, 1989b; Leath *et al.*, 1990).

Northern corn leaf blight (NCLB), an important maize disease worldwide, is caused by the ascomycete *E. turcicum* (teleomorph *Setosphaeria turcica*). One of the main disease control methods is the cultivation of resistant varieties (Galiano-Carneiro and Miedaner, 2017). Several qualitative resistance genes were reported to confer resistance against NCLB. The resistance phenotype conferred by *Ht1, Ht2,* and *Ht3* genes is related to chlorosis (Hooker, 1963, 1977, 1981). Instead of chlorotic lesions, the *Htn1* confers a longer latent period (Gevers, 1975). However, the resistance phenotype of some *Ht* genes may not be expressed under low post-inoculation temperatures and low light intensity (Thakur *et al.*, 1989a; Thakur *et al.*, 1989; Leath *et al.*, 1990). A loss in resistance function of the *Ht1* gene under high post-inoculation temperatures (26/22°C) was observed in the B37 line. However, the same observations were not noticed the H4460 line, indicating that genetic background may influence the function of the resistance gene *Ht1* under high temperatures (Thakur *et al.*, 1989). Conversely, the *Ht2* gene did not express resistance phenotype neither in B37 nor in H4460 lines when exposed to low pre- and post-inoculation temperatures of 22/18°C, and low light intensities of 324 and 162 µmol m^{-2} s^{-1}. Nonetheless, the *Ht3* gene led to a resistant phenotype in the B37*Ht3* and H4460*Ht3* lines when these lines were inoculated with a virulent isolate and maintained under high pre- and post-inoculation temperature regimes (26/22°C) and full light intensity (647 µmol m^{-2} s^{-1}) (Thakur *et al.*, 1989a; Leath *et al.*, 1990). In general, the genes *Ht1* and *Htn1* conferred stable phenotypes in the B37 line when compared to *Ht2* and *Ht3*, when inoculated with virulent and avirulent isolates, maintained under low light intensity (155 µmol m^{-2} s^{-1}), and exposed to distinct post-inoculation temperatures (15/10°C, 20/15°C, 25/20°C and 30/25°C) (Hanekamp, 2016).

The resistance conferred by the *Ht2* gene is most sensitive to changes in environmental conditions compared to other *Ht* genes. This gene is located on chromosome 8 in the maize genome and is associated with the position bin 8.06 (Chung *et al.*, 2010). The *Ht2* gene is race-specific (Chung *et al.*, 2010), and confers small chlorotic lesions as a resistance phenotype. Chlorotic lesions can persist for some days without necrosis (Hooker, 1977), and decrease the lesion area (Abadi *et al.*, 1989). Moreover, the delay in lesion formation by about 2.6 – 6.8 days and reduction in diseased leaf area by about 12 – 22% were observed for the maize hybrid DK888 bearing the *Ht2* (Chung *et al.*, 2010). In general, the resistance associated with the *Ht2* suggests a lower level of resistance when compared to the *Ht1* (Hooker, 1977; Cota *et al.*, 2013). Moreover, maize plants bearing the *Ht2* did not show reduced pathogen sporulation (Navarro *et al.*, 2021a).

In a proteomic study analyzing defense responses conferred by the *Ht2* gene in the line A619*Ht2,* in an incompatible interaction with a race 13 isolate, differences on proteins expression were identified. Of the 87 up-regulated proteins, 18% were responsible for

disease defense (Zhang *et al.*, 2014). Moreover, ATP synthesis was suppressed in susceptible hosts by phytotoxins, resulting in the suppression of resistance mechanisms (Zhang *et al.*, 2014). *E. turcicum* produces monocerin, a non-specific phytotoxin, which was shown to be phytotoxic to Johnson-grass and cucumber (Robeson and Strobel, 1982). Moreover, another study showed that *E. turcicum* produces the host-specific HT-toxin, referring to *Helminthosporium turcicum* (*syn. E. turcicum*) (Dong and Li, 1996). Maize plants HZS*Ht2* infiltrated with HT-toxin prevented phytotoxin damage by up-regulating energy metabolism and signaling pathogen invasion (Wang *et al.*, 2010). Resistance mechanisms for the detoxification of the HT-toxin, conferred by the *Ht2* gene, might involve a translation elongation factor or an upregulated QM-like protein. The QM-protein is related to stress response pathways, which mediates proline levels in the plant (Zhang *et al.*, 2014). Proline is an amino acid that acts as an osmolyte, playing an important role as a metal chelator, anti-oxidative defense molecule and signaling molecule (Hayat *et al.*, 2012). Moreover, resistance mechanisms associated with the *Ht2* gene, such as HT-toxin detoxification, are not well explained.

Post-inoculation temperatures affect plant resistance responses, pathogen growth and disease development. Low post-inoculation temperatures and low light intensities seems to affect the NCLB development probably by changes on expression of resistance phenotype conferred by *Ht2* (Thakur *et al.*, 1989a; Leath *et al.*, 1990). However, the effect of pre-inoculation temperatures (i.e. the pre-exposition of plants to different temperatures before pathogen inoculation), which may affect only plant responses to the pathogen infection, has not been described yet in the interaction between the maize lines carrying the *Ht2* and *E. turcicum*. The objective of this study was to prove the efficacy of the *Ht2*-resistance gene against *E. turcicum* by applying different pre-inoculation temperatures. Therefore, the disease severity and the content of *E. turcicum* DNA were measured in susceptible maize plants of the line B37 (without resistance genes), and in resistant plants (B37*Ht2*) exposed to warm (30/25°C) and moderate (20/15°C) pre-inoculation temperatures.

Material and Methods

Plant material, inoculation and disease assessment

Maize plants from the differential set of the line B37, without resistance genes, and from the line B37*Ht2,* with the resistance gene, were cultivated in a soil mixture containing compost, clay and sand in proportions of 3:3:1, respectively. Two seeds of each line were sown per pot, totaling four pots per treatment (11 cm x 11 cm x 10 cm). Seeds from the differential set were provided by KWS Saat SE (Einbeck, Germany). All plants were cultivated in the greenhouse for 30 days (Figure 1). In order to exclude the influence of temperature on

pathogen development, maize plants were exposed to different pre-inoculation temperature regimes. Therefore, when the fifth leaves were unfolded, every eighth plant was moved to a climate chamber set to warm temperature conditions of 30/25°C, and moderate temperature conditions of 20/15°C. Both chambers (RUMED® Rubarth Apparate GmbH, Germany) were exposed to a light/dark photoperiod of 14/10 h with light intensity of 120 ±10 µmol m^{-2} s^{-1}, and relative air humidity of 70%.

After 10 days under different temperature conditions, plants were inoculated with one of the three avirulent (race 0) isolates of *E. turcicum* from a warm region in south Brazil (isolates B38-1, B42-2, and B53-3) or one of the three avirulent (race 0) isolates from a region with mild temperatures in Germany (isolates: D6-4, H151, Nes18-4); characterized in previous works (Hanekamp, 2016; Navarro *et al.*, 2021b). Following the nomenclature proposed by Leonard *et al.* (1989), isolates named race 0 are able to infect and cause disease only in maize plant bearing no *Ht* resistance genes. Each plant received 7 ml of conidia suspension and was maintained in under high relative air humidity (>95%) for 24 h. After inoculation, all plants were maintained under day/night temperature conditions of 25/20°C, with a light/dark photoperiod of 14/10 h, relative air humidity of 70%, and light intensity of 120 ±10 µmol m^{-2} s^{-1}. The experiment was replicated once. The disease severity was evaluated visually based on diagram assessment (Pataky, 1992), considering necrosis, at 14 and 21 days post inoculation (dpi).

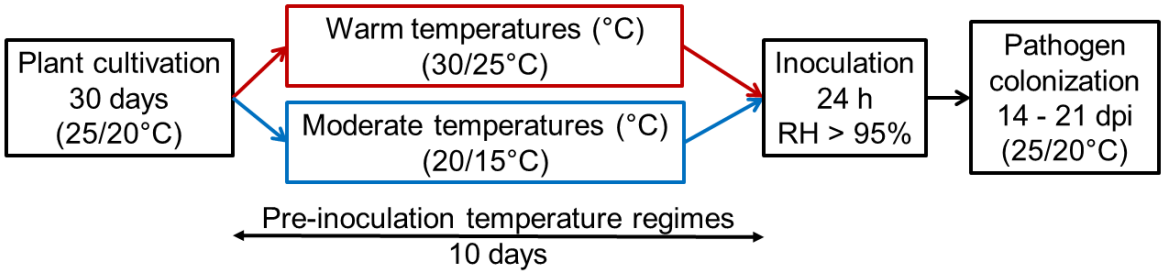


Figure 1. Outline of temperature conditions during the experimental procedure comparing the disease severity and fungal DNA content caused by different *Exserohilum turcicum* isolates on the maize lines B37 and B37*Ht2*. RH relative air humidity; dpi – days post-inoculation.

Fungal DNA quantification in planta

Exserohilum turcicum DNA was isolated from eight infected leaves sampled at 21 dpi. The fifth leaf from two plants of the same line cultivated in the same pot were pooled and frozen at -20°C, totaling four replications. Three technical replicates were performed for each biological replicate. Additionally, the race 0 isolate B38-1 was cultivated in liquid Czapek

Dox Medium at 22°C in the dark in order to obtain a DNA standard. The mycelial culture was shaken at 100 rpm for 14 days. Then, it was filtered by vacuum suction. Mycelia were frozen at -20°C, lyophilized (Zimbus technology, Germany), and ground with a swing mill (Retsch® MM400, Germany). Genomic DNA (gDNA) was extracted using a cetyltrimethylammonium bromide (CTAB)-based method. Therefore, 1 ml of CTAB-buffer (20 mM Na- ethylenediaminetetraacetic acid (EDTA), 0.13 M sorbitol, 30 mM N-laurylsarcosine, 20 mM CTAB, 0.8 M NaCl, 10 nM Tris – pH 8.0 adjusted with NaOH) was added to 50 mg ground leaf sample. Each sample received 0.6 U of proteinase K (Roth®, Germany). The samples were treated in an ultrasonic bath for 5 s prior to10 min incubation at 42°C and a 10 min incubation at 65°C (tubes were shaken three times during the incubation). After incubation, 800 µl of chloroform-isoamyl alcohol (24:1) was added and tubes were shaken. Samples were incubated for 10 min on ice, and then centrifuged at 13 000 *g* for 10 min (Hettich Zentrifugen Mikro 220R, Germany). The supernatant was transferred to another tube with 200 µl of 30% (w/v) polyethylene glycol (PEG) and 100 µl 5 M NaCl. The pellet was washed with 70% (v/v) ethanol, and then dried at room temperature for 60 min. The dry pellet was dissolved in 100 µl Tris-EDTA buffer pH 8.0 (0.1 M Tris, EDTA 10 mM) and stored at -20°C.

The DNA concentration was quantified using gel electrophoresis. As the DNA concentration was higher than 10 ng μl^{-1}, all samples were diluted 1:10. The dilution factor was considered in further calculations. A standard curve was obtained by diluting the extracted fungal DNA from 1000 to 0.01 pg μl^{-1} (1000, 100, 10, 1, 0.1, 0.01 pg μl^{-1}) to quantify the target gene by qPCR. Data were analyzed using the software BioRad CFX Maestro 1.1 (Bio-Rad, USA). The DNA content of *E. turcicum* was determined by quantitative polymerase chain reaction (qPCR) amplification of the internal transcribed spacer (ITS1 and ITS2) regions (Beck 1998). Therefore, the forward primer JB 586 (5'-TGGCAATCAGTGCTCTGCTG-3') and the reverse primer JB 595 (5'-TCCGAGGTCAAAATGTGAGAG-3') were used resulting in an amplicon size of 485 base pairs. qPCR reactions were performed with 5 µl of the premix qPCR BIO SyGreen Mix Lo-ROX (PCR Biosystems, London, UK) with a primer concentration of 0.4 µM and 1 µl of the DNA sample in the CFX384 Thermocycler (Biorad, Rüdigheim, Germany) in 384 well microplates (SARSTEDT AG and Co. KG, Nümbrecht, Germany). The final volume of the reaction was 10 µl. The qPCR was performed starting with an initial denaturation step at 94°C for 3 min, followed by 40 cycles of 94°C for 5 s , 63.5°C for 15 s, and 72°C for 15 s, with a final elongation of 5 min at 72°C. Data analysis was performed with the software BioRad CFX Maestro 1.1 (Bio-Rad, USA).

Data analysis

Data of disease severity and fungal DNA content were analyzed with software Statistica 13.0 (Statsoft, Tulsa, USA), and graphs were plotted with the software Microsoft Excel 2016. Data of disease severity and fungal DNA content were transformed for Box-Cox and fitted to a linear mixed model, with maize lines and temperature as fixed factors and replications and isolates as random effects (p-value ≤ 0.05) provided in the package lme4 from the R software 3.6.0 (Core Team 2019).The homogeneity of variance for transformed data of disease severity between repetitions was analyzed by Bartlett's test (p-value ≤ 0.05). Correlations between fungal DNA and disease severity were fitted to the exponential model for each maize.

Results

Disease symptoms in the susceptible line B37 kept at moderate and warm temperatures before inoculation were characterized by strong necrosis or dead leaves, with an average of 66.8% for plants kept at moderate temperatures and 70.0% for plants kept at warm temperatures (Figure 2). However, there were no significant differences in the disease severity between plants of the line B37 maintained under different pre-inoculation temperatures (Table 1, Figure 3A). The fungal DNA content in plants of the line B37 was in average 5943.62 ηg DNA/ g DW for plants kept at moderate temperatures and 5570.89 ηg DNA/ g DW for plants kept at warm temperatures (Figure 3B). As observed for disease severity, no significant differences were observed in the DNA content of *E. turcicum* in plants of the line B37 maintained under different pre-inoculation temperatures (Table 1). The variance in DNA content was explained by the exponential relationship with disease severity for the susceptible line B37. The exponential model was fitted (p-value ≤ 0.01) to data of fungal DNA content as a function of disease severity for B37 ($r^2 = 0.31$) (Figure 4A).

The resistance phenotype in the line B37*Ht2* were characterized by chlorosis and necrosis for plants kept at moderate and warm temperatures before *E. turcicum* inoculation (Figure 2). No significant differences were observed between the pre-inoculation temperatures for the data of disease severity of the line B37*Ht2* (Table 1). The average disease severity of B37*Ht2* was 4.9% for plants kept at moderate temperatures and 15.4% for plants kept at warm temperatures before inoculation of *E. turcicum* (Figure 3A). No significant differences were observed in the DNA content of *E. turcicum* for plants of the line B37*Ht2* maintained under different pre-inoculation temperatures (Table 1). The fungal DNA content in plants of the line B37*Ht2* was in average 93.59 ηg DNA/ g DW for plants kept at moderate temperatures and 138.58 ηg DNA/ g DW for plants kept at warm temperatures (Figure 3B).

No relationship was observed between the data of fungal DNA content and disease severity in the resistant line B37*Ht2* (Figure 4B).

The disease severity and the fungal DNA content were higher for the line B37 when compare to the line B37*Ht2* (*p*-value ≤ 0.001) (Table 1). However, there was no significant differences between the pre-inoculation temperatures for data of disease severity (*p*-value = 0.19) and for the data of fungal DNA content (*p*-value = 0.65). The interaction between pre-inoculation temperatures and maize lines was not significant different for the data of disease severity and fungal DNA content.

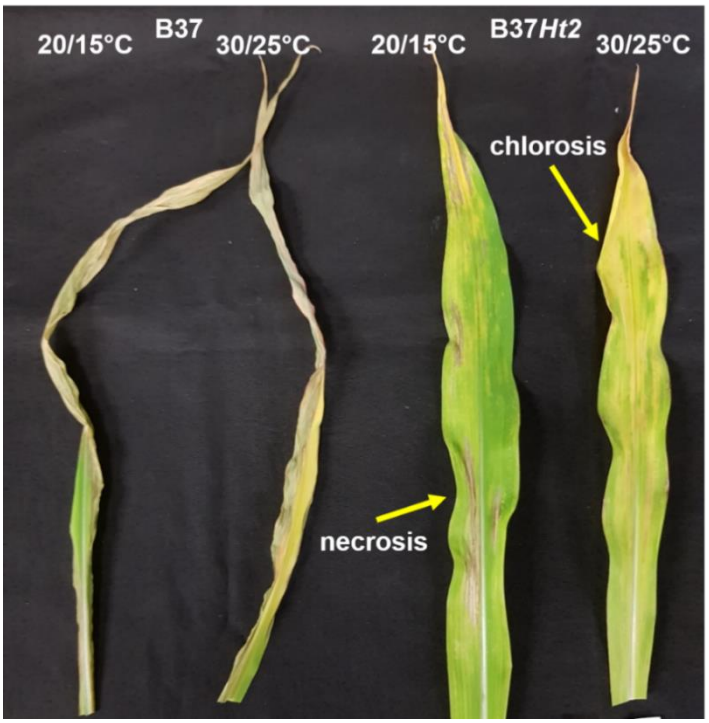


Figure 2. Symptoms of *E. turcicum* on the maize lines B37 and B37*Ht2* maintained during 10 days at different pre-inoculation temperatures (day/night regimes of 20/15°C and 30/25°C), and followed by inoculation with Brazilian isolate B38-1.

