High Fumonisin Content in Maize: Search for Source of Infection and Biological Function

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Life is not empty

Kindness, apples, faith

Aye,!

While there are peonies one can live on…

In my heart, there is something like a blaze of light, like a morning dream

And so restless am I that I feel like running

To the far end of the plains, up to the mountain top

A voice keeps calling me from afar……..

“Sohrab Sepehri”, Iran (1928-1980)

“I dedicate this thesis to my parents”

Contents

Chapter 1

General Background

Introduction

Maize (*Zea mays*) is one of the most important cereal crops in the world, and is widely grown in diverse climatic and ecological conditions. Worldwide production of maize is 690 million tons, of which 273 million tons (around 40%) is grown by the United States (FAO, 2013). China and Brazil, with productions of 208 and 71 million tons respectively, are the next largest producers in the world. Maize plays an important role in the diet of both humans and animals. Therefore, the growing demand from a rising human population has been causing a steady increase in the global production of maize (Shiferaw et al., 2011; Nuss & Tanumihardjo, 2010).

Fungi in the genus *Fusarium* are well known as economically important maize pathogens. They cause ear and kernel rot, stalk rot and seedling blight, with subsequent yield reductions of 10 to 30%, while the quality of the products is also influenced (Logrieco et al., 2002; Gilbertson et al., 1985; Pintos Varela et al., 2013). Among the large number of *Fusarium* spp. reported from maize infected tissues, relatively few are considered to be of major significance. The main *Fusarium* pathogens associated with maize ear rot are divided into two groups. The members of the *Gibberella fujikorui* species complex, including *Fusarium verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsushima) Nirenberg, *F. subglutinans* (Wollenweb. & Reinking) P. E. Nelson, T. A. Toussoun & Marasas and *F. temperatum* are responsible for *Fusarium* ear rot or pink fusariosis. These species are representatives of *Liseola* section. Recently, *F. temperatum* has been separated from *F. subglutinans* (Scauflaire et al., 2011) and shown to be a pathogen of seedling blight and stalk rot of maize in Spain (Pintos Varela et al., 2013). The second group contains species of the *Discolour* section, mainly *F. graminearum* Schwabe and *F.culmorum* (W. G. Smith) Sacc, which cause red fusariosis or *Gibberella* ear rot (Munkvold, 2003). Although these *Fusarium* species are of the greatest overall significance, other toxigenic *Fusarium* spp. including *F. avenaceum, F. poae, F. equiseti, F. tricinctum, F. cerealis, F. sporotrichioides* and *F. semitectum* also contribute to the infection of maize ear tissue in particular situations. *F. oxysporum* has been found to be a less common

species in maize and other cereal crops. *F. venenatum* is another species that sporadically has been isolated from maize tissues (Logrieco et al., 2002; Kosiak et al., 2003; Yli-Mattila, 2010). The distribution and prevalence of different *Fusarium* spp. largely depends on environmental conditions and agricultural practices (Arino & Bullermann, 1994). Simultaneous occurrence of several species of *Fusarium* on maize kernels can affect the final level of infection and mycotoxin contamination (Munkvold, 2003).

Maize *Fusarium* diseases have received more attention because of their association with kernel mycotoxin contamination. Mycotoxins are secondary metabolites produced by fungi such as *Fusarium* spp. that are capable of contaminating food and feed products. Occurrence of mycotoxins in maize had always been a serious concern for both human and animal consumers. Low doses of mycotoxins could also be dangerous when consumed over prolonged periods of time (Pestka & Smolinski, 2005). The main *Fusarium* mycotoxins, including trichothecenes, fumonisins, zearalenone, enniatins and moniliformin are usually reported from infected maize tissues (Glenn, 2007; Placinta et al., 1999). They are formed in rotted stalks (zearalenone, zearalenols) (Bottalico et al., 1985), infected leaves (nivalenol) and in entire plants (zearalenone) (reviewed in Logrieco et al., 2002). Many countries have established threshold values for *Fusarium* mycotoxin contamination in cereals and products and samples exceeding this limit are not allowed to be marketed (D'Mello et al., 1999).

Epidemiology of Maize *Fusarium* Diseases

F. verticillioides is the predominant *Fusarium* pathogen, known as causal agent of maize pink ear rot. It is a heterothallic species and forms fruiting structures less readily than homothallic ones. Therefore, it usually produces thickened hyphae to be able to survive the absence of host plants during the off season. Although sexual reproduction is considered important for the genetic recombination of the fungi, in epidemiology it does not play a major role. For this heterothallic *Fusarium* spp., formation of asexual spores on plant residues is considered as the main source of inoculum. Infection of the silk channel by airborne conidia leads to symptomless kernel infection. The primarily infectious propagules, thus, are microconidia, although dispersion of macroconidia by wind has been reported as well (Munkvold, 2003).

In contrast, reports show *F. graminearum* isolates (teleomorph: *Gibberella zeae* (Schweinitz) Petch, which are a causal agent of both head blight (scab) of wheat and red ear rot of maize, are able to produce brownish perithecia in nature (Parry et al., 1995). In Europe, this species (*F. graminearum*, lineage 7 *sensu stricto*) has been replacing the closely related *F. culmorum* (Yli-Mattila, 2010). The perithecia are formed on the surface of crop debris and the ascospores start the primary infection. The optimal temperature for releasing the ascospores is around 16°C (Tschantz et al., 1976). Reports show that the perithecial drying during the day followed by high relative humidity at night can stimulate discharging of spores, but ascospore release will be inhibited under heavy rainfall conditions (Paulitz, 1996). In addition to sexual spores as the principal inoculum source of fungus, asexual spores originating from sporodochia also are important for the infection of maize plants (Paul et al., 2004). Dispersal of ascospores is usually by air (Paulitz et al., 1999); while macroconidia are disseminated by water splashes or wind (Parry et al., 1995). No secondary infection by the fungus is demonstrated (Fernando et al., 1997). Since *F. graminearum* is primarily a monocyclic disease, the role of primary inoculum in disease epidemics is outstanding.

Source of *Fusarium* Inoculum

Fusarium species that invade cereals are able to survive saprophytically on crop residues (Parry et al., 1995). This residue in or on the soil where the crop is grown or in nearby fields, is considered to provide the primary source of inoculum for the infection of the plants during the growing season (Munkvold, 2003). Different reports show that the maize plant debris could be the principal source of inoculum for both maize and wheat crops when they are grown in rotation (Seaman, 1982; Clear & Abramson, 1986; Teich & Nelson, 1984). Further studies in Europe and North America confirmed that, compared to other debris, maize residues are more effective inoculum contributing to the incidence of fusarium head blight of wheat crop (Dill-Macky & Jones, 2000; Schaafsma et al., 2001). In Uruguayan production systems, however, maize debris contributes a lower propotion of inoculum production for *F. graminearum* epidemics (Pereyra & Dill-Macky, 2008). Maize debris provides a long-term reservoir of inoculum, particularly if they form a surface residue (Cotten & Munkvold, 1998). Re-colonization of surface residue by airborne inoculum leads to long-term survival of fungus, production of new spores, and subsequently infection of new maize plants. In this case, survival of the fungus is affected by the

size and residue depth (Cotten & Munkvold, 1998). Infected debris generates different types of infectious propagules including sexual or asexual spores (Munkvold, 2003).

In addition to wheat, barley and maize debris, some weed species and wild plants have been demonstrated as inoculum source of *F. graminearum* (Jenkinson & Parry, 1994); while lack of perithecia production by *G. zeae* on sunflower residues indicated this substrate could not contribute to the primary inoculum of the fungus (Pereyra & Dill-Macky, 2008). On the other hand, the potential inoculum of *Fusarium* spp. in the soil is affected by the previous crop and the system of soil preparation, as well. Therefore, organic farming systems have indicated a significantly lower level of inoculum in the soil compared to the fields under integrated management (Meier et al., 2001).

Most of the *Fusarium* species in maize such as *F. verticillioides*, *F. graminearum* and *F. subglutinans* are seedborne pathogens. Therefore, seeds are another source of inoculum in the fields (reviewed in Munkvold, 2003). Symptomless systemic infection of maize by *F. verticillioides* has been reported. The fungus transmits from infected seeds to the upper parts of plants (Munkvold et al., 1997; Munkvold & Carlton, 1997). Furthermore, Desjardins et al. (1998) also demonstrated symptomless systemic infection of maize kernels by spores originating from the root rhizosphere.