Table 2. Effect of pre-inoculation temperature, maize lines and their interactions on the Box-Cox transformed data of disease severity and on the Box-Cox transformed data of fungal DNA content based on a mixed model analysis of variance.

Effect	Disease severity (Box-Cox transformed)				Fungal DNA content (Box-Cox transformed)			
	df_n[1]	df_d[2]	*F*-value	*p*-value	df_n[1]	df_d[2]	*F*-value	*p*-value
Temperature	1	381.19	1.69	0.1937	1	182.02	0.20	0.65
Line	1	381.22	768.13	< 0.001	1	182.04	871.02	< 0.001
Temperature x Line	1	381.05	2.74	0.0990	1	182.04	0.91	0.34

[1] Numerator degrees of freedom (df_n), [2] denominator degrees of freedom (df_d); degrees of freedom calculated using the Satterthwaite formula for a mixed model; *F*-value for testing effect and probability (significance) level of *F* -value (*p*-value).

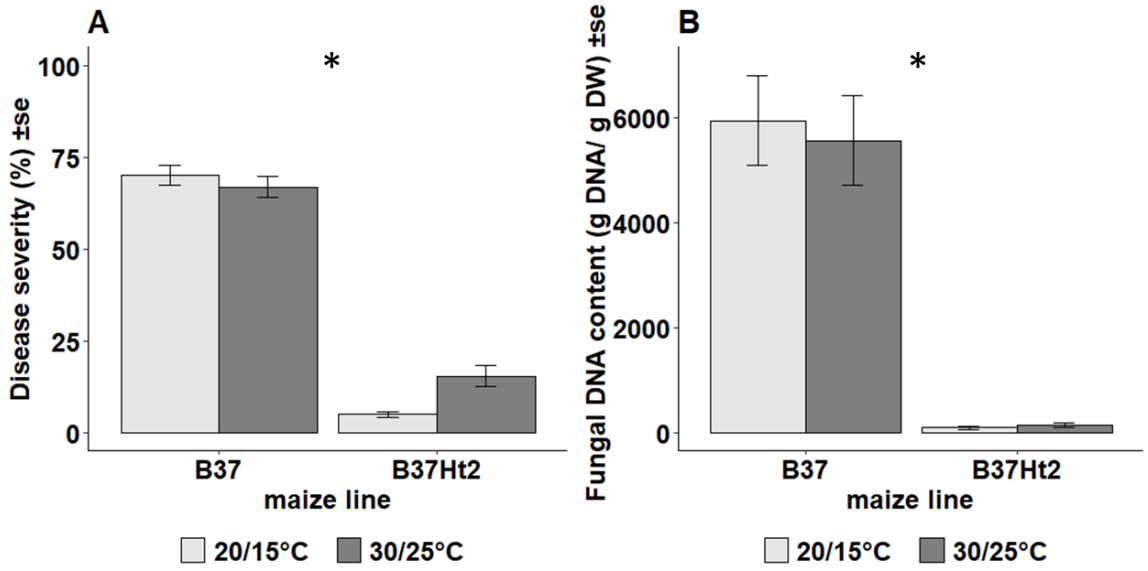


Figure 3. NCLB severity (n = 8 plants) in percentage (A) according to the diagrammatic scale of Pataky (1992), and *Exserohilum turcicum* DNA content per dry weight (ηg DNA/ g DW) (n = 4 pools of two leaves from two different plants) (B) in maize plants maintained under different pre inoculation temperature regimes (day/night temperatures of 20/15°C and 30/25°C) 10 days before inoculation with 3 *E. turcicum* isolates from Brazil (B38-1, B42-2, B53-1) and 3 *E. turcicum* isolates from Germany (D6-4, H151, NES18-4). The asterisk represent significant differences between lines for the evaluated parameters (*: *p*-value ≤ 0.05). Bars indicate standard error.

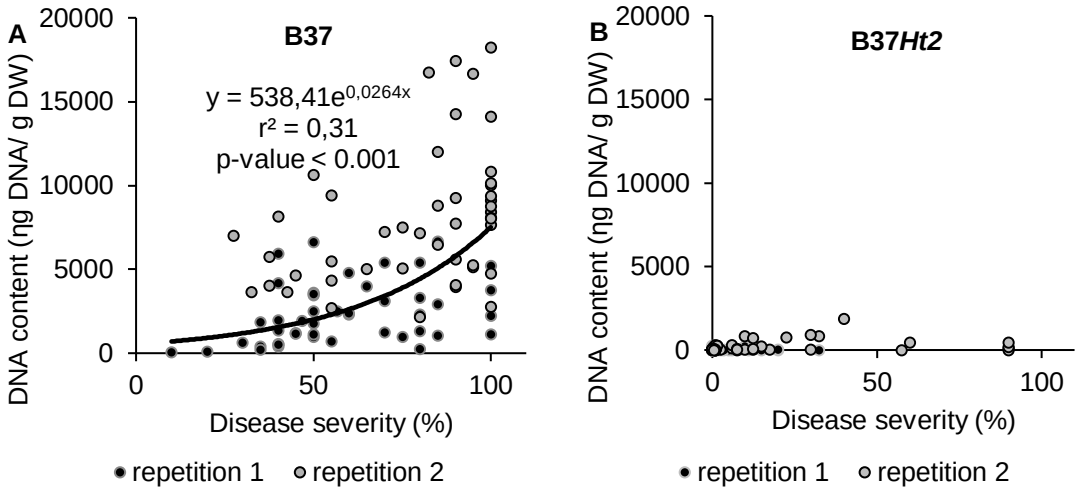


Figure 4. DNA content of *Exserohilum turcicum* per dry weight (ng DNA/ g DW) in function to NCLB severity (%) in maize plants from the susceptible line B37 (A) and for the resistant line B37*Ht2* maintained under different pre inoculation temperature regimes (day/night temperatures of 20/15°C and 30/25°C) 10 days before inoculation with 3 *Exserohilum turcicum* isolates from Brazil (B38-1, B42-2, B53-1) and 3 *E. turcicum* isolates from Germany (D6-4, H151, NES18-4). Black points represent the first experimental repetition and grey points represent the second experimental repetition.

Discussion

In our experiments, pre-inoculation temperatures (Figure 1) had no effect on the disease severity of NCLB, or on *E. turcicum* DNA content *in planta* (Figure 3). Therefore, pre-inoculation temperature did not influence the efficacy of resistance promoted by the *Ht2* gene. A correlation was established between the fungal DNA content and disease severity in the compatible interaction. In susceptible plants, fungal DNA content increased exponentially with the increase in disease severity. However, no correlation was observed between the fungal DNA content and the disease severity in the line B37*Ht2.* The disease severity was only assessed by estimating the necrotic area as observed in the diagrammatic scale from Pataky (1992), while chlorosis was not considered in the disease quantification. The necrosis is usually correlated to phytotoxin production (Robeson and Strobel, 1982), whereas chlorosis has been described as mostly related to resistance (Bigirwa *et al.*, 1993; Hanekamp, 2016). The necrosis observed at 30/25°C on B37*Ht2* might be related to a resistance reaction or heat stress, as a low content of fungal DNA was quantified for those samples (Figure 2).

Pathogen virulence to the *Ht2* gene is difficult to characterize by disease phenotyping. Navarro *et al.* (2020) reported chlorosis and small necrotic lesions in avirulent isolates

inoculated on B37*Ht2* (incompatible interaction). The assessment of disease phenotype is described by a categorical scale of different symptoms, or infection types (Bigirwa *et al.*, 1993; Hanekamp, 2016). Pathogen virulence or avirulence are characterized according to the given score from this categorical scale (Navarro *et al*. 2021b). In this scale, when a plant reaction is classified in the scores 1, 2 and 3, the isolate is designated as avirulent (incompatible interaction), whereas in a plant reaction that is classified in the scores 4, 5 and 6, the isolates will be designated as virulent (compatible interaction). Thus, categorical data (scale from 1-6) are transformed into binary data (avirulent/virulent), and information regarding host and/or pathogen fitness are not taken in consideration (Kosman *et al.*, 2019). The methodology for data analysis considering the infection type, which was proposed by Kosman *et al.* (2019) may improve disease phenotyping, especially for the line B37*Ht2*, by avoiding misinterpretations between incompatible and compatible interactions. The incompatible interaction with B37*Ht2* can be confused as this line displays small necrotic lesions and chlorosis.

The DNA quantification was suitable for distinguishing between resistant and susceptible lines, as observed for *Colletotrichum graminicola* in maize (Weihmann *et al.*, 2016). However, fungal DNA content is not necessarily positively correlated with disease symptoms, and disease severity (necrosis) (Mahlein, 2016; Nutter Jr, 2001). Additionally, the diagnosis by qPCR is destructive, more laborious and requires skills in molecular biology (Mahlein, 2016). Recently, hyperspectral sensors have been studied for the identification of resistant plants. The screening for resistance requires a hyperspectral microscopic approach that is able to detect early and marginal changes in the host tissue. However, multiple observations during the crop cycle are required to evaluate barley resistance types to *Blumeria graminis* f. sp. *hordei* (Mahlein, 2016; Kuska *et al.*, 2015), which demonstrate that the method need improvements. With the advance of new technologies, hyperspectral sensors to detect diseased plants may be improved. These approaches might be tested for hemibiotrophic pathogens, such as *E. turcicum,* especially for improving the assessment of physiological races.

Lower levels of resistance reported for the *Ht2* gene (Hooker, 1977; Cota *et al.*, 2010; Navarro *et al.*, 2021a) might be due to the suppression of resistance expression of the *Ht2* gene by the *Sht1* gene, and/or by incomplete dominance of the *Ht2*, as described by Ceballos and Gracen (1989) and Chung *et al.* (2010), respectively. The incomplete dominance of the *Ht2* gene can be associated with a gene dosage effect (Chung *et al.*, 2010). In homozygous individuals, the higher expression of resistance genes confers higher resistance levels, due to the more effective perception of pathogen invasion and activation of host resistance responses (Chung *et al.*, 2010). Maize plants homozygous for the *Hm2*

resistance gene (*Hm2/Hm2*) to *Cochliobolus carbonum,* which encodes a HC-toxin reductase, presented higher levels of resistance and *Hm2* transcript levels when compared to heterozygous plants (*Hm2/hm2*) (Chintamanani *et al.*, 2008). Curiously, heterozygous plants (*Ht1/ht1*) for the dominant resistant gene *Ht1* were less resistant than homozygous plants (*Ht1/Ht1*) (Dunn and Namm, 1970). The decrease on spore germination was observed for homozygous plants due to increase on phytoalexin production (Calub *et al.*, 1973). These reports about the gene dosage effect by *Ht1*, basically exclude the hypothesis of lower levels of resistance for *Ht2* due to seed contamination, as postulated by Weems and Bradley (2018).

The influence of different environmental conditions, such as post-inoculation temperatures and/or light intensity, is often reported in the pathosystem maize-*E. turcicum* (Thakur *et al.*, 1989a; Leath *et al.*, 1990; Carson and van Dyke, 1994). High temperatures can increase the production of aggressiveness factors, such as phytotoxins, increasing the damage caused by the pathogen in the host tissue (Cuq *et al.*, 1993). The increase on phytotoxins concentration resulted on an increase on lesion size and infection efficiency (Bashan *et al.*, 1992). Moreover, temperature can also affect host physiology by altering plant resistance mechanisms, such as the detoxification of phytotoxins (Pedras *et al.*, 2001). Lastly, *E. turcicum* has been described as more aggressive as post-inoculation temperatures increase (Navarro *et al.*, 2021). However, our experiments prove that variations in pre-inoculation temperatures do not influence the efficacy of resistance carried by the *Ht2* gene against *E. turcicum*. Besides methodological advances in isolate screening and/or resistance phenotyping, more studies are necessary to describe how expression of the *Ht2* gene is up- and down-regulated, and consequently what pathways are activated to trigger resistance. The recognition of pathogen invasion, which is activated by signaling cascades, is not well understood. Moreover, studies regarding the expression of avirulence genes, phytotoxin production and detoxification might be conducted to elucidate the role of pathogen under higher temperatures. Weaker resistance levels might be correlated to the presence of the dominant gene *Sht1*, and to the incomplete dominance of the *Ht2* gene. Therefore, the epistatic effect promoted by the *Sht1* gene and the *Ht2* gene dosage effect needs to be elucidated in more detail.

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Chapter 6. *Fusarium* spp. causing maize ear rot in Germany and Brazil: a comparison of pathogen aggressiveness and host resistance

Abstract

Diseases caused by *Fusarium* spp. infecting maize ears can be classified in two main groups: Gibberella ear rot (GER) and Fusarium ear rot (FER). GER is mainly caused by *F. graminearum* species complex, while FER is caused by species from the *F. fujikuroi* species complex. GER is prevalent in regions with colder temperatures and high precipitation, especially during the flowering period. FER occurs at a higher frequency in warm and dry areas, especially with dry conditions during grain-filling. The prevalence of *Fusarium* spp. in Germany and Brazil can be strongly related to diverse weather conditions. In Germany, the most prevalent species is *F. graminearum* and the most commonly detected mycotoxins are DON and ZEA. Conversely, the most prevalent species in Brazil is *F. verticillioides,* with samples being contaminated with high levels of fumonisins. The main objective this study was to compare GER and FER in regard to European and South American maize production conditions. For this purpose, (1) the aggressiveness levels of German and Brazilian isolates were compared by inoculating two reference lines (Dent 21 and Dent 4); (2) the resistance of European (temperate: A12, A3, A5 and A8) and South American (tropical: T3 and T4) maize lines was tested under greenhouse conditions; and (3) the resistance of tropical lines (T3 and T4) were tested for two pre-inoculation temperature regimes: warm (30/25°C) and mild (20/15°C). The resistance of temperate and tropical maize lines; and the effect of the pre-inoculation temperature to the tropical lines were tested for *F. graminearum* and *F. verticillioides* isolates originating in both countries. Inoculations were performed by the method of silk-channel injection, and the disease severity was estimated in the stage of full maturity. In general, *F. graminearum* was more aggressive than *F. verticillioides*. The German *F. culmorum* and *F. graminearum* were more aggressive than were the respective Brazilian strains. German isolates were more aggressive than Brazilian isolates when inoculated in the tropical lines. It is essential to preselect highly aggressive strains before starting to select for resistance. In addition, plants pre-exposed to higher temperatures presented higher disease severity when compared to plants exposed to mild temperatures.

Keywords: Gibberella ear rot, Fusarium ear rot, *Fusarium graminearum*, *Fusarium verticillioides*, pre-inoculation temperature, tropical and temperate lines

Introduction

Fusarium spp. are soilborne pathogens that are spread worldwide, mostly due to their broad host range (Miedaner *et al.*, 2001). Besides the large number of hosts, *Fusarium* spp. can infect distinct plant organs. For instance, *Fusarium* spp. can cause symptoms on maize roots, seedlings, stalks and ears (White, 2010; Oldenburg *et al.*, 2017). Yield losses caused by *Fusarium* spp. infecting maize ears and stubbles are estimated to be between 10 and 30% (Logrieco *et al.*, 2002), especially when considering qualitative yield losses due to mycotoxin contamination. The consumption of maize products with a high mycotoxin content can be poisonous to humans and animals leading to several diseases (Blacutt *et al.*, 2018). *Fusarium* spp. can cause two different ear rots: Gibberella ear rot (GER or red ear rot) and Fusarium ear rot (FER or pink ear rot). GER symptoms are usually characterized by a red or pink mold, which covers large areas of the ear, whereas FER symptoms are usually white to light pink mold in random kernels (Munkvold, 2003b). The colonization of maize kernels by FER can sometimes be symptomless. Besides asymptomatic kernels, FER can have systemic colonization of the entire maize plant (Munkvold, 2003d; Gai *et al.*, 2018). Additionally, GER is caused by *F. graminearum* species complex (FGSC), while FER is mainly caused by *F. fujikuroi* species complex (FFSC) (Munkvold, 2003b; White, 2010; van der Lee *et al.*, 2015).

Fusarium spp. populations can also vary according to their chemotypes. FGSC produces deoxynivalenol (DON), 3-acetyl- and 15-aceytil-deoxynivalenol (3-ADON and 15-ADON), Nivalenol (NIV), which are toxins for the type B trichotecenes, and Zearalenone (ZEA) (Logrieco *et al.*, 2002; van der Lee *et al.*, 2015). FFSC are fumonisin producers, which includes B series fumonisins (FB1, FB2, FB3 and FB4) (van der Lee *et al.*, 2015; Blacutt *et al.*, 2018). The frequency of FGSC and FFSC can vary according to the location and environment (Reid *et al.*, 1999). FGSC usually is predominant in areas with colder temperatures and higher precipitation during the crop season. High humidity levels during flowering (more precise silking) favors fungal infection (Munkvold, 2003b). Moreover, species prevalence may change according to the weather conditions of different seasons (Miedaner *et al.*, 2010; Goertz *et al.*, 2010; Pfordt *et al.*, 2020). Colonization is stimulated by moderate temperatures and high levels of precipitation during maturity (Munkvold, 2003b). Conversely, FER is observed in warm and dry areas, especially with drier weather during the grain-filling stage (Munkvold, 2003b; Oldenburg *et al.*, 2017). The detection of distinct mycotoxins reinforces the incidence of distinct *Fusarium* spp., according to weather conditions. In Germany, the most prevalent *Fusarium* spp. in maize samples are *F graminearum, F. verticillioides* and *F. temperatum* (Pfordt *et al.*, 2020), and the most commonly detected mycotoxins are DON and ZEA (Goertz *et al.*, 2010). In Brazil, the most

prevalent *Fusarium* spp. detected in maize kernels are *F. verticillioides*, and *F. graminearum* species (Ottoni, 2008; Stumpf *et al.*, 2013) and the samples are mostly contaminated with fumonisins (FB1, FB2, FB3) (Almeida *et al.*, 2002; Ottoni, 2008; Lanza *et al.*, 2014). Furthermore, a molecular analysis of Brazilian *F. verticillioides* isolates demonstrated that the *Fusarium* spp. population can be genetically diverse (Silva *et al.*, 2017) within the same species complex. In southern Brazil, *F. meriodionale* presenting the NIV genotype was the most frequently detected, followed by *F. graminearum* with 15-ADON, and *F. coraderiae* presenting NIV and 3-ADON genotypes (Kuhnem *et al.*, 2016). Moreover, *F. corderiae* is most frequent in elevated areas (>800 metres above sea level – m.a.s.l.), which have mild temperatures, whereas *F. meridionale* is more frequent in lower regions (<800 m.a.s.l.) (Kuhnem *et al.*, 2016). Therefore, *Fusarium* spp. and mycotoxin production may vary not only depending on the weather conditions of every season but also according to the location.