Weed Plants as Symptomless Alternative Hosts

Although weed plants provide suitable habitat conditions such as high humidity for the development of plant diseases, the role of weeds as hosts is probably more complex. For many years, wild plants, weeds as well as cultivated plants have been considered as bridges between seasons or between crops for the survival of fungal pathogens over the periods. These bridging hosts contribute to epidemics of plant diseases either as a source for production of additional inoculum or as a harbor for resting propagules of pathogens. Therefore, the pathogens will be able to remain active between seasons. On the other hand, with the presence of weeds and wild plants the possibility of potential danger of minor pathogens should not be ignored; even though the main cultivated crop has escaped from those (Dinoor, 1974).

The involvement of weed plants in epidemics of plant pathogens is a delicate subject. It is claimed that in fields under suitable agricultural management, the causal organism may be present and contribute to the buildup of inoculum, but disease epidemics would not occur. The transition from a minor or moderate level of disease to a major problem will only happen when farmers do not employ suitable cultural practices such as tillage, residue management or rotation. In such situations, wild plants may be contaminated by the pathogen and the development of disease will occur in wild hosts (Dinoor, 1974). Weed plants and natural vegetation are thought to be a reservoir of *Fusarium* pathogens over the winter (Jenkinson & Parry, 1994). The first report regarding weeds as *Fusarium* harbor indicated recovery of *Fusarium* spp. from 19 species of cereals and grasses as well as 24 species of common weeds (Gordon, 1959). In 1960, for the first time the importance of weed plants as a source of fungal inoculum was emphasized (Garrett, 1960). Garrett proposed that weed roots infected by soil-borne pathogens could directly act as a source of inoculum for the roots of susceptible plants. Viable and compatible inoculum is necessary to establish a destructive epidemic (Dinoor, 1974). It is believed the role of weed plants in the survival of *Fusarium* species such as *F. poae* which have no saprophytic growth on debris and no resting spore for overwintering is significantly important (Jenkinson & Parry, 1994).

Alternative hosts have been generally considered as the bridges between crops that support the fungal inoculum source. They include any hosts such as grasses or broad-leaved weeds in addition to the main host. Alternative hosts harbor the pathogens during the off season, when the economically main host is absent and, then, serve as bridges in the main growing season (Dinoor, 1974; Parry et al., 1995). Therefore, a fungal pathogen may have an entirely different adaptation to a range of hosts that would be distinctly unrelated. Jenkinson & Parry, (1994) demonstrated some new weed hosts for *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, and *F. sambucinum*. Studies in Southern Manitoba indicated some wild grasses such as *Bromus intermis*, *Calamagrostis canadensis*, and *Agropyron trachycaulum* were colonized more easily than others as symptomless carriers by *Fusarium* spp. (Inch & Gilbert, 2003). The gramineous weeds of *Digitaria sanguinalis*, *Setaria* spp., *Lolium multiflorum*, and *Cynodon dactylon* as well as wheat, barley and maize debris have also been cited as the sources of *F. graminearum* inoculum; while white clover and birdsfoot trefoil (the perennial pastures in

Uruguay) did not contribute to *G. zeae* inoculum (Pereyra & Dill-Macky, 2008). Several weed species have been found to host 14 different *Fusarium* spp. in Croatian fields (Postic et al., 2012). Most of the studies on weeds as alternative hosts for fungal pathogens have concentrated on *F. oxysporum* (Katan, 1971; McDonald & Leach, 1976; Haware & Nene, 1982; Helbig & Carroll, 1984; Altinok, 2013).

Studies have mostly revealed the symptomless colonization of weeds by different pathogenic fungi (Roy, 1982; Cerkauskas et al., 1983; Helbig & Carrol, 1984; Roy et al., 1994; Jenkinson & Parry, 1994; Postic et al., 2012; Altinok, 2013). Katan, (1971) reported that the contribution of a symptomless carrier for inoculum production of *Fusarium* tomato wilt is 1-4% of the propagules developed on a susceptible host. The exhibition of no symptoms by weed plants infected with *Fusarium* species has been remained a contentious issue. Some reports have claimed that only less aggressive strains can invade weeds and produce a symptomless infection (Helbig & Carroll, 1984). Lack of strong adaptation of *Fusarium* on alternative hosts showed that the passage through an alternative host causes the reduction of pathogenic fitness (aggressiveness); but increases overall fungal reproduction (saprophytic behavior). As a result of transition through an alternative host, a conversion of pathogen behavior may be occurred and colonization of primary host would be improved (Akinsanmi et al., 2007). Successful infection of weed plant tissues and lack of diseases symptoms on them would suggest that the contamination of weeds by *Fusarium* spp. may be endophytic (Jenkinson & Parry., 1994). The endophytic growth of *F. culmorum*, *F. graminearum* as well as *Microdochium nivale* (syn. *F. nivale*) has been demonstrated (Sieber at al., 1988). All these studies conclude that effective weed management can be a useful approach for the reduction of inoculum of *Fusarium* diseases in maize fields (reviewed in Fandohan et al., 2003).

Identification and Characterization of *Fusarium* Species

The current estimated number of described *Fusarium* species is between 70 and 500 (Leslie & Summerell, 2011), and the number is expected to increase in the coming years as more are discovered. The most common and widely applied method for identification of *Fusarium* species relies on morphological and other phenotypic traits. Typical criteria employed for traditional identification are the presence or absence of microconidia and chlamydospores, the

size and shape of micro- and macroconidia, morphological characters of conidiogenous cells as well as colony morphology and growth rate (Leslie & Summerell, 2006). However, the application of morphological characters alone for identification and differentiation of similar species may not be sufficient. The problem is clearly obvious when very closely related *Fusarium* species, such as members of the *F. avenaceum*/*F. acuminatum*/*F. tricinctum* species complex, are studied. Furthermore, description of species boundaries and subsequently detection of inter- and intra-specific variability by such classical methods is difficult or in some cases probably impossible (Harrow et al., 2010). Fortunately, alternative procedures have been introduced in recent years, in which DNA sequence analysis is used to evaluate and characterize the *Fusarium* species status. Performing these techniques as alternative or complementary approaches has reduced some of the disadvantages of conventional diagnostic methods.

As mentioned, the molecular techniques generally use the DNA sequences data to support classical phenotypic identification and to increase knowledge of the taxonomy of *Fusarium*. They provide the opportunity to distinguish unknown isolates and clarify the phylogenetic structure within closely related species. In addition, the DNA sequences of single-copy nuclear genes have successfully been used to determine the evolutionary history of secondary metabolites, and many toxin profiles have been mapped to the *Fusarium* species (O'Donnell et al., 1998b; Kristensen et al., 2005; Marín et al., 2012; Gräfenhan et al., 2013). In comparison with phenotypic variations, DNA sequence variations are more numerous and undergo fewer changes in culture collections (Leslie & Summerell, 2011). Several gene sequences have successfully been used to differentiate species in the genus of *Fusarium* including the ß-tubulin gene, the calmodulin gene (O'Donnell et al., 1998a; Yli-Mattila et al., 2002; Steenkamp et al., 2002), and the translation elongation factor 1-alpha (TEF-1α) gene sequence (O'Donnell et al., 1998b, 2000; Knutsen et al., 2004; Harrow et al., 2010; Marín et al., 2012; Gräfenhan et al., 2013).

The partial TEF-1α gene is taxonomically most informative sequence in the *Fusarium* genus. It is constantly single-copy in *Fusarium* and, due to its high sequence polymorphism among closely related species, has been a useful genetic marker. It was, therefore, used as a single-locus identification tool to create the Fusarium-ID v.1.0 database (Geiser et al., 2004). The ef1 and ef2 primer pairs, which can amplify a 700 bp region of TEF, were first designed to

evaluate the relationships within *F. oxysporum* species complex (O'Donnell et al., 1998b). Currently, these primers are used for a wide variety of filamentous ascomycetes. In some cases, the phylogenetically distinct species may show slight differences in the TEF-1α gene, which are not sufficient for their differentiation by Fusarium-ID database. Classification should, therefore, be done cautiously and extra sequence data from other gene markers is necessary to establish the phylogenetic relationships within such groups. According to previous studies, for example, differentiation between *F. avenaceum* and *F. arthrosporioides* strains is possible only when the combined ATP Citrate Lyase (acl1) and TEF-1α gene sequences are employed (Gräfenhan et al., 2013). One possible explanation would be the presence of a fair to poor representation of such species in the database (Geiser et al., 2004).