The main control practices applied for GER and FER are based on chemical control, cultural practices and host resistance. Chemical control consists of the application of fungicides, however, fungicide sprays are not very effective at controlling *Fusarium* spp. (Munkvold, 2003a; Lanza *et al.*, 2016), since the efficacy is related to the timing of the application. The best timing is during mid-flowering (Andriolli *et al.*, 2016), when infections through sink-channels may occur (Reid *et al.*, 1999). Besides fungicide applications, cultural practices, such as low fertilization, low irrigation (water availability), late planting date, tillage, crop rotation (Munkvold, 2003a), and insect control (Degraeve *et al.*, 2016), may affect disease incidence and mycotoxin production. Nitrogen fertilization, for example, is negatively correlated to fumonisin levels (Ono *et al.*, 2011). Water stress in the early season is related to the increase in stalk rot (Sumner and Hook, 1985). In Brazil, the increased risk of water deficit, especially during the second season (winter season or “safrinha”), may increase the susceptibility to *Fusarium* spp. infections (Stefanello *et al.*, 2012; Costa *et al.*, 2019). Moreover, tillage has a slight influence on *Fusarium* spp. stalk and ear rot in South Africa (Flett and Wehner, 1991; Flett *et al.*, 1998) and in Tanzania (Degraeve *et al.*, 2016). In Germany, ploughed fields have less ear rot infection by *F. graminearum* when compared to reduced tillage (Pfordt *et al.*, 2020). Furthermore, crop rotation apparently has no effect on *Fusarium* infections. In Germany, maize, wheat and sugar beet as previous or pre-crops show no differences on ear infection of *F. graminearum* and *F. temperatum* (Pfordt *et al.*, 2020). In Brazil, maize or soybean do not increase the risk of *Fusarium* head blight in wheat (Spolti *et al.*, 2015). Conversely, oats as the previous crops followed by no-till maize increased the levels of fumonisins (Ono *et al.*, 2011). However, when comparing tillage systems, no significant differences are observed between conventional and no-till systems

(Ono *et al.*, 2011). Briefly, tillage and previous crops have little influence on ear infection in Germany (Pfordt *et al.*, 2020), and in Brazilian fields (Ono *et al.*, 2011). Lastly, insect control may have an important effect on *Fusarium* infection, and, consequently on mycotoxin content (Degraeve *et al.*, 2016). Caterpillars can spread the pathogen and provoke wounds, which increase ear and stalk susceptibility (Munkvold, 2003b). In Europe, the European corn borer (*Ostrinia nubilatis*) has an important role in disease incidence and spread (Oldenburg *et al.*, 2017; Blacutt *et al.*, 2018). Furthermore, insecticide sprays reduce insect damage, and consequently, levels of fumonisins decrease for some treatments (Curtis *et al.*, 2011).

In addition to insecticide spraying, transgenic resistance is also associated with insect control, and consequently, host resistance against *Fusarium* spp. (Munkvold, 2003a). Transgenic maize hybrids (*Bt* maize) consist of the introduction of *cry* genes originating in *Bacillus thuringiensis*. The expression of *cry* genes results in the production of insecticidal crystalline proteins in the maize plant. *Bt*-maize plants confer some level of resistance to feeding by the European corn borer, so a consequent decrease in Fumonisin levels is observed (Munkvold *et al.*, 1999). Besides transgenic maize hybrids, host resistance plays an important role in GER and FER disease control. Susceptible maize hybrids, showing moderate levels of GER disease severity, showed up to 48% of yield losses (Vigier *et al.*, 2001). As *Fusarium* spp. have a broad range of hosts (Nicolli *et al.*, 2018), sources of qualitative resistance (major genes) are scarce. *In vitro* tests demonstrated that a dominant gene conferred resistance to infection via silk. However, the resistance was not effective under field conditions (Reid *et al.*, 1994). Thus, quantitative resistance is used in commercial hybrids (Munkvold, 2003a).

The quantitative resistance against *Fusarium* ear infection is classified into distinct types according to the resistance mechanism: silk-channel resistance and kernel resistance (Mesterházy *et al.*, 2012). The silk-channel resistance is correlated to a closed stylar canal, which avoids the conidia to achieve the pericarp (Reid *et al.*, 1992b; Duncan and Howard, 2010). Silk resistance can be tested by spraying a conidia suspension on the silk, or by injecting the inoculum through silk-channels using a syringe (Mesterházy *et al.*, 2012). The silk-channel injection permit to test for resistance in other plant tissues besides the silk-channels, such as husk leaves and kernel pericarp (Mesterházy *et al.*, 2012). In general, tighter husk leaves may increase moisture, which favors pathogen infection, and consequently the disease levels (Mesterházy *et al.*, 2012). *Fusarium* spp. are able to penetrate maize leaves through the stomata, trichomes and directly by forming an appressoria-like structure. The penetration usually occurs in the corner of cell walls (Nguyen *et al.*, 2016a, 2016b). In addition, pericarp resistance was shown by a thicker wax layer and

probably by its composition (presence of phenolic compounds) (Sampietro *et al.*, 2009; Lanubile *et al.*, 2017). The silk-channel injection method showed the best correlation between disease severity and mycotoxin concentration (Clements *et al.*, 2003; Mesterházy *et al.*, 2012). Lastly, kernel resistance was tested by wounding 3-4 kernels in the center of the ear with pins that were previously immersed on a conidia suspension (Reid and Hamilton, 1996). This method simulated transmission and infection through wounds caused by insects feeding (earworms) (Blacutt *et al.*, 2018).

As *Fusarium* spp. are known to cause important maize diseases in Germany and Brazil, the objective of this work was to compare GER and FER in a European and South American maize production scenario. Pathogen aggressiveness was compared between isolates collected in Germany and in Brazil. Moreover, the host resistance may influence disease intensity, thus the resistance of European (temperate) and South American (tropical) maize lines was compared under greenhouse conditions. The resistance of temperate and tropical maize lines was tested for *F. graminearum* and *Fusarium verticillioides* isolates originating from Germany and Brazil. Finally, the resistance of tropical lines was tested in warm (30/25°C) and mild (20/15°C) temperatures before inoculation. The effect of temperature in the resistance of tropical lines was also tested for *F. graminearum* and *Fusarium verticillioides* isolates originating in both countries.

Materials and Methods

Isolation and species identification

Samples from maize stalks and ears showing symptoms similar to stalk and ear rot were collected from fields in Regensburg, Bernberg, Gondelsheim (Germany) and Moncelice (Italy) in 2017 in Europe. In South America, samples were collected from maize-producing areas in Argentina (Pergamino) in 2015 and Brazil (States of Paraná, São Paulo and Minas Gerais) in 2017/2018. In total, 42 strains from *Fusarium* ssp. were isolated from Argentinian and Brazilian samples. The amount of sampling material from Europe was higher, as 716 strains were isolated from European material in total. Stalks were divided into three sub-samples: ower nodium, internodium and upper nodium; cobs were divided into three regions; upper, middle and lower. Each region was cut into 3 sub-pieces, totalling 9 subsamples per stalk and per cob. In addition, 30 kernels were randomly selected from each cob. All pieces and kernels were surface-sterilised for 10 min in a solution of 0.1% silver nitrate ($AgNO_3$) (Pfordt *et al.*, 2020). After surface-sterilisation, the material was washed with sterile water, dried in sterile filter paper and incubated on potato-dextrose-agar (PDA) containing two antibiotics, 200 ppm streptomycin and 40 ppm rifampicin (dissolved in methanol 100% and diluted in sterile water 1:1). After two to three days, when it was

possible to visualize some mycelia growth, a small piece of the colony was transferred to PDA plates and to synthetic low nutrition agar (SNA) for *Fusarium* species identifications. Single-spore cultures were produced from the selected isolates. Moreover, a spore suspension was prepared in a low concentration $6.25*10^4$ spores ml^{-1} to produce single spore colonies. A drop of 50 ml of the diluted spore suspension was transferred to a new PDA plate and distributed using a Drigalski spatula. After two days of incubation, small colonies were transferred to new PDA plates. Single-spore isolates were stored in SNA plates at 4°C for further experiments.

The *Fusarium* spp. complex was identified by analysing macroscopic characters, such as the colour and mycelia texture of PDA colonies, and microscopic characters such as macro- and/or microconidia production, conidiophores morphology, the presence or absence of chlamydospores, and fruiting bodies (sporodochia) (Leslie and Summerell, 2006). Macro- and microscopic characters were analyzed on 14 days-old PDA and SNA colonies (Figure 1). To distinguish *Fusarium* at the species level, it is necessary to conduct some molecular analysis to identify strains (O'Donnell *et al.*, 2004); therefore, in this work *F. graminearum* is referred to as FGSC (Figure 1A-B) and *F. verticillioides* is referred to as FFSC (Figure 1C-D). Further isolates tested in the aggressiveness experiments were indentified in the species levels according to the morphological criterion.

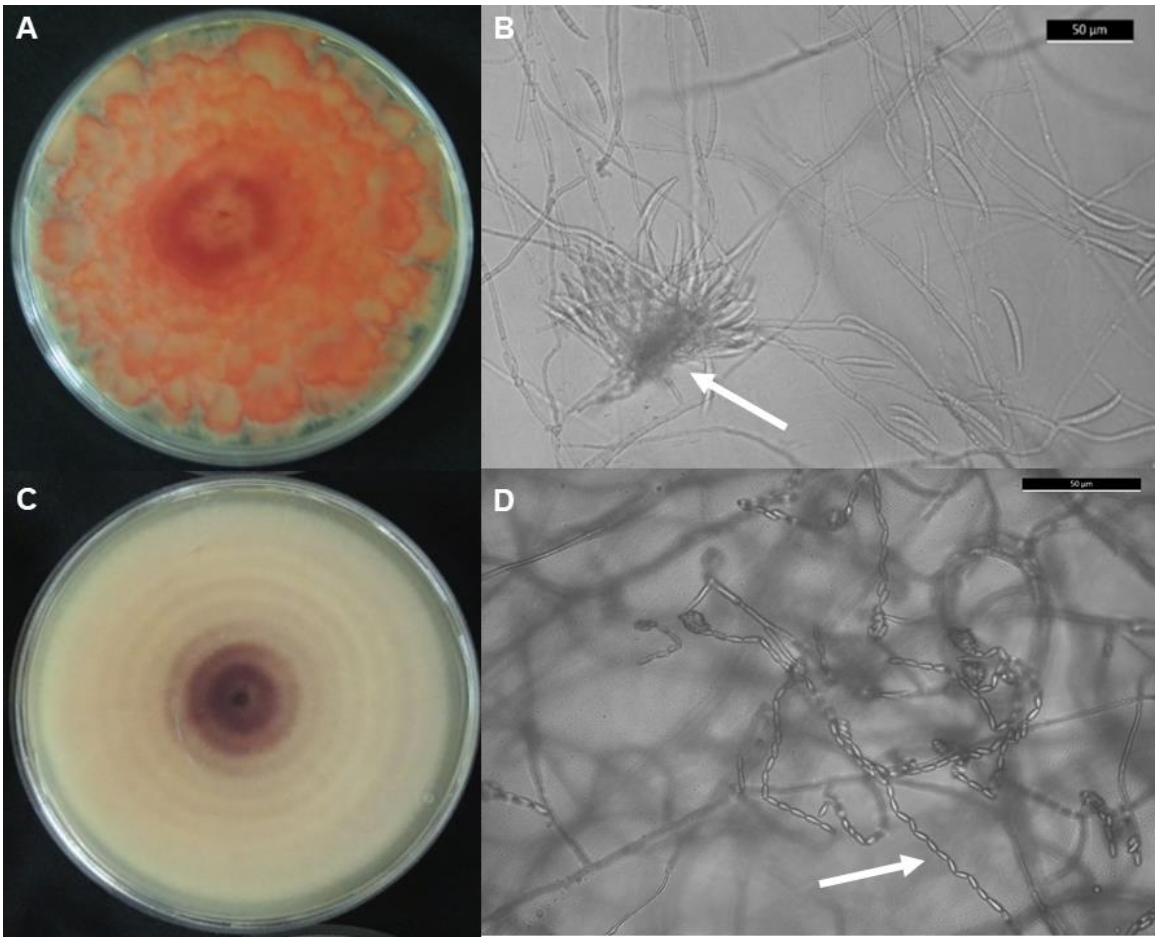

Figure 1. PDA plate (A,C), and conidia produced in SNA plate (B,D) by isolates of *Fusarium graminearum* species complex (A-B) and *Fusarium verticillioides* species complex (C-D) from 21 days-old colonies. *F. graminearum* produces fruting bodies, named sporodochia, which is represented by the white arrow (B). *F. verticillioides* conidiophores forms conidia in chains (D).

Cultivation of plant material, inoculation and disease assessment

Plants were sown in pots (18 cm x 18 cm x 18 cm) with soil mixtures with the following proportions: 3x compost, 3x substrate, 1x sand, and fertiliser Osmocote® Exact – Stander 3-4M (16% N, 9% P_2O_5, 12% K_2O, 2% MgO+TE) mixed in a concentration of 3 g l^{-1}. Seeds were provided by B. Kessel (KWS, Einbeck, Germany). Two ventilators were set-up in the greenhouse to simulate wind and allow pollination. The *Fusarium* spp. used in the experiments were chosen based on the most prevalent species in Germany (Pfordt *et al.*, 2020) and in Brazil (Ottoni, 2008; Stumpf *et al.*, 2013; Lanza *et al.*, 2014). As the main interest was to simulate natural conditions for *Fusarium* spp. infection in both conditions (cold and warm weather conditions as observed in Germany and Brazil, respectively), and to test silk-channel resistance, the inoculation method performed in our experiments was

silk-channel injection (Papst *et al.*, 2007; Nerbass *et al.*, 2015; Nerbass *et al.*, 2016). The first cob was inoculated with 1 ml of spore suspension (spore concentration of 1.5 10^4 spores $ml^{-1)}$ at flowering stage (BBCH 63-65), when silk-channels were apparent (Landcashire *et al.*, 1991). Disease assessment was based on a diagrammatic scale (EPPO Guidelines, 2015).

Aggressiveness between isolates from Germany and Brazil

The aggressiveness of German and Brazilian isolates was tested for the species *F. culmorum, F. equiseti, F. graminearum, F. tricinctum* and *F. verticillioides*. *Fusarium* spp. were chosen based on the presence of isolates from South America, as a smaller number of strains, and consequently *Fusarium* spp. were available. All isolates were inoculated in two reference lines: Dent-4, and Dent-21. In previews experiments conducted by breeders, Dent-4 and Dent-21 showed a disease intensity by *Fusarium* spp. of 5% (resistant line) and 44% (moderate susceptible line), respectively. The maize plants were cultivated in the greenhouse (temperature 21 ± 6°C, 65%± 15 air humidity, day/night light regime 14/10 h with light intensity of 600 ± 200 µmol m^{-2} s^{-1}). The experiment was performed twice (in 2018 and 2019).

Host resistance of temperate and tropical maize lines

The host resistance from four temperate (A12, A3, A5, and A8) and two tropical (T3, and T4) maize lines was tested by inoculating the two most frequent *Fusarium* spp. complex in Germany and Brazil: *F. graminearum* and *F. verticillioides*. Maize plants were cultivated in the greenhouse (temperature 21 ± 6°C, 65%± 15 air humidity, day/night light regime 14/10 h during with light intensity of 600 ± 200 µmol m^{-2} s^{-1}). Two experiments were conducted for the temperate lines. Difficulties with cob formation enabled only one experimental replication for the tropical lines. Therefore, all tropical lines were maintained in a growth chamber (day/night temperature regime of 28/24°C, day/night light regime 14/10 h, 70% air humidity, with light intensity of 115 ± 10 µmol m^{-2} s^{-1}) for the first 45 days. After this period, they were moved to the greenhouse under the same conditions as the temperate lines.