Detection and Quantification of Fungal Biomass

For many years, DNA-based methods, particularly real-time PCR (qPCR) have developed as potentially more reliable techniques for identification, detection and quantification of plant pathogens as well as studying plant systemic infections (McCartney et al., 2003). The qPCR assay allows the quantification of unknown samples, which means to determine the number of copies of the target gene present in a sample. Since monitoring of PCR products is possible either by fluorescent DNA-intercalating dye (such as SYBR Green I) or sequence-specific probe-based assays (Wittwer et al., 1997), measurement of the intensity of fluorescent signals during the exponential step of DNA amplification will lead to DNA quantification. For this purpose, standard curve needs to be constructed. It can be generated by running the qPCR for a serial dilution of pure genomic DNA of fungus. The average amount of threshold values (Ct) should, then, be plotted against the logarithmic scale of starting DNA quantity (SQ). Afterwards, the initial number of copies of the target gene in an unknown sample is measured by interpolating its Ct value to the standard curve equation (McCartney et al., 2004).

Quantitative and species-specific determination of *Fusarium* spp. biomass in plant tissues is essential in disease etiology and epidemiology research, as well as in resistance breeding. Both absolute and relative qPCR assays have been used with success for detection and quantification of the pathogens such as *F. solani* f.sp. *glycines* in soybean that are slow growing fungi with variable phenotypic characteristics (Gao et al., 2004). Since several studies have shown a

positive correlation between the fungal biomass and mycotoxin content (Waalwijk et al., 2004; Schnerr et al., 2002; Yli-Mattila et al., 2008; Fredlund et al., 2010), it is supposed that the qPCR can be used as a fast and cost-effective means to assess the risk of grain contamination. Although mycotoxin profile analysis should not be displaced by the qPCR procedure, it can be employed as a high throughput and low cost method in quarantine posts where batches and cargoes are initially inspected for the risk of mycotoxin contamination. This primitive process can help sorting the contaminated plant materials that likely exceed the legal thresholds, so they can undergo further chemical analysis. Simultaneous quantification of different target DNA in a single qPCR with small reaction volumes will make the assessments more applicable, faster and cost-effective.

Role of Mycotoxins in *Fusarium* Diseases

Possible involvement of toxins or other certain pathogen-produced molecules in plant diseases has always been of great interest for plant pathologists. Pathogenesis as a qualitative term has simply been defined as the ability of pathogen to cause disease; while virulence is a quantitative term which describes the amount or extent of disease caused. The economic or scientific importance of a virulence factor may be identical or even higher than the pathogenicity factor. Virulence factors should thus be regarded as significant as pathogenicity factors (Yoder, 1980). Fungal toxins may play a role in pathogenicity, virulence or no role in plant disease. To evaluate toxins as factors in pathogenesis, commonly used criteria include: i) Host specificity, ii) Present in infected plant, iii) Production at a key step in disease development, iv) Induction of typical disease symptoms (Yoder, 1980). A common and practical approach to find the toxin function is elimination of toxin from the biological system, leaving the rest of process the same and, then, monitoring how disease will be changed. For this purpose, several ways such as using metabolic inhibitors have been introduced (Yoder, 1980). For many years, genetic analysis was employed to evaluate toxins as pathogenicity factors by application of the toxin-producing strains against the natural variants that are unable to produce toxins. Such strains have different genetic backgrounds. They undoubtedly differ in many traits other than toxin production that may affect the virulence of the pathogen. Therefore, the results of research on this topic have been presented cautiously. In recent years, genetic manipulation via recombination or mutational

analysis has generated identical amended strains differing only in a gene that confers toxin production (Desjardins et al., 2002).

Many of the *Fusarium* species are well known to be aggressive plant pathogens. Since the mycotoxins produced by these species are phytotoxic, it is speculated that *Fusarium* mycotoxins should have a role in the pathogenicity of fungus (Yang et al., 1996). Among the most important *Fusarium* associated with maize, most researches have been focused on species that are known as producers of trichothecenes and fumonisins. This is due primarily to their contribution in the human and animal food chain.

All trichothecene-producing *Fusarium* species are principal pathogens that can infect a range of the plant species and cause destructive diseases. Wet weather at harvesting time and high humidity during storage can increase the trichothecene level in maize and wheat kernels (Desjardins et al., 1993). Trichothecenes have been known to be the virulence factors in some *Fusarium* spp. pathogens (Yoder, 1980). They can damage the protein synthesis in plants and/or suppress or delay the plant defense reaction (Harris et al., 1999). According to the high correlation between levels of trichothecene production, sexual fertility and the original isolation of the strain from diseased plant materials in *G. pulicaris* (anamorph: *F. sambucinum*), it is suggested that trichothecene production may be implicated in both pathogenicity and fertility (Beremand et al., 1991). Other reports show trichothecenes are not necessary for fungal growth in vitro. The growth rate and morphology of non-trichothecene producing strains were not distinguishable from those of the progenitor strains (Hohn & Desjardins, 1992). Since toxin production in vitro is affected by the physical environment and culture compounds, therefore, virulence may not be correlated with amount of toxin produced in culture (Yoder, 1980).

Trichothecenes act as virulence factors in some *Fusarium* spp. pathogens. They are able to produce different disease symptoms such as necrosis, chlorosis and wilting in a variety of plant species and affect the amount or extent of disease (Yoder, 1980; Desjardins et al., 1993). It has been shown that trichothecenes are host non-specific and different eukaryotic organisms such as plants are influenced through exposure even to the low concentrations of toxin (Desjardins et al., 1993). Although trichothecenes have been found in some *Fusarium* infected plant tissues (Snijders & Perkowski, 1990; Desjardins et al., 1989; Desjardins & Plattner, 1989), detection of

toxin in the infected tissues, however, have not always been successful (Bean et al., 1984). Toxins may fail to establish in infected tissues, although the infection may have resulted from toxin action. In this case, the plant enzymes may be responsible for toxin inactivation or it is not detectable due to the complex plant matrix effects (Yoder, 1980; Mitchell, 1984). The results of disruption of the *Tox*5 gene in *G. pulicaris*, responsible for trichothecene synthase, suggested the role of trichothecenes in virulence may be different from one plant species to another. It has been clearly observed to be the virulence factor of *F. sporotrichioides* and *G. pulicaris* on parsnip roots, while infection of potato tubers by *G. pulicaris* is independent of trichothecene (reviewed in Desjardins et al., 1993). Similarly, although trichothecenes contribute to the virulence of *F. graminearum* to cause fusarium head blight on wheat (Desjardins et al., 1996), they are not essential for the infection of maize tissue. Trichothecenes may act as a virulence factor to enhance the spread of the fungus on maize plants (Harris et al., 1999).

The results for finding the importance of naphthazarin production on the virulence of *F. solani* var. *martii* (teleomorph: *Nectria haematococca*) indicated that this toxic compound would not be a significant virulence factor for the infection of pea plants (Holenstein & Defago, 1983). Regarding the function of secondary metabolites in the infection process of *F. avenaceum* on potato tuber tissues, although the reports have suggested some additional pathogenicity factors, they obviously show the contribution of the enniatin toxin to the virulence of the pathogen (Herrmann et al., 1996).

Fumonisins as polyketide mycotoxins are produced by number of *Fusarium* spp. such as *F. verticillioides*. Toxicity of fumonisins to plants and field animals has been clearly demonstrated (Lamprecht et al., 1994); but there are controversial reports regarding the potential function of fumonisins in virulence on maize. In latest reports, fumonisin bioavailability to maize roots has been linked to the reduction in stalk weight and root mass, while the number of leaf lesions increased (Williams et al., 2006). These findings have supported the importance of fumonisins in plant pathogenesis. Recently studies have presented that the expression of foliar maize diseases is associated with fumonisin production, and this toxin can contribute to all aspects of *F. verticillioides* maize seedling diseases (Williams et al., 2007; Glenn et al., 2008). According to the field studies using *F. verticillioides* strains carrying gene disruptions, fumonisin production is not necessary for the fungus to cause maize ear rot (Desjardins et al., 2002). If

fumonisins have no role in maize ear rot, they may be important in other ecological aspects of *F. verticillioides*. Furthermore, they may distribute to enhance the fungal virulence on plant species other than maize (Proctor et al., 2002).