Influence of pre-inoculation temperatures on the efficacy of resistance in tropical lines

The effect of temperature on the resistance of tropical lines was tested by maintaining maize plants under different temperature regimes before inoculation (Figure 2). Maize plants were maintained in the greenhouse (temperature 21 ± 6°C, 65%± 15 air humidity, with a day/night light regime of 14/10 h and light intensity of 600 ± 200 µmol m^{-2} s^{-1}) for 45 days

after sowing, until they had the seven unfolded leaves (BBCH 17/32). In the growth chamber, all plants were submitted to the same environmental conditions (day night temperature regime 25/20°C, 70% air humidity, day/night light regime 12/12 h during with light intensity of 150 ± 50 µmol m^{-2} s^{-1}) until the first silk channels was visible (BBCH 63). As the plants were not flowering homogeneously, every plant was moved to a different chamber when its silk channel was visible. The warm temperature chamber was set to a day/night temperature regime of 30/25°C, and the chamber with moderate temperatures were set to 20/15°C. All plants were maintained in different chambers for 10 days. Before moving all plants back to the previous conditions (day night temperature regime 25/20°C, 70% air humidity, day/night light regime 12/12 h during with light intensity of 115 ± 10 µmol m^{-2} s^{-1}), plants were inoculated with strains of *F. graminearum* or *F. verticillioides* species complex from Germany or Brazil. After inoculations, all plants were maintained under the same environmental conditions until they reached full maturity (BBCH 99).

Data analysis

In the experiment to measure aggressivenes and host resistance of the temperate lines, data of disease severity were fitted to a linear mixed model, with replications as the random effect. The disease severity was compared by multiple comparison using the Tukey test (*p*-value ≤ 0.05) provided in the package lme4 from the R software 3.6.0 (Core Team 2019). For the tropical lines, in both experiments of host resistance and influence of temperature an analysis of variance (ANOVA) was conducted. *Fusarium* spp. or lines were compared by multiple comparisons applying Tukey's test (*p*-value ≤ 0.05). Additionally, the aggressiveness or resistance to German and Brazilian isolates was compared within the same *Fusarium* ssp. (*p*-value ≤ 0.05).

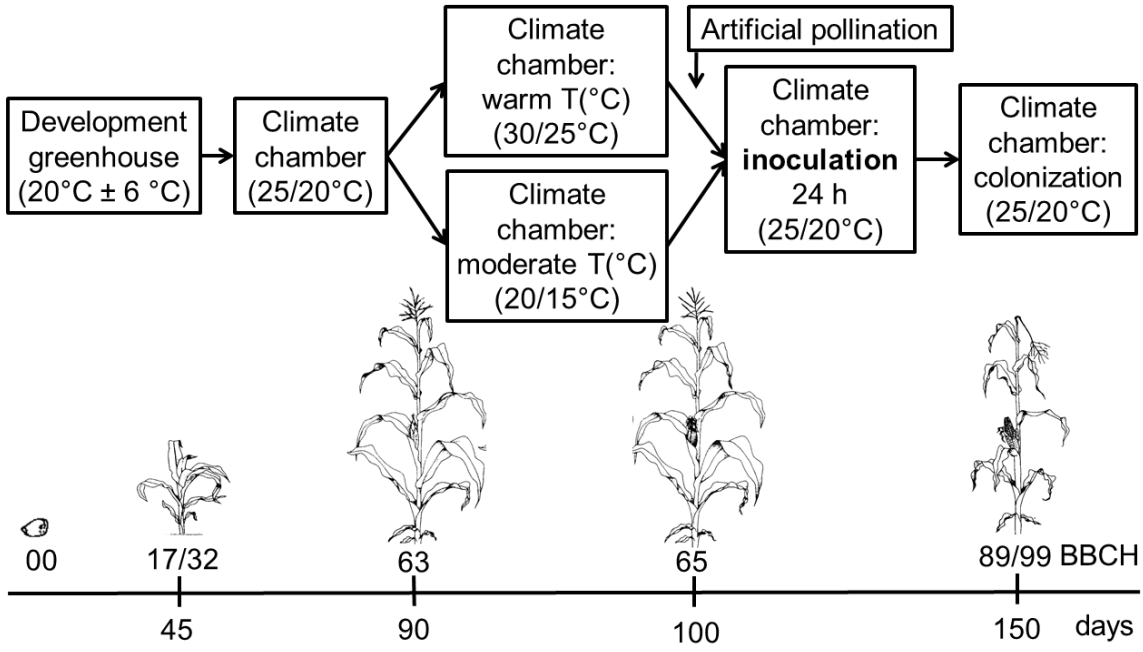

Figure 2. Scheme for the experimental conduction under different temperature regimes. Plant development was conducted in the greenhouse for 45 days. Plants were moved to the climate chamber under a day/night temperature regime of 25/20°C. When plants were showing the first silk channels, they were maintained under warm (30/25°C) and moderate (20/15°C) temperature regimes. After inoculation, all plants were moved back to the previous temperature regime (25/20°C) until harvest. Maize figures retract BBCH stages (Uwe Meier, 2001).

Results

Aggressiveness between isolates from Brazil and Germany

Higher disease severities among all tested *Fusarium* spp. were observed for the Brazilian *Fusarium equiseti* isolate and the German *F. culmorum* and *F. graminearum* isolates (Figure 3). Moreover, differences between Brazilian and German isolates were observed for *F. culmorum* and *F. graminearum.* The German isolates showed higher disease severity for both species: *F. culmorum* and *F. graminearum*. Moreover, the reference lines Dent-21 and Dent-4 showed significant differences in disease severity (p-value ≤ 0.001), confirming their distinct levels of resistance to *Fusarium* infections. There were no significant differences between German and Brazilian strains of *F. equiseti*, *F. tricinctum* and *F. verticillioides*.

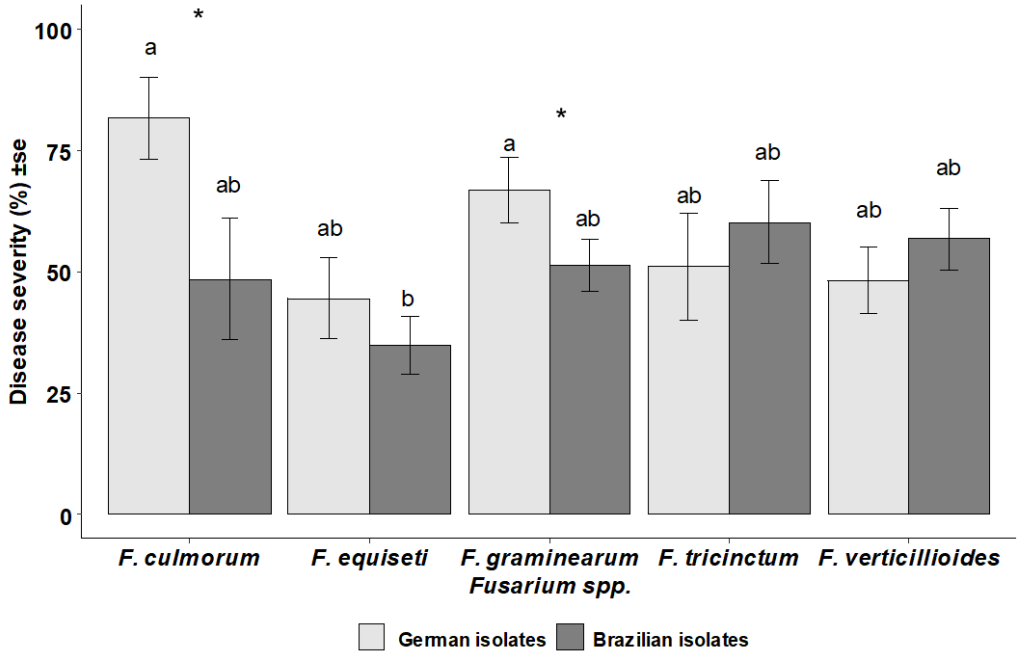

Figure 3. Comparison of aggressiveness between *Fusarium culmorum*, *F. equiseti, F. graminearum, F. tricinctum,* and *F. verticillioides* isolates originated from Germany and Brazil. One isolate per species and per country was inoculated in cobs from two maize reference lines Dent-21 (moderate susceptible line, and Dent-4 (resistant line). Cobs were inoculated by silk-channel injection in a spore concentration of $1.5*10^4$ spores ml^{-1}. Data of disease severity were fitted to a linear mixed model, with experiment replications as random effect. Means sharing same letter are not significantly different using Tukey-adjusted comparisons (*p*-value ≤ 0.05). *Fusarium* spp. showing an asterisk represent significant differences on aggressiveness between isolates from Germany and Brazil (*: *p*-value ≤ 0.05). Bars indicate standard error (n = 3 plants).

Host resistance of temperate and tropical maize lines

Temperate lines showed differences in resistance levels regarding *F. graminearum* and *F. verticillioides* (*p*-value ≤ 0.001) (Table 1). In general, *F. graminearum* presented higher disease severity than *F. verticillioides* (Figure 4). The resistance to Brazilian and German isolates was different between the temperate lines (*p*-value ≤ 0.001) (Table 1). For *F. graminearum,* lines A12 and A5 showed different resistance levels against German and Brazilian isolates (Figure 4A). Moreover, disease severity in the A12 line inoculated with the German *F. graminearum* isolate was higher than disease severities in lines A5 and A8 inoculated with the Brazilian isolate. For *F. verticillioides*, line A3 was more susceptible than the A12 and A5 line (Figure 4B). Moreover, within temperate lines, only line A8 showed differences at the level of disease severity between strains from Germany and Brazil.

Table 1. Effect of temperate maize lines inoculated with *F. graminearum* and *F. verticillioides*, obtained from maize samples in Germany and Brazil and their interactions on data of disease severity based on a mixed model analysis of variance.

Effect	Disease severity			
	df_n[1]	df_d[2]	*F*-value	*p*-value
Temperate lines (A12, A3, A5 and A8)	3	267	201.14	<0.001
Fusarium spp. (*F. graminearum* x *F. verticillioides*)	1	267	211.61	<0.001
Country (German isolates x Brazilian isolates)	1	267	260.84	<0.001
Line x *Fusarium* spp.	3	267	35.57	0.014
Line x country	3	267	0.99	0.396
Fusarium spp. x country	1	267	106.49	0.001
Line x *Fusarium* spp. x country	3	267	51.54	0.001

[1] Numerator degrees of freedom (df_n), [2] denominator degrees of freedom (df_d); degrees of freedom calculated using the Satterthwaite formula for a mixed model; *F*-value for testing effect and probability (significance) level of *F* -value (*p*-value).

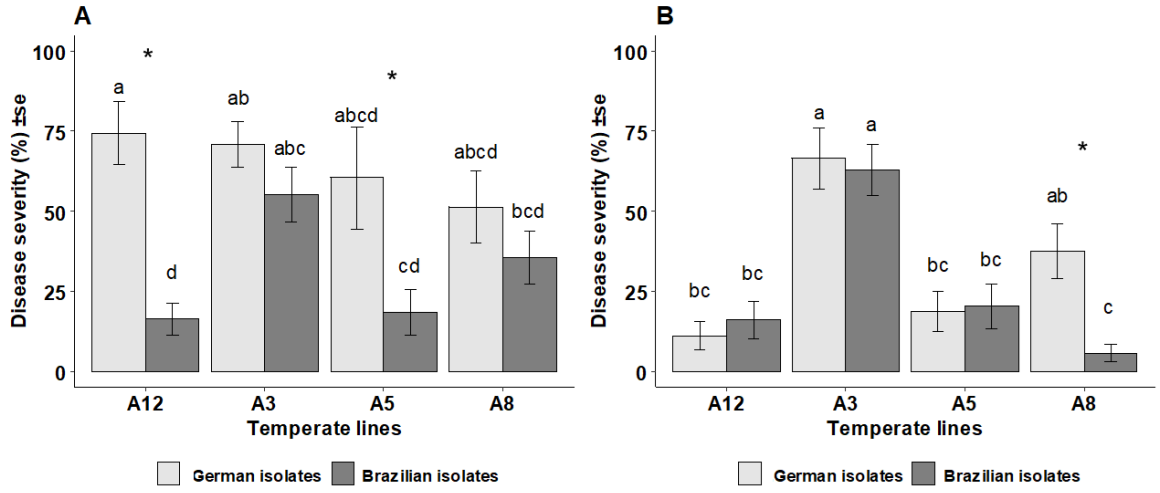

Figure 4. Comparison of resistance between 4 maize lines (A12, A3, A5, and A8) inoculated with *Fusarium graminearum* (A) and *F. verticillioides* (B) isolates originated from Germany (light grey) and Brazil (dark grey). One isolate per species and per country was inoculated in cobs by silk-channel injection in a spore concentration of $1.5*10^4$ spores ml^{-1}. Data of disease severity were fitted to a linear mixed model, with experiment replications as random effect. Means sharing same letter are not significantly different using the Tukey-adjusted comparisons (*p*-value ≤ 0.05). Maize lines showing an asterisk represent significant differences in resistance against *Fusarium* spp. isolates from Germany and Brazil (*p*-value ≤ 0.05). Bars indicate standard error (n = 3 plants).

As the tropical lines grew under different environmental conditions at the beginning of the development, tropical lines were only compared with each other. Differences in resistance levels were observed between the tropical lines T3 and T4 (*p*-value ≤ 0.01) (Table 1), presenting an average of 34% and 70% disease severity, respectively (Figure 5A). Moreover, tropical lines were more resistant to Brazilian isolates when compared to German isolates (*p*-value ≤ 0.01), showing 36 and 70% of disease severity respectively(Figure 5B). No significant differences in resistance levels were observed between tropical lines inoculated with *F. graminearum* and *F. verticillioides* (*p*-value = 0.45) (Table 2).

Table 2. Analysis of variance (ANOVA) for tropical lines T3 and T4 inoculated with two *Fusarium* spp., *F. graminearum* and *F. verticillioides*, collected from maize samples in Germany and Brazil.

Factor	***p*-value**
Tropical line (T3 x T4)	0.002**
Fusarium spp. (*F. graminearum* x *F. verticillioides*)	0.448
Country (German isolates x Brazilian isolates)	0.002**
Line x *Fusarium* spp.	0.887
Line x country	0.966
Fusarium spp. x country	0.231
Line x *Fusarium* spp. x country	0.968

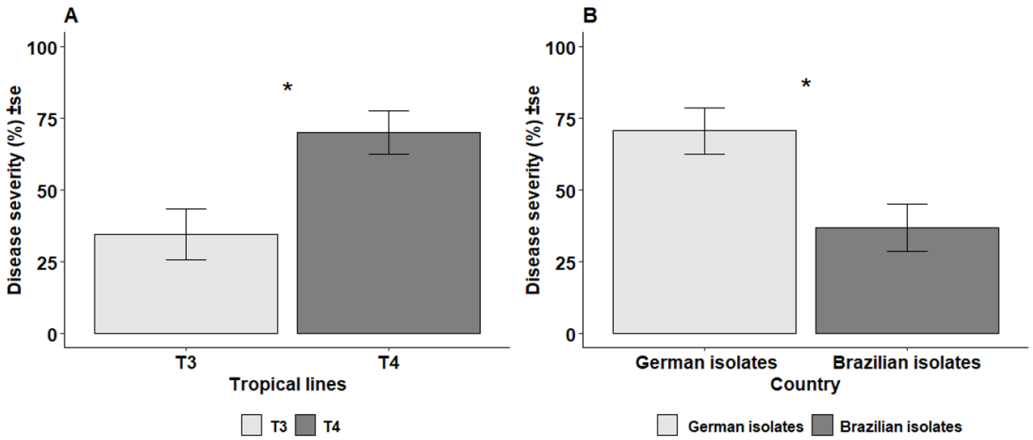

Figure 5. Comparison of resistance between tropical maize lines (T3 and T4) inoculated with *Fusarium graminearum* and *F. verticillioides* isolates originated from Germany (light grey) and Brazil (dark grey). One isolate per species and per country was inoculated in cobs by silk-channel injection in a spore concentration of $1.5*10^4$ spores ml^{-1}. Graphs showing an asterisk represent significant differences in resistance against *Fusarium* spp. isolates for maize lines or country of origin (*p*-value ≤ 0.05). Bars indicate standard error (n = 3 plants).

Effect of pre-inoculation temperatures on the efficacy of resistance in tropical lines

The tropical lines showed differences in resistance to *Fusarium* spp. when plants were maintained under different pre-inoculation temperature regimes. Plants pre-disposed to the higher temperature regime (30/25°C) showed a higher disease severity when compared to plants maintained under moderate temperatures (20/15°C) (Figure 6A). In this experiment, there were no differences in resistance levels between the tropical lines T3 and T4 (*p*-value = 0.23); therefore, data of both tropical lines were combined. Moreover, the German *F. graminearum* isolate showed a high disease severity compared to the Brazilian isolates (Figure 6B). No significant differences were observed between German and Brazilian *F. verticillioides* isolates.