Objectives of the Study

Develop a qPCR protocol for simultaneous quantification of the DNA of nine *Fusarium* species occurring in maize at a reaction volume of 4 µl. Furthermore, specific optimal conditions for each species should be established to maximize the performance of the analysis when a single pathogen is studied.

The second objective was to investigate the role of weeds in survival of *Fusarium* pathogens and assess their ability to produce mycotoxins. Motivation for this studey was an observation of rare occurrence of unusually high amounts of fumonisins and fungal colonization in well-controlled field trials, which was difficult to explain (Nutz & Karlovsky, unpublished). There is a hypothesis that particular species of weed plants that host *Fusarium* spp. increase the infection pressure locally and account for the high levels of *Fusarium* mycotoxins in organic maize fields. The project should address the following questions: Do the weed plants play a role as alternative hosts for *Fusarium* species pathogenic to maize? Can they provide a significant source of inoculum for maize plants and thus increase mycotoxin accumulation?

In the third part of this study, the aim was to identify differences between the aggressiveness of *F. verticillioides* strains, differring in the production of fumonisins, towards maize, sorghum, rice and beetroot seedlings in vitro. These differences would define a biological function for fumonisins in the virulence of *F. verticillioides* for maize and other hosts. We hypothesize that fumonisin synthesis originated on hosts other than maize and in plant tissues other than silks/cobs.

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Chapter 2

Real-time PCR (qPCR) for Simultaneous Quantification of Maize Pathogens: *Fusarium avenaceum*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. poae*, *F. proliferatum*, *F. subglutinans*, *F. tricinctum*, and *F. verticillioides* in 4-µl Reactions

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Abstract

Fusarium avenaceum, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. poae*, *F. proliferatum*, *F. subglutinans*, *F. tricinctum*, and *F. verticillioides* are mycotoxin producing pathogens of maize and small-grain cereals. We developed a species-specific real-time PCR (qPCR) assay for simultaneous quantification of genomic DNA of these nine *Fusarium* species in plant tissues in 384-well microplates in a total volume of 4 µl. The reactions are set by combining 1 µl sample DNA with 3 µl master mix containing SYBR Green; the wells are sealed with mineral oil instead of adhesive foil to prevent concentration changes due to the evaporation. The thermocycler program was optimized to allow for simultaneous quantification of all nine *Fusarium* species in the same microplate. The sensitivity of method ranged from 0.05-1.52 pg DNA per well and repeatability ranged from 0.81% to 1.71% RSD (relative standard deviation). The PCR efficiency of 92.15% on the average was achieved. The assay was used for the analysis of several thousands field samples of maize grain, wheat grain and whole plants. It can easily be extended to simultaneous, low-cost quantification of further pathogens with a throughput of over a thousand assays per day and thermocycler.

Additional keywords: qPCR, 384-well microplate, *Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium equiseti*, *Fusarium graminearum*, *Fusarium poae*, *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium tricinctum*, *Fusarium verticillioides*

Introduction

Real-time PCR (qPCR) has become the standard method for species-specific quantification of fungal biomass in plant tissues. The qPCR assays for major plant pathogens and decay fungi have been established and used extensively in the last decade. The majority of qPCR assays carried out in research laboratories and plant diagnostic services relied on real-time thermocyclers in 96-well format; both SYBR Green-based detection of PCR products and hybridization probes (e.g., TaqMan) were extensively used. In most published qPCR assays, thermocycler programs were optimized for each assay separately, making it necessary to carry out a separate thermocycler run for each template. The growing need for multiple qPCR assays and the availability of thermocyclers with 384-well blocks entailed the development of common thermocycler programs shared by several assays to be carried out simultaneously in the same microplate. 384-well blocks increased the throughput, permitting the analysis of over a thousand samples per day with a single-block machine. Special instrumentation allows reducing the reaction volume to several nanoliters (Brenan & Morrison, 2005; Dahl et al., 2007). Most laboratories, however, still work with standard thermocyclers and set their qPCR assays in 15 to 25 µl even when using 384-well thermocyclers.

Simultaneous quantification of several targets in a single qPCR, designated multiplexing, is not possible with low-cost assays based on intercalating dyes such as SYBR Green II. Several targets that occur in a mutually exclusive fashion can theoretically be quantified in a single reaction with SYBR Green II detection but only semi-quantitative data can be obtained for samples containing more than one target (Brandfass & Karlovsky, 2006). Detection based on doubly labeled hybridization probes such as TaqMan allows limited multiplexing (up to four simultaneous assays). The comparatively high costs of doubly-labeled probes, however, as well as high demands on qPCR optimization in a multiplex set up and competition among templates have limited the use of the method. Small reaction volumes and large well densities of new thermocycler blocks dwarfed the advantages of multiplexing as compared to a set of parallel assays with intercalating dyes. We envision a new paradigm in which growing number of qPCR assays will be adapted to few shared thermocycler programs, allowing for simultaneous quantification of different targets in the same microplate.

Genus *Fusarium* comprises economically important pathogens of crop plants, most of which are known to produce mycotoxins (Moretti, 2009). Maize (*Zea mays* L.) is a host of several *Fusarium* species that cause ear and kernel rot, stalk rot and seedling blight (Logrieco et al., 2002; Gilbertson et al., 1985; Pintos Varela et al., 2013). Some *Fusarium* species colonize maize without visible symptoms and can therefore be regarded as endophytes (Gelderblom et al., 1988). Major *Fusarium* pathogens reported to cause ear rot of maize can be divided into two groups. The first group contains members of *Gibberella fujikuroi* species complex, *Fusarium verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsushima) Nirenberg, *F. subglutinans* (Wollenweb. & Reinking) P. E. Nelson, T. A. Toussoun & Marasas and *F. temperatum*, the latter of which has recently been separated from *F. subglutinans* (Scauflaire et al., 2011). The second group consists of species of the *Discolour* section, most importantly *F. graminearum* Schwabe and *F.culmorum* (W. G. Smith) Sacc, which are responsible for red ear rot (*Gibberella* ear rot). A range of further *Fusarium* species have been reported to infect maize ears and cause tissue damage and mycotoxin accumulation, including *F. avenaceum*, *F. poae*, *F. equiseti*, and *F. tricinctum*. While the role of *G. fujikuroi* species complex and section *Discolour* in the accumulation of mycotoxins in maize grains is established, the relative importance of further species is the subject of ongoing research.

Quantitative and species-specific determination of *Fusarium* spp. biomass in plant tissue is indispensable in research on disease etiology and epidemiology as well as in resistance breeding. Because of positive correlation between fungal biomass and mycotoxin content in plant tissue (Waalwijk et al., 2004; Schnerr et al., 2002; Yli-Mattila et al., 2008; Fredlund et al., 2010), qPCR can be used as a fast and cost-effective means to assess the risk of grain contamination. In contrast to mycotoxin analysis, qPCR can be carried out with a high throughput and low costs. The assessment of mycotoxin risk based on fungal biomass does not replace mycotoxin analysis; but it may help identifying batches or cargoes in risk of exceeding legal thresholds, tagging them for chemical analysis of mycotoxin content or for exclusion from human consumption in growing areas where mycotoxin analysis is not available or bears prohibitive costs.

In this work we developed a protocol for the simultaneous quantification of DNA of nine *Fusarium* spp. occurring in maize by qPCR with SYBR Green detection in 384-well plates with

a reaction volume of 4 µl. Furthermore, specific optimal conditions for each species were established to maximize performance of the analysis when a single pathogen is studied.