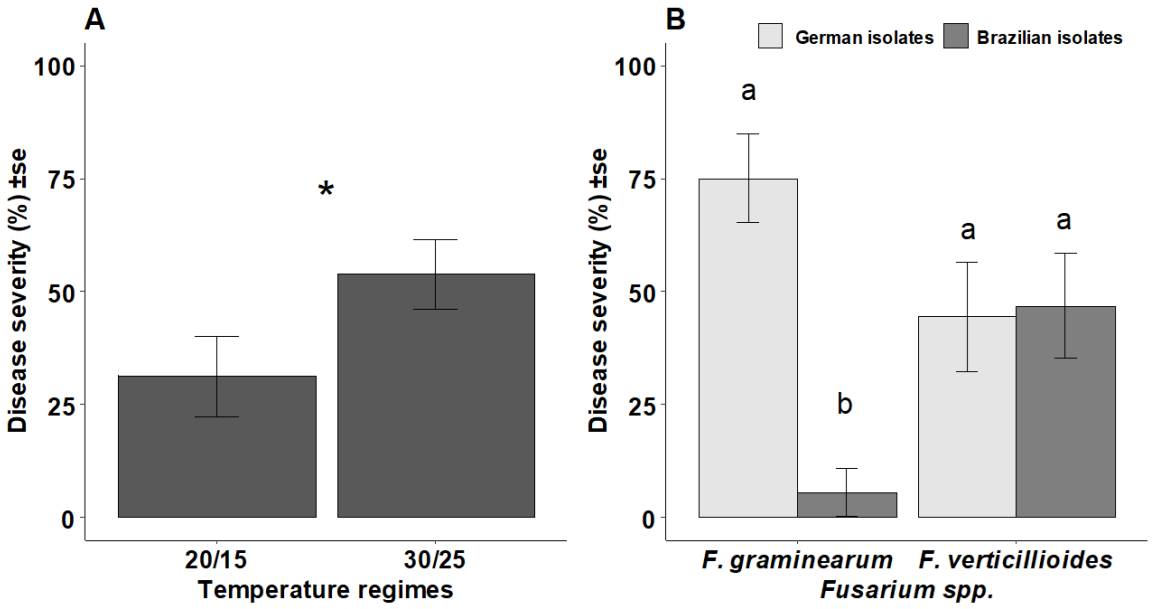

Figure 6. Disease severity of two tropical maize lines (T3 and T4) inoculated with *Fusarium graminearum* and *F. verticillioides* isolates originated from Germany (light grey) and Brazil (dark grey). One isolate per species and per country was inoculated in cobs (n = 3 cobs) by silk-channel injection in a spore concentration of $1.5*10^4$ spores ml^{-1}. Analysis of variance (ANOVA) was performed with the data of disease severity. Differences in host resistance to *Fusarium* spp. between tropical maize lines pre-disposed to two different temperature regimes (30/25°C) and (20/15°C) before inoculation are represented by asterisk (**p*-value ≤ 0.05) (A). Means sharing the same letter are not significantly different using Tukey-adjusted comparisons (*p*-value ≤ 0.05) (B). Bars indicate standard error (n = 3 plants).

Discussion

Differences in the importance of GER and FER in Germany and Brazil are strongly related to climate conditions, and consequently to the prevalence of *Fusarium* spp. and mycotoxins (Munkvold, 2003b). In Germany, the most prevalent species are from the FGSC (Goertz *et al.*, 2010; Pfordt *et al.*, 2020), whereas species with the highest prevalent in Brazil are for FFSC (Stumpf *et al.*, 2013a; Lanza *et al.*, 2014). Moreover, the prevalent FGSC chemotype in Germany is DON (Goertz *et al.*, 2010), while in Brazil it is NIV (Kuhnem *et al.*, 2016). Additionally, the cropping system of maize differs in many points between both countries. In Germany, maize is cultivated in ploughed soil or with reduced tillage, and previous crops are mainly wheat, maize and sugar beet (Pfordt *et al.*, 2020). In Brazil, however, no-till is vastly practiced due to its better soil conservation. No-till reduces erosion, increases organic matter, soil moisture retention and, promotes a slow decomposition (Ono *et al.*, 2011). Furthermore, Brazilian weather conditions allow two cultivating seasons per year. Areas of soybean production in the main season can be succeed by maize production in the second season (safrinha). Moreover, other succession crops can be wheat or oats (Spolti *et al.*, 2015). Even if tillage and previous crops may not have a strong influence on the prevalence of *Fusarium* spp. (Ono *et al.*, 2011; Spolti *et al.*, 2015; Pfordt *et al.*, 2020), the cultivation of *Bt*-transgenic maize can significantly reduce *Fusarium* infections by earworms (Munkvold *et al.*, 1999), such as *Spodoptera frugiperda,* in Brazil. Impressively, transgenic maize represents 92% of the area of maize production in Brazil (ISAAA, 2018).

Besides the two cropping systems, the *Fusarium* spp. populations can differ regarding their aggressiveness levels (Jardine and Leslie, 1999). In our experiments, the high disease severity of German *F. graminearum and F. culmorum* strains compared to the Brazilian strains can be related to the high prevalence of FGSC in Germany. In general, *F. graminearum* and *F. culmorum* are usually more aggressive than other *Fusarium* spp. (Schaafsma *et al.*, 1993; Iglesias *et al.*, 2010; Miedaner *et al.*, 2010). In addition, the Brazilian *F. verticillioides* strain did not show high disease severity because FFSC colonize maize plants asymptomatically (Gai *et al.*, 2018; Blacutt *et al.*, 2018). A symptomless colonization makes correlation between disease severity and mycotoxin production difficult, as shown for *F. verticillioides,* where the disease severity does not reflect the fumonisin production (Iglesias *et al.*, 2010). However, for *F. graminearum*, weaker correlations between disease severity and mycotoxin levels can be established (Mesterházy *et al.*, 2012). Therefore, resistance to *F. graminearum* might be easily detected (Presello *et al.*, 2006), whereas this might not be the case for *F. verticillioides*. However, tested temperate lines showed differences in resistance levels between *F. graminearum* and *F. verticillioides*.

Therefore, it is strongly recommended include a strain of *F. verticillioides* on breeding programs in order to test for resistance to this species.

In our experiments, some temperate lines and tropical lines showed higher disease severities when inoculated with the German *F. graminearum* isolate in comparison to the Brazilian isolates (Tables 1 and 2, Figures 4 and 5). The high aggressiveness of the German isolate was also observed for *F. graminearum* strains in the aggressiveness experiment (Figure 3), and for the experiment testing two pre-inoculation temperatures (Figure 6). In Germany, breeding programs select resistant hybrids by inoculating a highly aggressive *F. graminearum* isolate (for example the strain IFA66 obtained from Hohenheim University, data not shown). Regarding the pathogen, a better screening to select highly aggressive strains from Brazil is recommended, in order to allow better comparisons of resistance between tropical and temperate maize lines. Silk-channel resistance can be better detected with highly aggressive *F. graminearum* isolates (Reid *et al.*, 1993; Miedaner *et al.*, 2010). The pin inoculation has classified most of the tested Brazilian hybrids as susceptible and moderate susceptible to GER (Nerbass *et al.*, 2016), difficulting the detection of resistant plants. Thus, selection for resistance to *Fusarium* spp. may be focused on silk-channel resistance by performing the inoculation method of silk-channel injection (Nerbass *et al.*, 2015; Nerbass *et al.*, 2016). Under natural conditions, selection may favor less aggressive strains (Iglesias *et al.*, 2010). Thus, a pre-selection of isolates regarding their aggressiveness levels is fundamental (Mesterházy *et al.*, 2012), in order to find the best strain for inoculations in breeding programs.

The selection for resistance with strains originating from different regions showed that resistance has low specificity to the pathogen population (Presello *et al.*, 2006; Miedaner *et al.*, 2010). *F. graminearum* strains from the USA, Italy and South Africa showed considerable levels of disease severity and were therefore suitable for the selection of host resistance in German fields (Miedaner *et al.*, 2010). Similar results were observed for maize genotypes tested in Argentina and Canada; the resistance had low specificity towards *Fusarium* strains and location (Presello *et al.*, 2006). Therefore, the resistance carried by the tropical lines might be durable under European conditions. To confirm the efficacy of resistance under European conditions, tropical lines should be preferably tested by inoculating German strains under field conditions. In general, it is strongly recommended to conduct experiments in the field, especially when it is necessary to grow plants to full maturity, such as for the evaluation of GER and FER. The evaluation of mature plants provides more reliable data (Blacutt *et al.*, 2018). However, the environmental conditions in Germany (especially long day light during summer) do not allow cob formation of the tropical genotypes.

Silk-channels have shown ontogenic resistance to *Fusarium* spp. The susceptibility to *Fusarium* spp. infections is correlated to silk age. Reid *et al.*, (1992a) has shown that the susceptility of silks to Fusarium spp. infection is decreased with an increase in silk age. However, no correlation was detected between the increase in the maturity group and the increase on susceptibility to *Fusarium* spp. (Reid and Sinha, 1998). A study of European lines from the early maturity group shows that flint lines are more susceptible to *F. graminearum* and *F. verticillioides* (Löffler *et al.*, 2010). The increased susceptibility of flint maize compare to dent might be correlated to the early flowering time (Löffler *et al.*, 2010). The early flowering time and a high tolerance to cold contributed to its introduction in temperate regions (Hufford, 2016). In addition, Mesterházy *et al.* (2012) mentioned that correlations between flint and susceptibility can be ambiguous according to the population studied. Han *et al.* (2018) detected two quantitative trait loci (QTL) for DON in the pool of dent lines and six QTL in the pool of flint lines from European breeding programs. Morphologically, flint kernels are thicker, harder and have a vitreous outer layer, whereas dent shows a higher soft starch content and has characteristic indented kernels (Unterseer *et al.*, 2016). Moreover, flint has a narrower, looser and lower number of husk leaves, whereas husk leaves from dent are wider, tighter, greater in number and have a bottleneck (Troyer, 2001), which may increase humidity and favor infections (Oldenburg *et al.*, 2017). The cultivation of flint maize seems to be common in South America (Brown *et al.*, 1985; Tamagno *et al.*, 2015), corroborating the fact that tropical/subtropical lines shows a high allele frequency for resistance to FER when compared to temperate breeding pools (Zila *et al.*, 2013).

In our experiment, the increase in pre-inoculation temperature had a negative effect on the resistance carried by the tropical lines (Figure 6). However, quantitative resistance was shown to be more stable in other crops. The increase in post-inoculation temperature had no effect on the quantitative resistance of Canadian canola cultivars against blackleg (Hubbard and Peng, 2018). Further experiments should be conducted to better understand the effect of pre- and post-inoculation temperature on the quantitative resistance, and especially for GER and FER. The species prevalence and aggressiveness has shown differences between both countries. GER is more prevalent in Germany, whereas FER is more prevalent in Brazil. In general, FGSC are more aggressive than FFSC, and the German strain were more aggressive than the Brazilian strain. Therefore, temperate and tropical lines were more effective to Brazilian isolates. Besides the resistance, levels of different mycotoxins can vary between samples from both countries. Levels of fumonisins in Germany ranged from 25 to 20,690 ηg/g (Goertz *et al.*, 2010), whereas levels of fumonins ranged from of 5,000 to 2,390,000 ηg/g in Brazil (Lanza *et al.*, 2014). As mycotoxins have

different levels of toxicity (van der Lee *et al.*, 2015), studies monitoring the prevalence of species and a pre-selection of highly aggressive strains should be conducted in order to better select for resistance to GER and FER.

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III. General discussion

Maize production in tropical regions differs from that in temperate regions in several agricultural aspects, mainly because of distinct environmental conditions (Velásquez *et al.*, 2018). In recent decades, no-till practices have been increased in tropical regions. No-till practices increase organic matter, prevent surface sealing caused by heavy rain, and consequently reduce erosion (Cerri *et al.*, 2007). Besides cultural practices, the pathogen population and the cultivation of susceptible hybrids contribute to diseases epidemics in maize. Therefore, the main methods applied for disease control are related to changes in crop management, pesticides application and resistant hosts. Tropical maize is a good source of disease resistance as it has been shown to have a wealth of resistance alleles (Zila *et al.*, 2013). The advancement of modern breeding practices has allowed the introduction of new sources of resistance from tropical maize germplasm into temperate breeding programs. However, this process is still laborious and slow (Pollak *et al.*, 1991). Studies comparing pathogen populations and their aggressiveness from different regions add information that may help in the estimation of the effectiveness of maize resistance to diseases and breeding, as demonstrated for northern corn leaf blight (NCLB), Gibberella ear rot (GER) and Fusarium ear rot (FER). Differences in *Exserohilum turcicum* and *Fusarium* spp. populations and their aggressiveness were compared between European and South American isolates (Table 1). Additionally, host resistance from tropical and temperate lines was tested for *Fusarium* spp.. Lastly, sources of qualitative resistance to *E. turcicum* were compared according to their resistance responses, pathogen colonization, disease epidemiology, photosynthesis impairments and biochemical disorders (Table 2, Figure 1).

Table 1. Comparison of pathogens and resistance related to northern corn leaf blight (NCLB), Gibberella ear rot (GER) and Fusarium ear rot (FER) in Europe and South America. *Exserohilum turcicum* and *Fusarium* spp. populations and their aggressiveness were compared between regions. Host resistance to *E. turcicum* was compared between European and South American commercial hybrids, and the resistance of tropical and temperate lines was tested for *F. graminearum* and *F. verticillioides* isolates originating from Germany and Brazil.

Disease	**NCLB**		**GER / FER**	
Trait	**Europe**	**South America**	**Germany**	**Brazil**
Pathogen populations	high race diversity	low race diversity	GER prevalent	FER prevalent
Pathogen aggressiveness	less aggressive	more aggressive	GER is more aggressive	GER isolates less aggressive
Host resistance	Cultivated hybrids carry qualitative resistance	Cultivated hybrids may carry quantitative resistance	Resistance on temperate lines is more effective for Brazilian isolates	Tropical lines are more resistance to Brazilian isolates

A. Northern corn leaf blight (NCLB)

Assessments of physiological races of *Exserohilum turcicum* in Argentina and Brazil was conducted by inoculating the maize line B37 with the genes *Ht1, Ht2, Ht3* and *Htn1*. The race assessment identified a high frequency of race 0 isolates indicating that *Ht* genes are not widely introduced in cultivated commercial hybrids in South America (Table 1). Thus, resistance to *E. turcicum* present in Argentinian and Brazilian hybrids is probably from quantitative sources. In Europe, race monitoring demonstrated that 32% of the screened isolates were virulent to plants bearing the *Ht3* gene, a source of qualitative resistance (Hanekamp, 2016). Therefore, a large proportion of the hybrids cultivated in Europe may carry the *Ht3* gene. As quantitative resistance is more durable (Pilet-Nayel *et al.*, 2017), and it is advantageous to introgress the resistance from tropical lines in the European breeding programs (Galiano-Carneiro *et al.*, 2020). Additionally, information about the frequency of races may guide breeders regarding which *Ht* genes to breed into new cultivars. Consequently, a better management of resistant cultivars can be recommended specifically for each region according to the pathogen population, in order to increase the durability of resistance.

In addition to the frequency of races, information about population aggressiveness contributes to estimating the progress of disease epidemics and the effectiveness of

resistance. In our experiments, South American isolates were more aggressive than European ones (Table 1), showing that *E. turcicum* has some plasticity, and may adapt to changes in the environment, such as warmer seasons or cropping systems. In general, disease severities were higher and incubation periods were lower at higher post-inoculation temperatures (day/night temperature of 30°C/25°C), indicating that high temperatures favor pathogen colonization. The increase in post-inoculation temperatures may favor the pathogen by increase in the concentration of aggressiveness factors, such as phytotoxins. Non-host-specific toxins may inhibit active defense processes disfavoring the host (Buiatti and Ingram, 1991), such as the non-host-specific phytotoxin monocerin, which is known to be produced by *E. turcicum* (Robeson and Strobel, 1982). Monocerin was shown to reduce fluorescence intensity in cells, and to induce protoplast and cell mortality, which leads to necrosis (Cuq *et al.*, 1993). Other phytotoxic compounds isolated from *E. turcicum* culture filtrates inhibited chlorophyll formation, which provokes chlorosis in maize plants (Bashan and Levy, 1992; Bashan *et al.*, 1995), disturbed cell membrane permeability (Dong *et al.*, 2001), and increased lesion size (Bashan and Levy, 1992). Additionally, a host-specific toxin named HT-toxin was shown to be a virulence factor (Dong and Li, 1996) and it provoked overexpression of QM protein, a protein related to stress responses pathways, in plants carrying *Ht2* gene (Wang *et al.*, 2010). In our *in vitro* experiments, *E. turcicum* showed higher mycelium growth under 25°C. However, high diseases severities were observed at day/night temperatures of 30°C/25°C. At high post-inoculation temperatures, host defenses may be suppressed, and the pathogen may release more phytotoxins, especially South American isolates, which indicate that these isolates are more aggressive than European isolates.

High temperatures may contribute to plant susceptibility or they may induce defense for different pathosystems (Garrett *et al.*, 2006). The increase in disease severities under high post-inoculation temperatures might be related to negative effects of heat stress during the activation of host defenses for the pathosystem maize-*E. turcicum*. Furthermore, pre-inoculation temperatures were shown to have no effect on host resistance. Consequently, a reduction of the detoxification process may lead to high disease intensities. Studies regarding phytotoxin production under high temperatures should be conducted to identify the effect of toxins on disease severity under high temperatures. High temperatures may also reduce the activity of enzymes responsible for the detoxification process. Studies on up- and down-regulation of resistance genes under different temperatures may indirectly elucidate the role of aggressiveness factors by suppressing resistance mechanisms.