Material and Methods

Fungal Isolates and DNA Isolation

Fungal strains used in this study are listed in Table 1. The cultures were grown on potato dextrose broth (PDB) (Roth, Karlsruhe, Germany) in Erlenmeyer flasks in darkness at 25°C for 7 to 10 days. Mycelium was harvested by filtration onto sterile paper disks, frozen in -70°C, freeze-dried and stored at room temperature till extracting genomic DNA. Forty milligrams of lyophilized mycelium were ground in 2 ml Eppendorf tubes with round bottom containing 4-5 wolfram carbide spheres (diameter 3 mm, Retsch, Haan, Germany) in a reciprocal mill (Mixer Mill MM 200,Retsch, Haan, Germany). Genomic DNA was extracted by a CTAB method (Brandfass & Karlovsky, 2008), further purified by phenol extraction, precipitated and dissolved in TE buffer. The DNA quality was checked and concentration determined using electrophoresis in 0.8% (w/v) agarose gels (Cambrex, Rockland, ME, USA).

Preparation of DNA Standards for qPCR

DNA quantification was carried out by densitometry of DNA bands after electrophoretic separation; because calculation of DNA concentration from absorbance of the solution in UV light is error-prone (Wilfinger et al., 1997). For this purpose, a range of dilutions of genomic DNA was separated electrophoretically on 1.2% (w/v) agarose gels prepared in TAE buffer (40 mM Tris, 1 mM EDTA, pH set to 8.5 with acetic acid, Riedel-de Haen, Hanover, Germany) along with a dilution series of lambda phage DNA of known concentration (methylated, from *Escherichia coli* host strain W3110). The electrophoresis was carried out at 4 V cm^{-1} for 60 min. After staining with ethidium bromide (0.5 µg ml^{-1}) and destaining in demineralized water, DNA bands were visualized in UV light using a CCD camera (Vilber Lourmat, Marne La Vallee, France). The electrophoretic bands within the linear range of the densitometry were used for quantification using Multi Analyst-Software (BioRad, Hercules, CA, USA). Standards for qPCR were prepared in a range of 0.056 pg $µl^{-1}$ to 1111 pg $µl^{-1}$ by consecutive 1:3 dilutions, starting with the highest standard, in sterile distilled water.

Table 1. Fungal isolates used in this research

Fungal strain	Isolate code	Source
Fusarium graminearum	DSM 62722	Deutsch Sammlung fon Mikroorganismen und Zellkulturen, Braaunschweig, Germany
Fusarium graminearum	DSM 67638	Deutsch Sammlung fon Mikroorganismen und Zellkulturen, Braaunschweig, Germany
Fusarium graminearum	BBA 62048	H. Nirenberg (BBA, Berlin, Germany) via E. Möller
Fusarium graminearum	Fg 5	H. Nirenberg (BBA, Berlin, Germany) via E. Möller
Fusarium graminearum	Fg 71	T. Miedaner, University of Hohenheim, Germany
Fusarium graminearum	Fg 210. 1 wt	Plant Pathological Strain Collection of the University of Göttingen, Germany
Fusarium proliferatum	FPRO 1	A. Szecsi, Budapest, Hungary via E. Möller
Fusarium proliferatum	FPRO 2	A. Szecsi, Budapest, Hungary via E. Möller
Fusarium proliferatum	FPRO 4	A. Szecsi, Budapest, Hungary via E. Möller
Fusarium proliferatum	FPRO 5	A. Szecsi, Budapest, Hungary via E. Möller
Fusarium proliferatum	FPRO 6	A. Szecsi, Budapest, Hungary via E. Möller
Fusarium proliferatum	FPRO 7	A. Szecsi, Budapest, Hungary via E. Möller
Fusarium proliferatum	FPRO 8	A. Szecsi, Budapest, Hungary via E. Möller
Fusarium proliferatum	FPRO 9	A. Szecsi, Budapest, Hungary, via E. Möller
Fusarium proliferatum	FPRO 11	A. Szecsi, Budapest, Hungar,y via E. Möller
Fusarium proliferatum	FPRO 12	A. Szecsi, Budapest, Hungary, via E. Möller
Fusarium avenaceum	Fa 95	E. Möller, University of Hohenheim, Germany
Fusarium avenaceum	Fa 23	Department of Crop Sciences, University of Göttingen, Germany
Fusarium avenaceum	Fa 5-2	Department of Crop Sciences, University of Göttingen, Germany
Fusarium avenaceum	DSM 62161	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany
Fusarium culmorum	Fc 15	T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany, via E. Möller
Fusarium culmorum	Fc 2	H. Nirenberg (BBA, Berlin, Germany) via E. Möller
Fusarium culmorum	Fc 22	T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany, via E. Möller
Fusarium culmorum	FCH 69	Department of Crop Sciences, University of Göttingen, Germany
Fusarium tricinctum	FT 2	Department of Crop Sciences, University of Göttingen, Germany
Fusarium tricinctum	FT 3	Department of Crop Sciences, University of Göttingen, Germany
Fusarium verticillioides	A00102	J. F. Leslie, Kansas State University, Manhattan, KS, USA, via E. Möller
Fusarium verticillioides	1.34	Mykothek FAP (W.Winter), via E. Möller
Fusarium verticillioides	FV 234/1	P, Battilani, Faculty of Agriculture, UniversitaCattolica del SacroCoure, Piacenza, Italy, via T. Miedaner
Fusarium verticillioides	FM 8114	Fusarium Research Center, Pennsylvania State University, USA
Fusarium verticillioides	FV Ita 1	A. Prodi, University of Bologna, Italy
Fusarium oxysporum	Fo 125	Department of Crop Sciences, University of Göttingen, Germany
Fusarium oxysporum	Foxy 121	Department of Crop Sciences, University of Göttingen, Germany
Fusarium oxysporum	Foxy 436	Department of Crop Sciences, University of Göttingen, Germany
Fusarium oxysporum	Foxy 119	Department of Crop Sciences, University of Göttingen, Germany
Fusarium oxysporum	Foxy 6	Department of Crop Sciences, University of Göttingen, Germany
Fusarium oxysporum	Foxy 2	Mykothek FAP (W.Winter), via E. Möller
Fusarium poae	Fpoae 517	Department of Crop Sciences, University of Göttingen, Germany
Fusarium poae	Fpoae 369	Department of Crop Sciences, University of Göttingen, Germany
Fusarium poae	FP 2	T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany, via E. Möller
Fusarium poae	DSM 62376	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany

Table 1: continued

Fungal strain	Isolate code	Source
Fusarium subglutinans	Fsub 2210	E. Möller, Field isolates from Maize, Radzikow, Poland
Fusarium subglutinans	Fsub 2209	E. Möller, Field isolates from Maize, Radzikow, Poland
Fusarium subglutinans	B 01722	J. F. Leslie, Kansas State University, Manhattan, KS, USA, via E. Möller
Fusarium subglutinans	B 00278	J. F. Leslie, Kansas State University, Manhattan, KS, USA, via E. Möller
Fusarium subglutinans	B 038J	J. F. Leslie, Kansas State University, Manhattan, KS, USA, via E. Möller
Fusarium subglutinans	B 03821	J. F. Leslie, Kansas State University, Manhattan, KS, USA, via E. Möller
Fusarium subglutinans	No. 43.92	H. Lew and A. Adler, Linz, Austria
Fusarium subglutinans	CBS 215.76	CBS-KNAW Fungal Biodiversity Center, Utrecht, Netherland
Fusarium subglutinans	Fsub 2-17	P. Karlovsky, Shaam 6-39, China
Fusarium subglutinans	Fsub 2215	E. Möller, Field isolates from Maize, Radzikow, Poland
Fusarium subglutinans	Fsub 2213	E. Möller, Field isolates from Maize, Radzikow, Poland
Fusarium subglutinans	Fsub 2220	E. Möller, Field isolates from Maize, Radzikow, Poland
Fusarium subglutinans	CBS 215.96	CBS-KNAW Fungal Biodiversity Center, Utrecht, Netherland
Fusarium sacchari	B 03853	J. F. Leslie, Kansas State University, Manhattan, KS, USA, via E. Möller
Fusarium sacchari	B 03852	J. F. Leslie, Kansas State University, Manhattan, KS, USA, via E. Möller
Fusarium crookwellense	FCKW1	H. Nirenberg (BBA, Berlin, Germany) via E. Möller
Fusarium crookwellens	BBA 64545	H. Nirenberg (BBA, Berlin, Germany) via E. Möller
Fusarium equiseti	ICARDA 93532	International Center for Agricultural Research in the Dry Area, Aleppo, Syria
Fusarium equiseti	ICARDA 93715	International Center for Agricultural Research in the Dry Area, Aleppo, Syria
Fusarium acuminatum	ICARDA 93803	International Center for Agricultural Research in the Dry Area, Aleppo, Syria
Fusarium acuminatum	ICARDA 92099	International Center for Agricultural Research in the Dry Area, Aleppo, Syria
Fusarium acuminatum	ICARDA 93682	International Center for Agricultural Research in the Dry Area, Aleppo, Syria
Fusarium acuminatum	ICARDA 93831	International Center for Agricultural Research in the Dry Area, Aleppo, Syria
Ustilago maydis	PL2	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany
Ustilago maydis	DSM 3121	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany
Ustlago maydis	PL4	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany
Acremonium longisporum	AL	Department of Crop Sciences, University of Göttingen, Germany
Acremonium longisporum	AC2	Department of Crop Sciences, University of Göttingen, Germany
Microdochium nivale	GN25	T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany, via E. Möller
Cladosporium herbarum	CH4	Department of Crop Sciences, University of Göttingen, Germany
Rhizoctonia cerealis	SAGWJ7	Department of Crop Sciences, University of Göttingen, Germany
Alternaria alternata	A4.1.1	Department of Crop Sciences, University of Göttingen, Germany