B. Gibberella and Fusarium ear rot (GER and FER)

Differences in species prevalence in Germany and Brazil (Table 1) contribute to a better understanding of different mycotoxins contamination in maize. For instance, *F. proliferatum* was shown to produce 10 times more fumonisins than *F. verticillioides* (Zhou *et al.*, 2018). *F. graminearum* producing 15-aceytil-deoxynivalenol formed a higher number of perithecia in maize when compared to nivalenol and 3-aceytil-deoxynivalenol producers (Nicolli *et al.*, 2018), which may contribute to pathogen variability and dispersion. Generally, mycotoxins are not virulence factors (Desjandins *et al.*, 1995); however, these substances may influence pathogen aggressiveness and consequently the effectiveness of resistance. It is expected that resistance carried by temperate lines is more efficient in regard to isolates of the *Fusarium graminearum* species complex (FGSC). As these species are prevalent in cold and humid regions (Munkvold, 2003), the selection of resistant lines is mainly focused on the FGSC, while the resistance carried by tropical lines could be more efficient for the *Fusarium fujikuroi* species complex (FFSC), as its prevalence is higher in warmer and drier areas (Blacutt *et al.*, 2018). However, tropical lines did not show differences in resistance levels between *F. graminearum* and *F. verticillioides*, and temperate lines showed higher levels of resistance for *F. verticillioides*. In general, *F. verticillioides* has lower levels of severity than *F. graminearum* (Miedaner *et al.*, 2010), due to its asymptomatic colonization (Blacutt *et al.*, 2018). Levels of disease severity are not always correlated to levels of mycotoxins (Presello *et al.*, 2006). Thus, the resistance for *F. verticillioides* should be tested not only by disease rating but also by other methods, such as measuring the amount of fungal DNA content or mycotoxin production (Schnerr *et al.*, 2002; Atoui *et al.*, 2012; Preiser *et al.*, 2015).

Fusarium verticillioides can also infect via silk and colonize systemically, as typical symptoms are infections in random kernels (Blacutt *et al.*, 2018), which is distinct to the symptoms of GER caused by *F. graminearum*. Therefore, it is strongly recommended to test for resistance for FER, apart from the trails for GER, as resistance mechanisms involved in silk infection may differ from those in kernel infection (Mesterházy *et al.*, 2012). A pre-selection of highly aggressive isolates should also be applied to *F. verticillioides* isolates from Germany and Brazil, as highly aggressive isolates were shown to be more effective in selecting for resistance to *F. graminearum* (Miedaner *et al.*, 2010). Furthermore, a comparison of a larger number of isolates is recommended in order to obtain a robust comparison of aggressiveness between pathogen populations.

Experiments aimed at identifying new resistant lines have been conducted in several locations for several years (Miedaner, 2011), as quantitative resistance is sensitive and may

change in line with environmental conditions (Pilet-Nayel *et al.*, 2017; Galiano-Carneiro *et al.*, 2020). Variations in quantitative resistance, which are expressed by loci, may be difficult to detect under strong variations in environmental conditions (Weinig and Schmitt, 2004). However, genotypes that are able to adapt to different weather conditions and present some fitness to advantages are referred as “phenotypically plastic” (Weinig and Schmitt, 2004). In our experiment, the resistance to GER expressed by the tropical lines under moderate temperatures (20°C /15°C) may be an evidence of phenotypical plasticity, as these lines are more adapted to moderate temperatures. However, the efficiency of resistance under field conditions should be confirmed by field experiments in several locations.

C. *Ht*-resistance genes to *Exserohilum turcicum*

The symptomology demonstrates that resistance reactions differ among the resistance genes *Ht1*, *Ht2*, *Ht3* and *Htn1*. In order to identify differences between the resistance mechanisms conferred by each *Ht* gene, pathogen colonization, fungal and plant biomass, and epidemiological, physiological and biochemical traits were characterized in several experiments. A compilation of all variables evaluated for the susceptible line B37 (compatible interaction) and for the resistance reaction (incompatible interaction) with the lines B37*Ht1*, B37*Ht2*, B37*Ht3* and B37*Htn1* is exhibited in Table 2.

The susceptible line B37 revealed necrosis, high values of disease severity, xylem penetration efficiency (XPE), xylem colonization efficiency (XCE), mesophyll colonization efficiency (MCE), *E. turcicum* DNA content, pathogen sporulation, number of lesions and lesion length, as expected for a compatible interaction. All evaluated photosynthetic variables, CO_2 assimilation (A), transpiration (E), stomatal conductance (gs) and the instantaneous carboxylation efficiency (k), were strongly reduced in the susceptible line. As regards the biochemical parameters, a high percentage of penetration sites accumulated peroxide (H_2O_2) at 6 dpi, which resulted in high peroxidase activity (POX). Additionally, a decrease in leaf area was recorded for this line at 28 dpi. There was no reduction on aerial and root biomass due to evaluation at early timepoints after infection (14 and 28 dpi) and to low levels of disease severity (<12%) in this experiment. A strong reduction on photosynthetic leaf area caused by necrosis reduces drastically the radiation use efficiency by plants and may lead to biomass reduction and yield losses.

A severe resistance reaction was observed in the line B37*Ht1.* Symptoms were characterized by strong chlorosis and necrosis (Hooker, 1963), which conferred high disease severity for this line, especially in young plants. Disease severity was usually as high as that observed for the susceptible line B37. However, data on pathogen colonization confirmed that necrosis was caused by resistance reaction, as the XPE, XCE, MCE were

low and, consequently, fungal DNA was detected in low levels. In addition, pathogen sporulation decreased by 90% in the line B37*Ht1* compared to B37. The reduction on DNA demonstrate that chlorosis and necrosis were provoked by host resistance reactions, which reduced sporulation expressively. The resistance mechanism triggered by *Ht1* might be related to antifungal compounds (phytoalexins) and to a high H_2O_2 production causing chlorophyll degradation.

The incompatible interaction between *E. turcicum* and *Ht2* was characterized by chlorosis (Hooker, 1977) and small necrotic lesions. Thus, it was difficult to distinguish the incompatible interaction with *Ht2* from the compatible interaction for some traits, especially during experiments to assess physiological races of *E. turcicum* isolates. The disease severity was reduced in this line when compared to B37 (susceptible line), due to a reduction in the number and size of lesions. A reduced pathogen colonization was also observed by the low values of XPE, XCE, MCE and fungal DNA content. As the resistance reaction conferred by *Ht2* was related to chlorosis and necrosis, a slight decrease in the photosynthetic variables A, E, gs and k was observed for this line. At early stages of disease development, high H_2O_2 was accumulated in most penetration sites, however POX activity was high. In general, the resistance mechanism triggered by *Ht2* caused low damage to plant physiology. However, as small necrotic lesions are observed in the incompatible interaction, this resistance mechanism might be related to a delay on the activity of detoxicating enzymes. The resistance on maize to *Cochliobolus carbonum* race 1 occurs by the detoxification of HC-toxin (Walton and Panaccione, 1993). The breakdown of phytotoxin may also account for symptom expression (Bashan *et al.*, 1996). The mechanism regulating the resistance of *Ht2* to the HT-toxin involves the upregulation of QM-like protein, which is component of several stress response pathways (Wang *et al*., 2010).

The gene *Htn1* can confer distinct resistance reactions and leads to an extended disease latent period (Gevers, 1975). *Htn1* confers quantitative resistance (Hurni *et al.*, 2015), and its resistant effect could also be verified in the compatible interaction by slowing down pathogen colonization and reduced fungal DNA content, as demonstrated in the chapter 3 of this present study. In the incompatible interaction (Table 2), no disease severity, XCE and MCE were observed. In addition, the *Htn1* gene reduced pathogen XPE. A slight decrease in the photosynthetic variables A, E, gs and k was observed for *Htn1*. Pathogen sporulation was only observed in few small lesions. Resistance mechanisms of *Htn1* extent the disease latent period (Gevers, 1975) and may consequently slow down NCLB epidemics. An extended latent period usually affects the apparent infection rate, which confirm its quantitative background of resistance (Hurni *et al.*, 2015). Molecular studies demonstrated that *Htn1* is in a locus on the chromossom 8 and recognizes pathogen

invasion and cell wall disruption encoding some unusual wall-associated receptor-like kinases (RLK) (Hurni *et al.*, 2015). Thus, resistance mechanisms triggered by *Htn1* are related to the recognition of cell wall alterations and signaling to the cytoskeleton (Diener and Ausubel, 2005). In addition, the line carrying *Htn1* demonstrated the highest percentage of penetration sites with H_2O_2 accumulation and the highest values for POX activity (Shimoni *et al.*, 1991). High ROS can be correlated to yellow spots observed in lower leaves, which are described as wilt-type lesions (Gevers, 1975). Wilt-type lesions, also called “lesion-mimics”, might have an inappropriate activation of resistance mechanisms like hypersensitive response (HR), which demands energy (Balint-Kurti, 2019), corroborating the low values of CO_2 assimilation in healthy plants. Wilt-type lesions could explain the higher percentages of H_2O_2 detection and high peroxidase activity in plants carrying the *Htn1* gene.

The resistance reaction conferred by *Ht3* is related to chlorotic spots (Hooker, 1981). No necrotic lesions were observed in this line. Fungal DNA content was very low and pathogen colonization was restricted to the xylem (values of MCE were null). B37*Ht3* reveled low H_2O_2 accumulation and POX activity, similar to the susceptible line B37. As no necrosis was observed in this line, there were no reductions in the photosynthetic variables A, E, gs, and k for the line B37*Ht3*. It seems that the resistance mechanism triggered by *Ht3* confers a strong barrier, preventing pathogen colonization in xylem vessels and avoiding colonization from the sieve cells. *Ht3* may recognize pathogen invasion earlier than the other genes. *Ht3* was introgressed from *Tripsacum floridanum* (Hooker, 1981), which is not an alternative host for *E. turcicum*. Chlorotic spots conferred by *Ht3* might be related to a resistance mechanism similar to non-host resistance.

Table 2. Traits and parameters/variables evaluated for northern corn leaf blight (NCLB) in the interaction with the maize line B37 and *Ht1, Ht2, Ht3* and *Htn1* resistance genes.

Trait	Parameter / Variable	B37 (no *Ht*-gene)	B37*Ht1*	B37*Ht2*	B37*Ht3*	B37*Htn1*
Symptomology	phenotype	necrosis	strong chlorosis and necrosis	chlorosis or small lesions	chlorotic spots	no lesions or wilt-type lesions, extended latency
	disease severity	++	++	+	0	0
Pathogen colonization	xylem penetration efficiency	+++	++	++	+	+
	xylem colonization efficiency	+++	+	+	0	0
	mesophyll colonization efficiency	+++	+	+	0	0
	E. turcicum DNA content	+++	+	+	+	+
Epidemiology	number of lesions	+++	++	+	0	0
	lesion length	+++	++	++	0	+
	sporulation	+++	+	+++	+	+
Physiology	CO_2 assimilation	+++	+++	+	0	++
	transpiration	++	++	++	0	++
	stomatal conductance	++	++	++	0	++
	instantaneous carboxylation efficiency	+++	++	+	0	++
	chlorophyll fluorescence	0	++	0	0	0
Biochemistry	H_2O_2 localization	++	+	+++	++	+++
	peroxidase activity	+	++	++	++	+++
Biomass	aerial biomass	0	0	0	0	0
	root biomass	0	0	0	0	0
	leaf area	-	0	0	0	0

0: 0%, +: 1-30%, ++: 31-60%, +++: 61-100% disease / strong reduction

A principal component analysis (PCA) was conducted to identify patterns among the resistance responses of *Ht* genes using selected variables related to the host (host variables) and to the pathogen colonization and epidemiology (pathogen variables) obtained from experiments conducted in the present study (Table 2). Two cluster analysis were performed to identify similarities between the lines according to host variables and to pathogen variables (Figure 1). More details regarding statistical approach for this extra analysis are described in the supplementary material.

The susceptible line B37 was clustered in distinct groups for the analyses with host variables and with pathogen variables (Figure 1). The cluster analysis for host variables clustered the line B37*Ht1* and B37*Htn1* as both lines showed some reduction in the photosynthetic variables. In contrast, the lines B37*Ht2* and B37*Ht3* did not exhibit high decrease on photosynthetic variables and formed a distinct cluster. Photosynthetic costs of resistance are low or almost absent for B37*Ht2* and B37*Ht3*, whereas photosynthetic activity of the line B37*Ht1* was not recovered in the late stages of disease development (Figure 1A).

In the analysis of pathogen variables, pathogen growth was almost absent in the lines B37*Ht3* and B37*Htn1* (Figure 1B). The xylem and mesophyll colonization were absent and values of DNA content, number of lesions and sporulation were strongly reduced in these lines. Thus, it is strongly purported that resistance mechanisms are acting in earlier stages of infection for these lines were compared to B37*Ht1* and B37*Ht2*, which were clustered in a distinct group. Conversely, resistance mechanisms triggered by B37*Ht1* and B37*Ht2* were not so efficient in reducing pathogen colonization. The most effective resistance mechanism has shown to be triggered by *Ht3,* as pathogen colonization and damage to photosynthetic apparatus was reduced for this line. The plant can compensate for photosynthetic costs associated with resistance mechanisms and might recover faster from pathogen infection. In contrast, the highest values of pathogen variables were observed for *Ht1.* This gene was not so efficient on prevent pathogen colonization, and beyond that, B37*Ht1* plants did not recovered from pathogen infection at 14 dpi. In general, it has been demonstrated that *Ht* genes trigger several resistance mechanisms, which leads to a complex host-pathogen interaction.

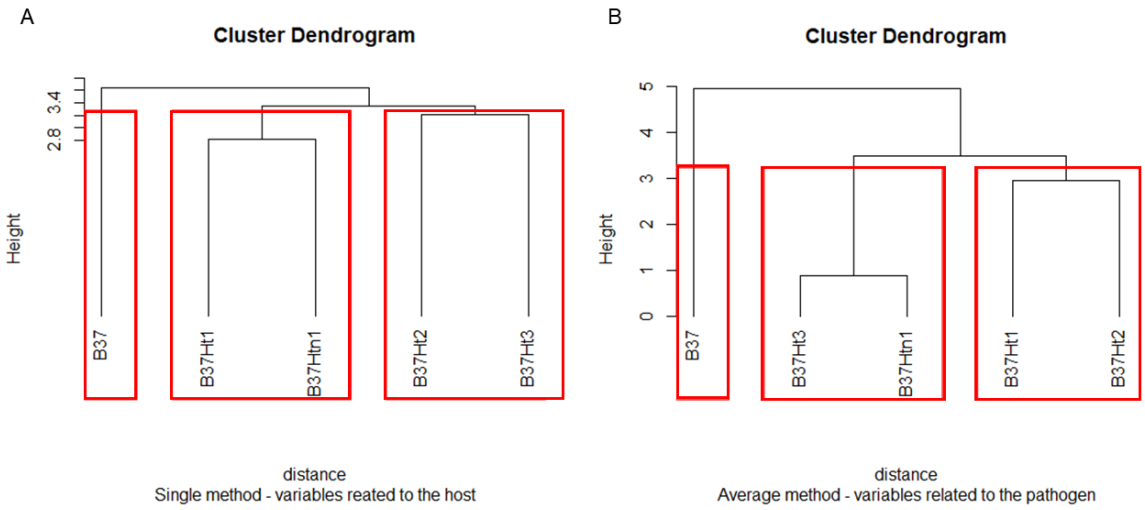

Figure 1. Dendrograms for the maize lines B37, B37*Ht1*, B37*Ht2*, B37*Ht3* and B37*Htn1,* which were inoculated with a race 0 isolate of *Exserohilum turcicum*, clustered in groups according to variables related to the host (A) and to pathogen colonization and epidemiology (B) based on standardized Euclidean distance by the average method. Cluster analyses were performed for the host variables aerial biomass (Dax/Dao), leaf area (Lax/Lao), H_2O_2 localization (H2O2), peroxidase activity (POX), CO_2 assimilation (Px/Po), transpiration (Ex/Eo), stomatal conductance (gsx/gso) and instantaneous carboxylation efficiency (kx/ko), and for the pathogen variables xylem penetration efficiency (XPE), xylem colonization efficiency (XCE), mesophyll colonization efficiency (MCE), disease severity (sev), fungal DNA content (DNA), number of lesions (n.les), lesion length (les.lngth), and sporulation.

The cultivation of breeding materials bearing *Ht* genes remains a good strategy for NCLB control. Pathogen colonization and sporulation was strongly reduced in the incompatible interactions. Additionally, the delay in infection progress in compatible interactions with some resistant lines implies that *Ht* genes may be associated with, or can confer, additional quantitative resistance to *E. turcicum*, as observed for *Htn1* (Hurni *et al.*, 2015). However, *Htn1* may no display any lesions, which leads to a misclassification as a source of qualitative resistance. *Ht1* and *Ht2* are still classified as qualitative forms of resistance, however, *Ht2* may be classified as a source of quantitative resistance, since the number and size of lesions are reduced. *Ht1* reduced sporulation and therefore, could be classified as quantitative resistance based on epidemiological approach (*sensu* Vanderplank 1968). Additionally, *Ht* genes have been shown to reduce fungal DNA content in the compatible interaction. For the genetic trait, the resistance triggered by *Ht* genes is governed by few genes. In fact, the genetic background leads to confusion for most resistance genes, as qualitative sources of resistance can be oligogenic (Hallmann and Tiedemann, 2019).

Nevertheless, it is important to point out that all related genes may have a gene-for-gene interaction with *E. turcicum* (Flor, 1971, Parlevliet and Zadoks, 1977) and a weaker gene-for-gene interaction might be classified as a mechanism of quantitative resistance (Poland *et al.*, 2009). Based on the hypothesis of resistance mechanisms triggered by the quantitative resistance, the *Ht* genes might be evolving into weaker forms of R genes, as xylem penetration is not completely blocked by the resistance mechanisms and the pathogen is able to reach the vascular tissue.

D. Conclusions

Pathogen populations have been proven to be different between regions in several aspects. The prevalence of species or the frequency of races affects the efficiency of resistance and consequently disease control. The main conclusions obtained in each chapter of the present work are expressed below.

- The race assessment in Argentina and Brazil demonstrated that qualitative resistance to *Exserohilum turcicum* is not being widely introduced in commercial hybrids and might be combined with sources of quantitative resistance though breeding programs in those countries.
- The plasticity of Brazilian *E. turcicum* isolates in adapting to high temperatures indicates that the durability of resistance may be decreased.
- The diversity of resistant phenotypes conferred by *Ht* genes is reflected in pathogen colonization due to distinct resistance mechanisms. The reduction in disease levels in the compatible interaction indicates that these genes may be associated with, or confer, additional quantitative resistance.
- Physiological, biochemical and epidemiological studies confirmed and emphasized that resistance mechanisms triggered by *Ht* genes are phenotypically different leading to distinct plant resistance responses.
- The resistance conferred by the *Ht2* gene is not affected by changes in the pre-inoculation temperature. Changes in resistance phenotypes under distinct post-inoculation temperatures might be related to the influence of temperature on the activity of detoxicating enzymes, which may increase necrosis due to high phytotoxins concentrations.
- In general, the German *Fusarium graminearum* was more aggressive than the Brazilian strain in both the temperate and tropical lines. This finding indicates that a pre-selection of highly aggressive *Fusarium verticillioides* strains is required to select maize lines for resistance. Moreover, low pre-inoculation temperatures

decreased disease severity in tropical lines, indicating that tropical lines have phenotypical plasticity.

Resistance mechanisms involved in the reduction of the number of lesions, lesion size and sporulation may decrease epidemics in the field, especially by decreasing secondary inoculum. The cultivation of resistant hybrids remains a successful strategy for controlling NCLB. The same strategy is strongly recommended for controlling GER and FER, and consequently decreasing mycotoxin levels, as the timing of fungicide applications for an efficient control of GER and FER is still difficult to predict (Munkvold, 2003; Lanza *et al.*, 2016). In general, estimations demonstrated that methods applied for disease control reduced yield losses by 32% (Oerke, 2006). Nevertheless, it is important to improve the efficiency of disease control methods, especially the increase in the durability of resistance, in order to reduce yield gap and optimize maize production.

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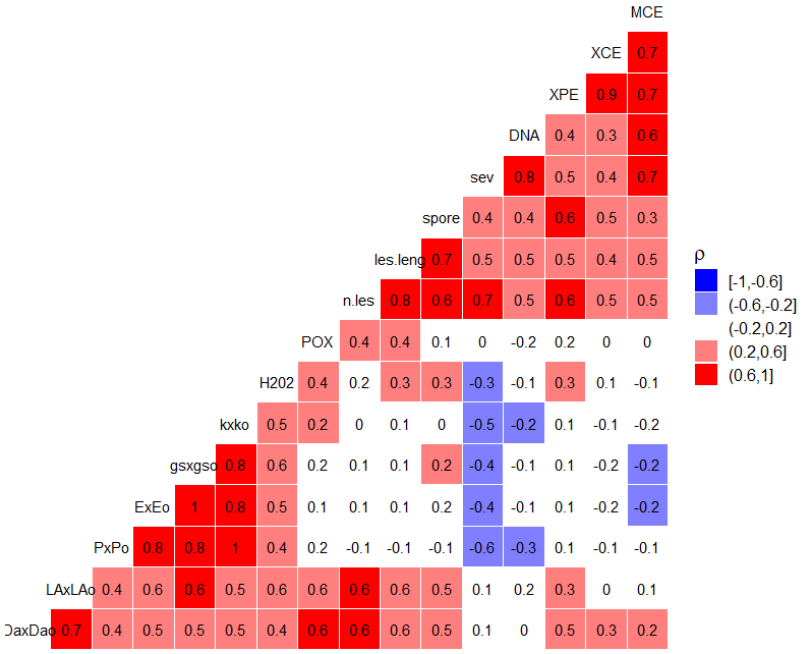
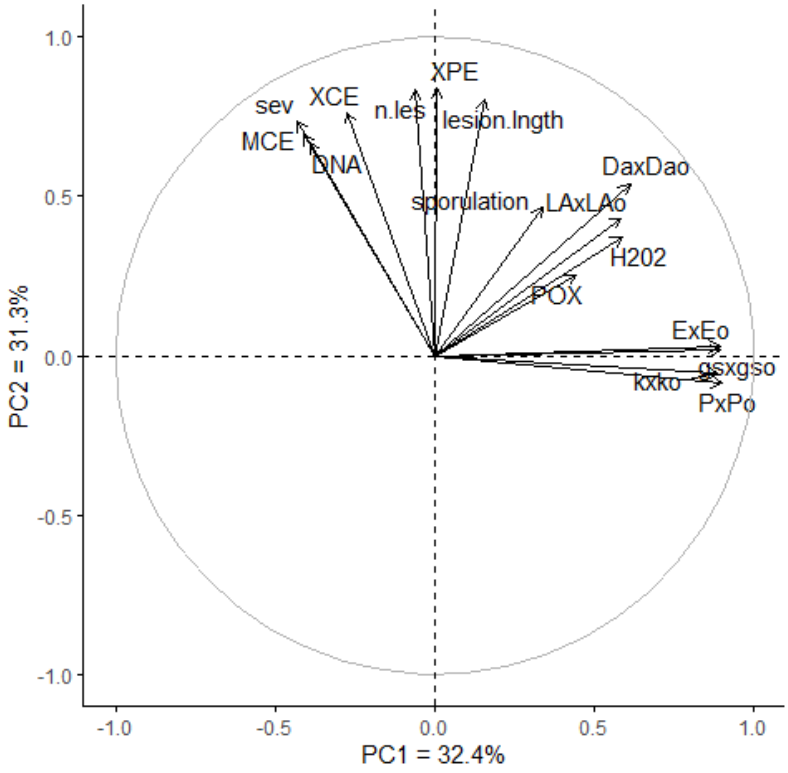
Supplementary Material

In the multivariate analysis was conducted with all evaluated variables in order to demonstrate the correlations between phenotypical similarities triggered by the *Ht* genes. Data represented in Table 2 for the traits symptomology, pathogen colonization, epidemiology and physiology were collected at 14 dpi, and the biochemical and biomass traits were quantified at 6 and 28 dpi, respectively. The selected host variables were peroxidase activity (POX), H_2O_2 localization (H_2O_2), instantaneous carboxylation efficiency (kx/ko), stomatal conductance (gsx/gso), transpiration (Ex/Eo), CO_2 assimilation (Px/Po), leaf area (Lax/Lao), and aerial biomass (Dax/Dao); and the pathogen variables were mesophyll colonization efficiency (MCE), xylem colonization efficiency (XCE), xylem penetration efficiency (XPE), fungal DNA content (DNA), disease severity (sev), sporulation (spore), lesion length (les.lngth), and number of lesions (n.les). A spearman correlation was

conducted to check for correlations between the evaluated traits (Supplementary figure 1). To obtain a balanced sample size for all variables, the correlations coefficients and the PCA were conducted with the average, maximum and minimum values of each variable. The cluster analysis was conducted only with average values of each variable (further information regarding the analysis can be found in the supplementary material). The principal component analysis demonstrated that host variables were positioned on the right-hand side and the variables related to the pathogen variables were positioned in the upper part of the graph (Supplementary figure 2). The photosynthetic variables were negatively correlated to the variables related to the pathogen (sev, DNA and MCE). In this pathosystem, the increase in disease severity caused a reduction in photosynthesis. Biochemical and biomass parameters correlated better to variables related to the host and therefore they were added to the cluster analysis for host variables (Supplementary figures 1 and 2).



Supplementary figure 1. Spearman correlation coefficients (ρ) for the variables mesophyll colonization efficiency (MCE), xylem colonization efficiency (XCE), xylem penetration efficiency (XPE), fungal DNA content (DNA), disease severity (sev), sporulation (spore), lesion length (les.leng), number of lesions (n.les), peroxidase activity (POX), H_2O_2 localization (H2O2), instantaneous carboxylation efficiency (kx/ko), stomatal conductance (gsx/gso), transpiration (Ex/Eo), CO_2 assimilation (Px/Po), leaf area (Lax/Lao), and aerial biomass (Dax/Dao) for the maize lines B37, B37*Ht1*, B37*Ht2*, B37*Ht3* and B37*Htn1* inoculated with a race 0 isolate of *Exserohilum turcicum,* which has incompatible interaction with lines carrying *Ht* genes.



Supplementary figure 2. Principal component analysis for the variables xylem penetration efficiency (XPE), xylem colonization efficiency (XCE), mesophyll colonization efficiency (MCE), disease severity (sev), fungal DNA content (DNA, number of lesions (n.les), lesion length (les.lngth), sporulation, aerial biomass (Dax/Dao), leaf area (Lax/Lao), H_2O_2 localization (H2O2), peroxidase activity (POX), CO_2 assimilation (Px/Po), transpiration (Ex/Eo), stomatal conductance (gsx/gso) and instantaneous carboxylation efficiency (kx/ko) measured on the maize lines B37, B37*Ht1*, B37*Ht2*, B37*Ht3* and B37*Htn1* inoculated with a race 0 isolate of *Exserohilum turcicum,* which has incompatible interaction with lines carrying *Ht* genes.

For the cluster analysis, the standardized Euclidean distance was used as a measure of dissimilarity. The following agglomerative hierarchical methods were applied: single linkage, complete linkage, average linkage (also called the “unweighted pair groups method using arithmetic average” – UPGMA), centroid and Ward’s method. The method that best fitted to the data was chosen using Gower’s distance. The number of clusters was selected using the silhouette method and visual analysis of the grouping patterns (Quinn and Keough, 2002). Two dendrograms were obtained to visualize the lines within groups (Figure 2). Cluster analysis was performed using the function hclust in R 3.6.0 software (R Core Team, 2019).

IV. Outlook and future studies

Our studies demonstrated that *Exserohilum turcicum* races and the aggressiveness of European isolates are different from South American isolates. Further molecular studies could be conducted in order to compare the genetic diversity between both populations and check for mixed reproduction modes. Information about genetic diversity and the reproduction system may provide clarity about pathogen plasticity, and consequently the durability of resistance introduced to these areas.

The resistance to *E. turcicum* was conferred by preventing xylem and mesophyll colonization. Additionally, epidemiological, physiological and biochemical studies demonstrated that resistance mechanisms triggered may differ according to the *Ht* genes. Further studies are necessary to identify virulence factors (effectors) and understand the mechanisms involved in resistance, such as transcriptomic studies or expression of resistance and/or avirulent genes.

Other microscopy techniques such as transmission electron microscopy and/or fluorescence microscopy could be applied to identify structural resistance mechanisms. Moreover, mechanisms of detoxification could be investigated, as they might be involved on resistance. The effect of phytotoxin under high temperatures remain to be investigated, in order to explain the role of aggressiveness factors under high post-inoculation temperatures. The mechanisms behind the detoxification of phytotoxins produced by *E. turcicum* remain to be studied.

The resistance gene *Ht2* was not influenced by the pre-inoculation temperatures. The reasons for the weak resistance of the *Ht2* gene are speculative. The location and sequence of the *Sht1* gene, which has epistatic effect to *Ht2*, remains to be identified. Moreover, the incomplete dominance of *Ht2*, and its gene dosage effect remain to be elucidated in more detail.

The number of *Fusarium* spp. isolates used in this study was restricted. An analysis with a larger number of isolates is suggested to draw conclusions regarding the aggressiveness of *Fusarium* spp. populations in Brazil and Germany. Moreover, experiments conducing plants until full stage are extremely laborious under greenhouse conditions. In general, field experiments provide more trustful results. The conduction of experiments in Germany and Brazil would allow broader comparisons regarding GER and FER epidemics in both countries.

V. Summary

In the last 60 years, maize production has increased worldwide, reaching 1.14 billion tons in 2018. Maize production in Europe and South America was about 110 and 130 million tons in 2018, respectively. The demand for highly productive maize is observed in both tropical and temperate zones. Thus, the selection of plants from different maturity groups and high yield production are required from breeding programs. Besides highly productive plants, other agronomical traits such as resistance to pest and diseases needs to be considered during selection. Globally, some of the most important diseases affecting maize are northern corn leaf blight (NCLB), and Gibberella and Fusarium ear rot (GER and FER, respectively). Host resistance to *E. turcicum* is based on qualitative or quantitative sources, while for GER and FER only quantitative resistance is available in commercial hybrids. The quantitative resistance is more durable; however, it is more laborious to introgress into breeding lines.

Northern corn leaf blight (NCLB) is an important disease in maize-producing areas worldwide. The symptoms of NCLB, whose causal agent is the ascomycete *Exserohilum turcicum* (teleomorph *Setosphaeria turcica*), are characterized by elliptical grey-green lesions. High disease severity can cause yield losses up to 40% (Levy und Pataky 1992). The main control methods applied for NCLB control are fungicide applications and the cultivation of resistant hybrids. Qualitative resistance has been widely used to control NCLB in many countries through the deployment of *Ht* genes. The race assessment from isolates collected in Argentina and Brazil during 2017, 2018 and 2019 revealed a high frequency of race 0 isolates (83% and 65% in Argentina and Brazil, respectively). In those countries, *Ht* genes are not being used extensively to control NCLB. This information is important for breeding programs and may help with disease management.

Favorable weather conditions for NCLB development are long dewy periods and moderate temperatures. These optimum conditions for disease development can be observed in temperate regions as well as in mid-altitude regions in the tropics. The comparison of *E. turcicum* isolates in response to temperatures varied *in vitro* and *in vivo* between 15 and 30°C demonstrating that the aggressiveness of South American isolates was higher than that of European isolates. The multivariate analysis confirmed that South American isolates are better adapted to higher temperatures by grouping them separately. In conclusion, *E. turcicum* populations may adapt quickly to environmental changes. The plasticity in adapting to environmental conditions of *E. turcicum* may decrease the durability of resistance.

Studies on the pathogenesis of *E. turcicum* in the differential maize line B37 with and without the resistance genes *Ht1*, *Ht2*, *Ht3* and *Htn1* were conducted for different stages of infection and disease development from penetration (0-1 dpi), until full symptom expression (14-18 dpi). Symptomological analysis demonstrated that *Ht1* expressed necrotic lesions with chlorosis, *Ht2* displayed chlorosis and small lesions, *Ht3* resulted in chlorotic spots and *Htn1* express wilt-type lesions. Histological studies conducted with Chlorazol Black E staining indicated that the pathogen was able to penetrate xylem vessels at 6 dpi in compatible interactions and strongly colonized the mesophyll at 12 dpi, which is considered the crucial process differentiating susceptibility from resistance. Additionally, lower disease levels, low fungal DNA content at 10 and 14 dpi, and the delayed progress of infection in compatible interactions with resistant lines imply that the *Ht* genes are associated with or confer additional quantitative resistance. Physiological studies showed a reduction in the photosynthetic rate, transpiration, stomatal conductance and instantaneous carboxylation efficiency in the incompatible interaction at 5 dpi. At 14 dpi, the strong necrosis displayed in the resistance reaction by B37*Ht1* resulted in the reduction of photosynthesis as observed for B37. However, leaf area, aerial and root dry biomass were not reduced in inoculated plants at 28 dpi. Additionally, high rates of peroxide localization were observed in inoculated plants at 3 and 6 dpi, corroborating data on peroxidase activity. In fact, *Ht1*, *Ht3* and *Htn1* reduced pathogen sporulation whereas *Ht2* reduced the number and size of lesions. All phenotypical studies demonstrated that *Ht* genes confer distinct resistance mechanisms.

The resistance phenotype expressed by *Ht2* may change according to environmental conditions. There are reports on the influence of low post-inoculation temperature (22/18°C) and low light intensity (324 and 162 µmol m^{-2} s^{-1}) on resistance expressed by this gene. Our objective was to prove that temperature has no influence on the resistance conferred by the *Ht2*-gene against *E. turcicum.* Therefore, maize plants were pre-exposed to warm (30/25°C) and moderate (20/15°C) temperature regimes for 10 days before inoculation. There was no influence of pre-inoculation temperature on the expression of resistance by *Ht2*. The resistance conferred by the *Ht2* gene was confirmed by quantifying the fungal DNA *in planta* at 21 dpi. Changes in resistance phenotypes may be related to pathogen aggressiveness factors.

GER and FER can cause qualitative yield losses due to mycotoxin production. GER is mainly caused by *Fusarium graminearum* and FER by *F. verticillioides*. GER is more frequent in regions with colder temperatures and high precipitation, and is more prevalent in Germany, while FER occurrence is favored by warm and dry weather conditions and is more prevalent in Brazil. In general, *F. graminearum* was more aggressive than *F. verticillioides*, which support affirmations about systemic colonization by *F. verticillioides*.