Selection of Primers and Evaluation of their Specificity

PCR primers selected for the work are shown in Table 2. Although the specificity of these primers was tested by the authors who designed them (rightmost column in Table 2), we carried out additional specificity tests for *F. avenaceum*, *F. subglutinans* and *F. poae* primers with DNA of a range of *Fusarium* species (64 isolates belonging to 13 species) and 9 isolates of other fungal species commonly associated with cereals (Table 1).

Table 2. Characteristics of primers used in this study for detecting of nine *Fusarium* spp.

Primer name	Primer sequence (5´-3´)	Target species	Product size (bp)	Reference
MGBF MGBR	CCATCGCCGTGGCTTTC CAAGCCCACAGACACGTTGT	*F. avenaceum*	58	Waalwijk et al., 2004
OPT18 F OPT18 R	GATGCCAGACCAAGACGAAG GATGCCAGACGCACTAAGAT	*F. culmorum*	472	Schilling et al., 1996
198F2 198R1	GACAGCAAGATTGACCTTTTGG GACATACTCTACAAGTGCCAA	*F. equiseti*	96	Wilson et al., 2004
Fg16N F Fg16N R	ACAGATGACAAGATTCAGGCACA TTCTTTGACATCTGTTCAACCCA	*F. graminearum*	280	Nicholson et al., 1998
Fp82F Fp82R	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGTT	*F. poae*	220	Parry & Nicholson, 1996
Fp3-F Fp4-R	CGGCCACCAGAGGATGTG CAACACGAATCGCTTCCTGAC	*F. proliferatum*	230	Jurado et al., 2006
SUB 1 SUB 2	CTGTCGCTAACCTCTTTATCCA CAGTATGGACGTTGGTATTATATCTAA	*F. subglutinans*	631	Mulè et al., 2004
Tri1 Tri2	CGTGTCCCTCTGTACAGCTTTGA GTGGTTACCTCCCGATACTCTA	*F. tricinctum*	215	Kulik, 2008
VER 1 VER 2	CTTCCTGCGATGTTTCTCC AATTGGCCATTGGTATTATATATCTA	*F. verticillioides*	578	Mulè et al., 2004

Real-time PCR (qPCR)

Thermocycler CFX384 (BioRad, Hercules, CA, USA) with 384-wells microplate (Kisker Biotech GmbH, Steinfurt, Germany) was used. The qPCR was performed in a total volume of 4 µl. Prior to distributing master mix, 5 µl of mineral oil (SIGMA, Tauflcirchen, Germany) were pipetted into each well. Every reaction contained 1 µl template DNA or 1 µl sterile water for negative controls. The reaction mixture consisted of 16 mM $(NH4)_2SO_4$, 67 mM Tris-HCl, 0.01% (v/v) Tween-20; pH was set to 8.8 at 25°C. Varying concentrations of deoxynucleotide triphosphates and $MgCl_2$ (Bioline, Lükenwalde, Germany) was used. *Taq* DNA polymerase (BIOTaq, Bioline, Lükenwalde, Germany) or hot start *Taq* DNA polymerase (Immolase DNA

Pol., Lükenwalde, Germany) was employed in the activity 0.1 u/reaction. 0.1x SYBR Green I (Invitrogen, Karlsruhe, Germany) and primers at a concentration of 0.3 µM were used. Fluorescent data were obtained during the annealing phase to construct a melting curve at the end of each assay. The qPCR was completed by carrying out a melting curve analysis according to the following protocol: PCR products were denatured at 95°C for 1 min, annealed at 55°C for 1 min and gradually heated from 55°C to 95°C at a rate of 0.05°C/s while the fluorescence was continuously recorded.

Determination of qPCR Sensitivity and Efficiency

Sensitivity was defined as the lowest amount of standard DNA (see **Preparation of DNA standards for qPCR**) that generated PCR products with the expected melting temperature in at least 7 out of 8 replicates. Each standard DNA was run in at least eight replicates on three different days (Supplemental Table 1). The PCR efficiency was calculated in a common way (Nutz et al., 2011).

Results

Selection and Specificity of Primers

The primers used in this work were designed and tested previously for species-specific detection of *Fusarium* spp. (Table 2). Apart from specificity tests carried out by the researchers who designed them, we conducted additional specificity tests for three primer pairs of *F. subglutinans* (Nicolaisen et al., 2009; Möller et al., 1999; Mulè et al., 2004), two primer pairs of *F. avenaceum* (Turner et al., 1998; Waalwijk et al., 2004) and one primer pair of *F. poae* (Parry & Nicholson, 1996). We carried out these tests under conditions recommended in publications in which the primers were described but reduced the reaction volume to 10 µl (*F. subglutinans*) or 4 µl (*F. avenaceum, F. poae*).

In our thermocycler, primer pair Fsub565/Fsub622A described by Nicolaisen et al. (2009) as specific for *F. subglutinans* generated products with different melting temperatures for different *F. subglutinans* strains; moreover, DNA of the strains *F. graminearum* Fg 71 and *F. verticillioides* Fv Ita 1 as template were amplified, too. The primer pair 61-2 F/61-2 R (Möller et al., 1999) turned out not to be specific for *F. subglutinans* because it generated products with

the genomic DNA of strains *F. graminearum* Fg 71 and *F. verticillioides* Fv Ita 1. The primers SUB 1/SUB 2 designed by Mulè et al. (2004) for *F. subglutinans* were specific and have, therefore, been selected.

For *F. avenaceum* we tested primers MGBF/R (Waalwijk et al., 2004) and JIAF/R (Turner et al., 1998). When pure fungal DNA was tested, both primer pairs were specific for *F. avenaceum* and exhibited the same sensitivity. When they tested on DNA extracted from infected plants, however, primers MGBF/R were more sensitive (data not shown). We, therefore, selected primers MGBF/R for the detection of *F. avenaceum*.

The qPCR with primer set Fp82F/R designed for *F. poae* (Parry & Nicholson, 1996) was negative for all *Fusarium* strains described in Table 1 except for *F. poae*.

Optimization of a Protocol for Simultaneous Quantification of Nine *Fusarium* spp. in a Single Microplate

The PCR assays were carried out in a total volume of 4 µl overlaid with the mineral oil. Using adhesive foil instead of oil lead to the identical results. Loading small volumes in 384-well microplates can conveniently be carried out with pipetting robots. Because most plant pathology laboratories do not possess this equipment, we designed a simple loading scheme for manual loading with multichannel pipettes, consisting of three steps (Fig. 1). The composition of the reaction mixture and a thermocycler protocol suitable for all nine *Fusarium* species were designed based on the conditions optimized for individual assays (see below). For a convenient printout, these conditions are summarized on Fig. 2.