With regard to tropical and temperate hosts, the German isolates were more aggressive than the Brazilian isolates when inoculated in the tropical lines. Additionally, tropical lines pre-exposed to higher temperatures presented higher disease severity when compared to plants exposed to mild temperatures. In general, the cultivation of resistant hybrids remains a successful strategy for controlling NCLB, GER and FER. The optimization of resistance resources is fundamental for maintaining the durability of resistance.

VI. Acknowledgements

My deepest acknowledgements to Prof. Dr. Andreas von Tiedemann for the opportunity to do my PhD at the Georg-August Universität Göttingen. I am glad he trusted me and answered to my first E-mail. These words gave me hope and made me believe that my goals could be reach. I am thankful for the trust and independence he gave me during this time, for the patience and advices not only related with my scientific work but also regarding science and a scientific career. I am learning a lot by working with him. His large expertise on phytopathology and applied research, his leadership and teaching skills, contribute to not only to improve my basic knowledge but made me reflect about the importance of social skills.

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My lovely acknowledgements to Dr. Antonio Fernandes Nogueira Júnior. He is always so patient with me. He trusted me and decide to combine our dreams. He instigates me to study phytopathology and always stimulates me to conduct my research and try new ideas. He also advised me and encouraged me to improve my work and writing skills even more. I am glad for all time we have been spending together.

Gracias a mi amiga Marta por la compañía, diálogos y el tándem. I was lucky to have Marta as a colleague and meet her in the beginning when I just moved to Göttingen. I appreciate her attention on introducing me to her friends, which also become my friends later. My thanks to all ‘sobremensa’ friends Natalia, Amelie, Domingo, Davide, Jose, Diogo, Petra,

Martina, Sarah, Dima, Marcel, Marta, Virginia and Antonio, which I spent nice time not only in the Mensa but also having dinners and doing excursions together.

My special thanks to Dr. Renate Krause Sakate for her advices and exchange. She is more than my godmother, she inspires me in the career as a scientist woman, as a plant pathologist, and as a professor.

Muito obrigada aos meus pais Edson e Veronica e a minha irmã Betina pelo amor incondicional. Minha familia é a minha base de perseverança, do saber, da educação, da personalidade, da curiosidade, da paixão pelo trabalho, do respeito ao próximo, e acima de tudo, do amor. Sua paciência, compreensão e incentivo contribuíram para que eu alcançasse esse título.

I am thankful to all my family and friends in Brazil, which support me on the decision to study abroad. I always remember and miss good moments we spent together.

Students supervisions:

Feb.2019 – Jun. 2019 Internship supervision with the topic: “Comparação da agressividade entre isolados sul americanos e europeus de *Setosphaeria turcica”* - Bachelor student Raphael de Araújo Campos

Apr.2019 – Jul.2019 Internship supervision with the topic: “Maize - *Setosphaeria turcica* interaction studies. Measurement of peroxidase activity and fungal DNA content*”* - Master student Christos Tsoukas

Jul.2019 – Sep.2019 Internship supervision with the topic: “Pathogenic traits of *Exserohilum turcicum*: *In vivo* and *in vitro* tests” - Master student Miguel Angel Garza Jacinto

Oct.2019 – Jan.2020 Trainee supervision as technical assistant - Gülsen Aydin

Jul.2020 – Mar.2021 Co-supervision of bachelor thesis with the topic: “Einfluss der Resistenzgene *Ht1*, *Ht2*, *Ht3* und *Htn1* auf den Biomasseertrag von Mais (*Zea mays* subs. *mays*) unter Befall mit der Turcicum-Blattdürre (*Exserohilum turcicum*)” – Bachelor student Lea Pichler

Aug.2020 – Dez.2020 Co-supervision of bachelor thesis with the topic: “Crescimento micelial e esporulação *in vitro* de isolados sul-americanos e europeus de *Exserohilum turcicum* sob diferentes temperaturas” – Bachelor student Richard Willian Verza de Lima

VII. Curriculum vitae

❖ Personal data

Full name: **BARBARA LUDWIG NAVARRO**

M.sc. phytopathology
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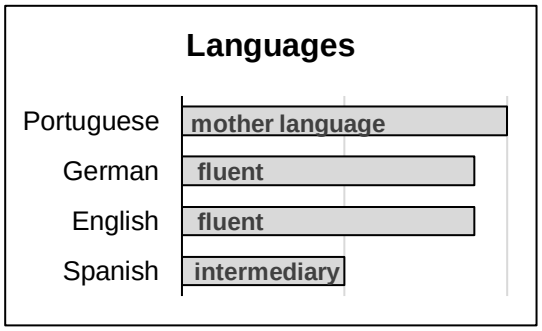
❖ Education

Apr. 2017 – Jan. 2021	**PhD in Plant Pathology**, Division of Plant Pathology and Crop Protection in George-August-University of Göttingen, Germany PhD thesis: "Studies on pathogenicity and host resistance of *Exserohilum turcicum* and *Fusarium* spp. on maize cultivated in tropical and temperate climate zones"
Feb. 2015 –Apr. 2017	**M.Sc. in Plant Pathology** – University of São Paulo (ESALQ-USP) – Piracicaba, São Paulo, Brazil Master dissertation: "The grapevine rust in *Vitis vinifera* and *Vitis labrusca*: epidemiological components and host responses"
Feb. 2010 – Jan. 2015	**Degree in Agricultural Engineering -** University of São Paulo (ESALQ-USP) – Piracicaba, São Paulo, Brazil Bachelor thesis: "Sensibility of *Phakopsora euvitis* isolates to the fungicide pyraclostrobin and characterization of the fungi *Sphaceloma ampelinum* in different medias"
Jul. 2012 – Aug. 2013	**International experience -** Hochschule Neubrandenburg, Neubrandenburg, Germany
Jan. 1999 – Dez. 2009	**Secondary education:** Colégio Cristo Rei – Marília, São Paulo, Brazil



Working experiences

Apr. 2017 – **Research scientist**
Georg-August University of Göttingen – Germany

- Project implementation and development
- Direct communication and exchange with stakeholders
- Data management and report of results
- Literature research and preparation of scientific publications
- Development of project proposals for resource acquisition for scientific grants
- Supervision of technical staff, interns, bachelor, and master students

Internships and other Activities

Nov. 2021 – Feb. 2021 **Tutor in the course Botany** for bachelor students in agricultural science
Georg-August University of Göttingen – Germany

- Supervision of activities on botanical identification

May 2020 – Jul. 2020 **Tutor in the course Plant Protection** for bachelor students in agricultural science
Georg-August University of Göttingen – Germany

- Supervision of activities on plant disease diagnosis and fungi identification

Feb. 2016 – Jun. 2016 **Tutor in the course Microbiology** for bachelor students in agricultural engineering
University of São Paulo – Brazil

- Supervision of activities and experiments developed on the practical part of the course
- Preparation of the material used during the practical work: cultivation of plants and microorganisms

Jun. 2016 – Dec. 2016 **Student representative** of Department of Plant Pathology and Nematology
University of São Paulo – Brazil

Jul. 2014 – Oct. 2014 **Intern in the company Vigna Brasil,** *Piracicaba (SP) - Brazil*

- Cost estimation for investments on poultry production in Cuando -Cubango – Angola - Africa

Aug. 2014 – Nov. 2014 **Tutor in the course Plant Pathology** for bachelor students in agricultural engineering
University of São Paulo – Brazil

- Supervising activities of plant disease diagnosis and fungi identification

Jan. 2014 – Dec. 2014 **Intern** in the working group **Plant Epidemiology** - Department of Plant Pathology and Nematology
University of São Paulo – Brazil

- Research on host specificity of *Puccinia psidii* to Myrtaceae
- Research on fungicide resistance of grapevine rust (*Phakopsora euvitis*) and antracnose (*Eusinoe ampelina*) to the fungicide pyraclostrobin

Jul. 2013 – Aug. 2013 **Intern on fruit orchards: Schneider Baumschulen,** *Edingen - Germany*

- Harvest of apple and other fruits, fruit selection to the market
- General work at the nursery

Aug. 2013 – Mar. 2013 **Intern in the company Saatzucht Steinach**, *Bocksee - Germany*

- Inoculation von grasses with *Microdochium nivale* and *Puccinia coronate, p*reparation of materials for field experiments
- Research on a fast assay to identify toxic substances of lupines seeds

Aug. 2011 – Jun. 2012 **Finance management at ESALQ Junior Consultoria**, *Piracicaba (SP) - Brazil*

- Agricultural consulting for small farmers

Jul. 2011 – Aug. 2012 **Intern at the Division of Horticulture and Quality (CQH) at the storage hall (CEAGESP)**, *São Paulo (SP) - Brazil*

- Questionnaire about changes on the size of potato sacks
- Tests about pesticides residuals on fruits and vegetables

May. 2010 – Jun. 2011 **Intern at the group of research on oil plants** (GPO)
University of São Paulo – Brazil

- Field experiments with single, double and crossed rows on soybean production

Grants and scholarships

Apr. 2015 – Mar. 2017 Grant for **Master studies** in Plant Pathology (CNPq)

Mar. 2016 – Jun. 2016 **Scholarship for monitor in bachelor course** - PAE Program

Aug. 2014 - Dec. 2014 **Scholarship (PIBIC/CNPq) for developing the scientific project** on Host specificity of *Austropuccinia psidii* to Myrtaceae

Jul. 2012 – Aug. 2013 **Grant for studying abroad** - Program “Science without borders“ - (CNPq)

Publications

➢ "Peer-reviewed" Manuscripts

- **Navarro BL,** Streit S, Pichler L, Nogueira Jr. AF, von Tiedemann A (2021) Physiological and biochemical responses of *Ht*-resistance genes in the maize line B37 against *Exserohilum turcicum*. *Manuscript in preparation.*
- Edwards Molina JP, **Navarro BL**, Allen TW, Godoy CV (2021) The re-emergence of soybean target spot in the American continent. *Manuscript submitted for publication* (TPPA-D-21-00186).
- **Navarro BL**, Edwards Molina JP, Nogueira Júnior AF (2021) Infection by Botryosphaeriaceae species in avocado, guava and persimmon during postharvest. *Manuscript submitted for publication* (JPHY-21-189).
- Shirado EV, Marques JPR, Alves RF, Appezzato-da-Glória, B, **Navarro BL**, Fischer IH, Spósito, MB (2021) Cercospora species cause pint spot disease on guava fruit in Brazil, *Manuscript accepted for publication at the Journal of Phytopathology* (JPHY-21-184.R1).
- **Navarro BL**, Ramos-Romero L, Kistner MB, Iglesias J, von Tiedemann A (2021) Assessment of physiological races of *Exserohilum turcicum* isolates from maize in Argentina and Brazil. *Tropical Plant Pathology* - https://doi.org/10.1007/s40858-020-00417-x
- **Navarro BL**, Campos RA, Gasparotto MCG, von Tiedemann A (2021) *In vitro* and *in planta* studies on temperature adaptation of *Exserohilum turcicum* isolates from maize in Europe and South America. *Pathogens* ***10***(2), 154.- https://doi.org/10.3390/pathogens10020154
- **Navarro BL**, Hanekamp H, Koopmann B, von Tiedemann A (2020) Diversity of expression types of *Ht* genes conferring resistance in maize to *Exserohilum turcicum. Frontiers in Plant Science***11,** 607850 - https://doi.org/10.3389/fpls.2020.607850
- **Navarro BL**, Marques JPR, Appezzato-da-Glória, B, Spósito, MB. (2019) Histopathology of *Phakopsora euvitis* on *Vitis vinifera*. European Journal of Plant Pathology 154, 1185–1193 - https://doi.org/10.1007/s10658-019-01719-w
- **Navarro BL**, Nogueira Júnior AF, Ribeiro RV, Spósito MB (2019) Photosynthetic damage caused by grapevine rust (*Phakopsora euvitis*) in *Vitis vinifera* and *Vitis labrusca. Australasian Plant Pathology* 48, 509–518 - https://doi.org/10.1007/s13313-019-00654-y
- Costa JO, **Navarro BL,** Soares JN (2016). Climate risk for occurrence of rust on the coffee plant. *Revista GEAMA*, v.2, n.1, p.8-14.

➢ Book Chapter

- **Navarro BL**, Blumenstein K, Nordzieke DE. Peroxiporins in plant-microbial interactions. In: Bienert GP, Medrano Fernandez I, Sitia, R. (Eds.) *Peroxiporins: Redox Signal Mediators In and Between Cells.* CRC Press/Taylor & Francis, Boca Raton, Florida. (*in press*)

➢ Contributions in non-reviewed journals

- **Navarro BL**. A cultura do milho no Brasil e na Alemanha: enfoque nas doenças. Agroadvance, 26.02.2021.https://agroadvance.com.br/a-cultura-do-milho-no-brasil-e-na-alemanha-enfoque-nas-doencas/

- **Navarro BL**, Lima CK. Sacaria de batata: qual o tamanho ideal? *Revista Batata Show*, Brasil, p. 71-72, 10.08.2011.
- **Navarro BL**, Lima CK. Sacaria de batata: qual o tamanho ideal? *Jornal Entreposto*, São Paulo, SP, p. 30, 03.08.2011.

➢ **Proceedings**

- **Navarro BL**, Streit S, Pichler L, Nogueira Jr AF, von Tiedemann, A (2021) Charakterisierung der Wirksamkeit von Ht-Resistenzgenen in Mais gegen *Exserohilum turcicum 62. Deutsche Pflanzenschutztagung "Gesunde Pflanzen in Verantwortung für unsere Welt", 21. - 24. September 2021, Georg-August-Universität Göttingen.*
- **Navarro BL**, Ramos-Romero L, Kistner MB, Iglesias J, von Tiedemann A (2021) Assessment of physiological races of *Exserohilum turcicum* isolates from maize in Argentina and Brazil. 5° Congreso Argentino de Fitopatólogos. 22-23.09.2021.
- **Navarro BL**, Ramos-Romero L, Kistner MB, Iglesias J, von Tiedemann A (2021) Vergleich zwischen Turcicum-Blattdürre (*Exserohilum turcicum*) an Mais (*Zea mays* L.) in Europa und Süd-Amerika. 34. Tagung des DPG-Arbeitskreises „Krankheiten im Getreide und Mais“ am 25.01.2021 als online Veranstaltung- Brauschweig - Germany
- Lima RWV, **Navarro BL**, Gasparoto MCG (2020) Crescimento micelial in vitro de isolados americanos e europeus de *Exserohilum turcicum* sob diferentes temperaturas. XXXII Congresso de Iniciação Científica da Unesp, 24 and 25. 11.2020, Registro (SP) - Brazil
- **Navarro BL**, Hanekamp H, Koopmann B, von Tiedemann A (2019) Understanding the compatible and incompatible host-pathogen interaction of *Setosphaeria turcica* races with maize leaves by histological studies. Plant Health 2019. 03 – 07.08.2019. Cleveland (OH) - USA.
- **Navarro BL**, Hanekamp H, Tiedemann Av (2018). Histologische Untersuchungen zur Pathogenese von *Setosphaeria turcica* in Mais *61. Deutsche Pflanzenschutztagung "Herausforderung Pflanzenschutz – Wege in die Zukunft", 11. - 14. September 2018, Universität Hohenheim - Kurzfassungen der Vorträge und Poster*, Julius-Kühn-Archiv 454, S.511-512. doi:dx.doi.org/10.5073/jka.2018.461.000
- **Navarro BL,** Nogueira Jr. AF, Bergamin Filho A, Amorim L, Spósito, MB. “Disease Efficiency: a study case with *Phakopsora euvitis* in *Vitis labrusca*”. 12th International Epidemiology Workshop (IEW 12), 10 – 14.06.2018, Lillehammer - Norway.
- **Navarro BL**, Marques JPR, Appezzato-da-Glória, B, Spósito, MB (2017). Histopathology of *Phakopsora euvitis* on *Vitis vinifera* cv. Moscato. 40° Congresso Paulista de Fitopatologia, 07 – 09.02.2017, IAC – Campinas (SP) - Brazil
- **Navarro BL**, Nogueira Jr. AF, Spósito MB. “Pathogenic specificity of *Puccinia psidii* from Myrtaceae species”.48 Congresso Brasileiro de Fitopatologia 10 - 14.08.2015 São Pedro (SP) - Brazil
- **Navarro BL**, Spósito MB., Nogueira Jr. AF, Amorim L. (2015) “Infeccíon de *Phakopsora euvitis* en *Vitis labrusca*”. Congresso Internacional de Fitopatología 2015 (Mexico City) - Mexican Journal of Phytopathology, v.33, S101.

Voluntary work

April.2020	**Video record for the international Master in Crop Protection -** "Plant Health Management in Tropical Crops" *University of Göttingen - Germany*
06.01.2020	**Organization of the Brazilian cultural evening** at the *University of Göttingen - Germany*
12.12.11 - 22.12.11	Member of the **Expedition** in the Project Bandeira Científica 2011 in *Belterra (PA) - Brazil* • Cultivation of vegetable gardens in schools and communities - Education about "healthy eating" • Social and economic advices for small farmers

VIII. Statutory declaration

I, hereby, declare that this dissertation was undertaken independently and without any unaccredited aid.

Place, Date: Göttingen, 22nd October 2021.

Barbara Ludwig Navarro