The performance of the method was tested on serial dilutions of pure genomic DNA for each *Fusarium* spp. Calibration curves are shown in Fig. 3. The coefficient of determination (R^2), ranging from 0.991 to 0.999, indicated a high accuracy of three-fold dilution series of standard DNA. The sensitivity of qPCR assays ranged from 0.05 pg (*F. proliferatum*) to 1.5 pg (*F. equiseti* and *F. verticillioides)* for pure fungal DNA (Table 3a). Melting curves of PCR products for all nine species showed single transition points at temperatures 81°C to 90°C, indicating that a single product was amplified in each assay. PCR amplification efficiency of 92.15% on the average was accomplished.

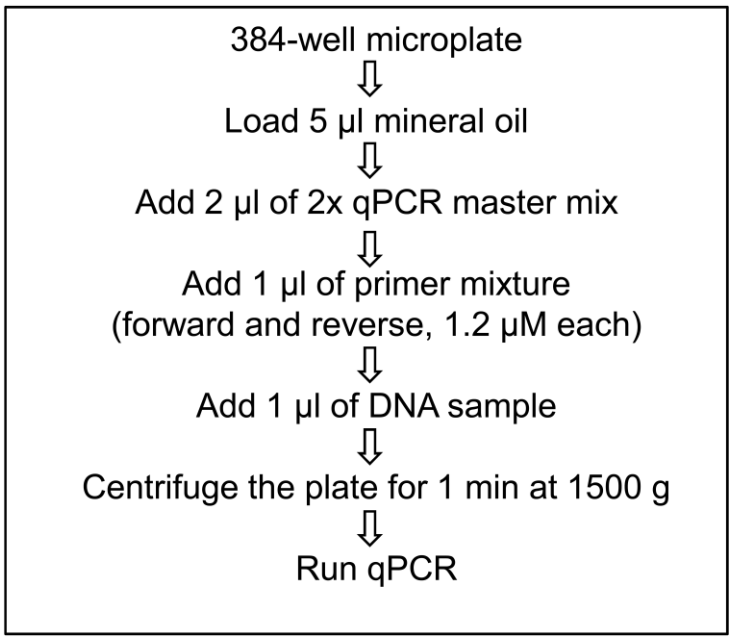

Fig. 1. Pipetting flowchart for multi-species qPCR detection of *Fusarium* spp. in a 384-well real-time thermocycler

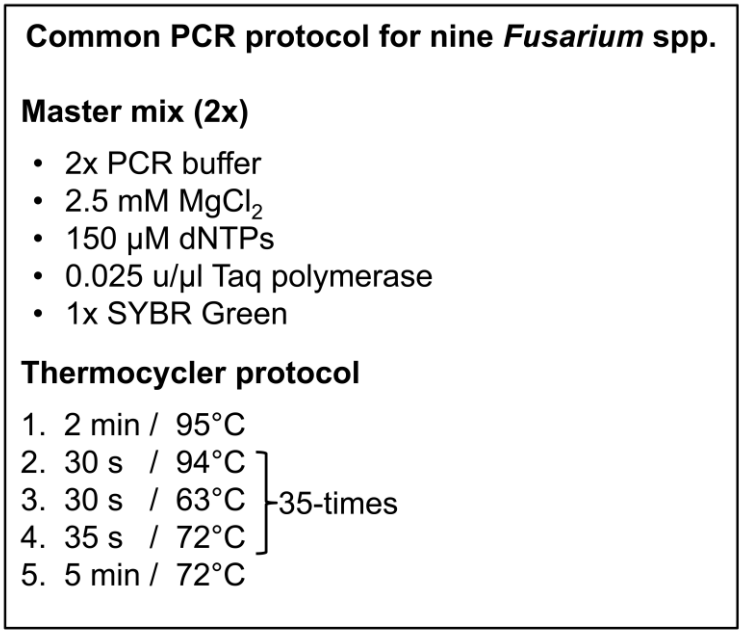

Fig. 2. Scheme of common PCR protocol for the nine *Fusarium* spp.

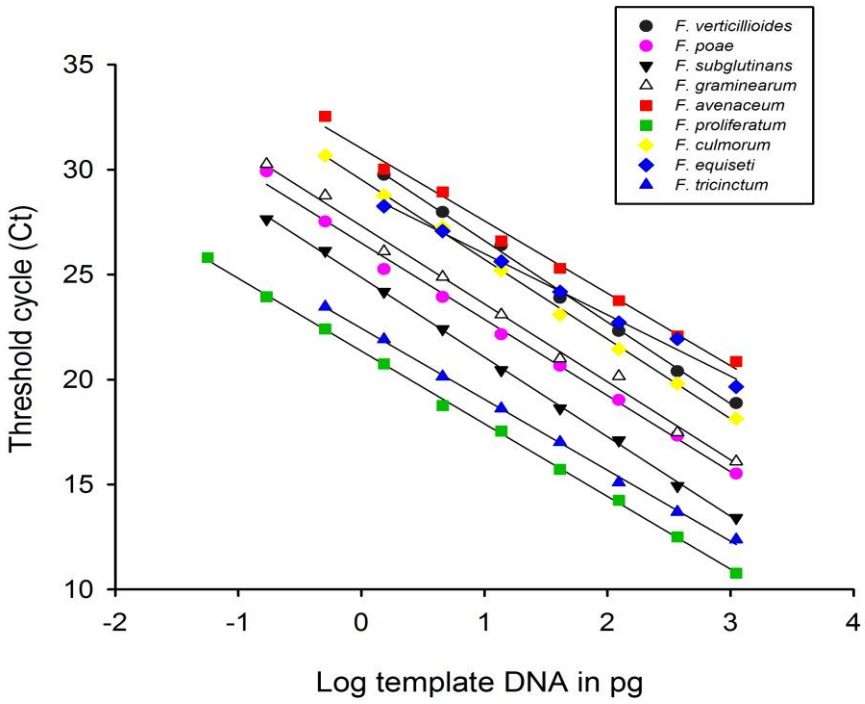

Fig. 3. Standard curves for simultaneous quantification of nine *Fusarium* spp.

Optimization of the qPCR Assays for Individual *Fusarium* Species

Similarly as in multiplex PCR assays, any protocol for parallel analysis of different species is a compromise among optimal conditions for the individual assays. In order to compare the performance of the multi-species assay with individual qPCR assays run under optimal conditions, we optimized the qPCR for each species, individually. Protocols for species-specific qPCR with SYBR Green detection, using the selected primers, were published for 4 out of the 9 *Fusarium* spp. (Brandfass & Karlovsky 2006; Nutz et al., 2011). Because these protocols performed sub-optimally in 4 µl reaction volumes (data not shown), we re-optimized the PCR conditions for all species in 384-well microplate. Simultaneous optimization of the annealing temperature and $MgCl_2$ concentration was achieved with the help of a temperature gradient block of the thermocycler. The experiments were conducted in four different quantities of the template DNA between 185 pg μl^{-1} and 0.475 pg μl^{-1}. The samples and the negative controls were run in duplicate.

F. avenaceum. The qPCR assay was performed in the range 59-65°C of annealing temperature and $MgCl_2$ concentrations 1.5, 2.0 and 2.5 mM. The optimal conditions were annealing

temperature of 63°C and 2.5 mM $MgCl_2$. The threshold values (Ct) indicated a delicate fluctuation for annealing temperatures 60-63°C and 2.0-2.5 mM $MgCl_2$ for standard DNA amounts higher than 10 pg. Therefore, considering the lowest amount of Ct in different quantities of template DNA, the suitable qPCR conditions were selected. The annealing time was lowered from the recommended time of 60 s (Waalwijk et al., 2004) to 25 s. Considering the length of PCR amplicon, the best time for elongation step was 10 s.

F. culmorum. Performance of annealing gradient qPCR assay between 58-66°C and 2.5, 3.0, 3.5 mM of Mg^{++} concentrations indicated that temperature of 62°C and 2.5 mM $MgCl_2$ can produce suitable Ct values in all DNA quantities, in contrast to the annealing temperature of 64°C and 4 mM of Mg^{++} concentration which are suggested by Brandfass & Karlovsky (2008). The time for annealing and extension steps was 40 s and 45 s, respectively.

F. equiseti. The qPCR assay was performed in range 58.2-66.8°C of annealing temperature and $MgCl_2$ concentrations 1.5, 2.0 and 2.5 mM. Initial experiments showed that 63°C of annealing temperature and 2.5 mM $MgCl_2$ would be the optimal qPCR conditions. But there was unspecific peak especially in low concentrations of DNA. Therefore the PCR condition was improved by using a high quality *Taq* DNA polymerase. In this case the optimal annealing temperature was increased to 65°C for 20 s. The time for extension step was 20 s.

F. graminearum. Annealing temperature in the range 59.0-66.5°C and Mg^{++} concentrations 2.0, 2.5 and 3.0 mM were tested. The optimal conditions were annealing temperature of 61°C and 2.5 mM $MgCl_2$, similar to Brandfass & Karlovsky (2008) who found 64°C and 2.5 mM $MgCl_2$ to be optimal for qPCR with the same primers in a reaction volume of 25 µl. We found no difference in the threshold cycle for annealing temperatures 60.0-62.5°C and 2.0–3.0 mM $MgCl_2$ for standard DNA amounts higher than 40 pg. The time for annealing and elongation steps were reduced from 45 s recommended by Brandass & Karlovsky (2008) to 30 s.

F. poae. Testing annealing temperature in the range 60-65°C and 1.5, 2.0 and 2.5 mM Mg^{++} concentration indicated that the qPCR could be operated in a wide range of annealing temperature and $MgCl_2$. The amount of Ct value did not show a big fluctuation in different conditions. Therefore, the annealing temperature of 62.5°C and 2 mM of $MgCl_2$ were selected as

the best qPCR conditions. Parry & Nicholson (1996) had suggested the annealing temperature of 60°C for the primer set Fp82 F/R. Time for annealing and extension steps was optimized on 30 s and 35 s, respectively.

F. proliferatum. The qPCR assay was carried out in the range 59-65°C of annealing temperature and 2.0, 2.5, 3.0 mM of Mg^{++} concentration. The optimal conditions were annealing temperature of 64°C and 2 mM $MgCl_2$, similar to Brandfass & Karlovsky (2008). There was no difference in threshold cycles for the annealing temperatures 60-65°C and 2.0-2.5 mM $MgCl_2$ while standard DNA amounts were less than 20 pg. The best time for annealing and extension steps was 30 s and 35 s, respectively.

F. subglutinans. Annealing temperature in the range 54-68°C and Mg^{++} concentration 2.0-4.5 mM were tested. An annealing temperature of 65°C yielded specific products, in contrast to the annealing temperature of 56°C suggested by the primer designers (Mulè et al., 2004). The optimal concentration for $MgCl_2$ was 3.0 mM. We found no difference in Ct values for 64-66°C and 2.5-3.5 mM $MgCl_2$. Considering the lowest Ct value and specificity for primer annealing, the best qPCR conditions were selected. Desired time for annealing and extension steps was 30 s and 40 s, respectively.

F. tricinctum. Performance of annealing gradient qPCR assay between 58.2-66.8°C and 2.5, 3.0, 3.5 mM of Mg^{++} concentrations indicated that temperature of 65°C and 2.5 mM $MgCl_2$ can produce suitable Ct values in all DNA quantities. The annealing conditions were similar to Kulik (2008); but the time for extension step was reduced of 55 s (Kulik, 2008) to 25 s.

F. verticillioides. Annealing temperature in the range 56-65°C and Mg^{++} concentrations 2.0, 2.5 and 3.0 mM were evaluated. The optimal conditions were annealing temperature of 62.5°C and 2.5 mM $MgCl_2$. There was no difference in Ct values for annealing temperatures 60.0-62.5°C and 2.5-3.0 mM $MgCl_2$ for the standard DNA amounts higher than 10 pg, but the best qPCR conditions were selected considering the suitable conditions for lower DNA quantities. The time for annealing and elongation steps were lowered from the recommended time of 50 s and 60 s (Nutz et al., 2011) to 30 s and 40 s, respectively.

The optimal qPCR conditions were tested for the overall performance of assay by using the data generated from a serial dilution of pure genomic DNA for each *Fusarium* spp., individually. The average amount of Ct values was graphed versus the logarithm of the correspondent starting DNA quantity (SQ) to construct the standard curves. Comparing of R^2 indicated the accuracy of the dilution series and the overall assay sensitivity. The coefficient of determination ranged from 0.990 to 0.999. Typical calibration curves generated for *Fusarium spp.* DNA are given in Fig. 4. The sensitivity of qPCR assays was estimated to be 0.05 pg for pure DNA of *F. proliferatum,* 0.17 pg for *F. avenaceum, F. graminearum, F. poae, F. subglutinans* and 0.50 pg for *F. culmorum, F. equiseti, F. tricinctum* and *F. verticillioides* in a background free of plant tissue and other contamination. The average repeatability varied from 0.84% RSD (relative standard deviation) for *F. verticillioides* to 2.00% RSD for *F. tricinctum* (Table 3b). The main point in this protocol was the least possible number of samples which are included in each qPCR assay. Considering amount of *Taq* DNA polymerase, the master mix would be prepared at least for 50 samples to decrease the pipetting error.

As it is presented above, the qPCR optimization tests indicated flexibility in some cases. In such conditions, qPCR assay could be operated in a wide range of annealing temperature and $MgCl_2$ concentrations. Nevertheless, concerning low Ct values and specificity of primer annealing, the best conditions were selected for each *Fusarium* spp. Optimized qPCR conditions for each *Fusarium* spp. are summarized in Table 3b. The performance of new optimized assays for nine species of *Fusarium* in 4 µl volumes was gratified and low concentrations of genomic DNA were also detectable by qPCR assay without creating unspecific peak.

The results also show that the difference between qPCR efficiency in single species and multi-species detection of *Fusarium* spp. is not high and the Ct values did not change by more than approximately 1 Ct. Furthermore, we compared PCR sealing film and the mineral oil as a coating layer for qPCR microplate. There was no difference in Ct values and efficiency of assay. Therefore, we would suggest using the mineral oil instead of other sealing film.

Table 3a. PCR conditions and performance parameters of multi-species qPCR assay

Fusarium spp.	$MgCl_2$ (mM)	$dNTP_s$ (µM)	Ta (°C)	Ann. time (s)	Ext. time (s)	PCR Efficiency (%)	Sens.[1] (pg)	Ct for 1 pg	Ave of %RSD[2]	Tm[3] (°C)
F. avenaceum						94.8	0.508	31.01	1.57	81
F. culmorum						83.4	0.508	29.52	1.71	87
F. equiseti						120.1	1.524	28.91	1.00	86
F. graminearum						85.9	0.169	27.31	1.40	82
F. poae	2.5	150	63	30	35	88.5	0.169	26.49	1.69	83
F. proliferatum						94.4	0.056	21.35	1.58	89
F. subglutinans						83.5	0.169	24.85	1.50	88
F. tricinctum						97.6	0.508	22.44	0.81	90
F. verticillioides						81.0	1.524	30.05	1.62	88

Table 3b. PCR conditions and performance parameters of single species qPCR assays

Fusarium spp.	$MgCl_2$ (mM)	$dNTP_s$ (µM)	Ta (°C)	Ann. time (s)	Ext. time (s)	PCR Efficiency (%)	Sens.[1] (pg)	Ct for 1 pg	Ave of %RSD[2]	Tm[3] (°C)
F. avenaceum	2.5	150	63.0	25	10	98.19	0.169	30.15	1.42	81
F. culmorum	2.5	150	62.0	40	45	94.28	0.508	28.27	1.11	87
F. equiseti	2.5	150	65	20	10	110.1	0.508	29.18	0.96	86
F. graminearum	2.5	150	61.0	30	30	100.3	0.169	28.09	1.79	82
F. poae	2.0	100	62.5	30	35	99.83	0.169	25.93	1.47	83
F. proliferayum	2.0	150	64.0	30	35	98.64	0.056	21.53	1.56	89
F. subglutinans	3.0	100	65.0	30	40	97.75	0.169	24.49	1.56	88
F. tricinctum	2.5	150	65	25	30	106.0	0.508	21.62	2.00	90
F. verticillioides	2.5	100	62.5	30	40	96.37	0.508	29.40	0.84	88

1. Sensitivity ("Sens."): The lowest standard in a 3-fold dilution series starting with 1111 pg that was amplified in at least 7 out of 8 replicates
2. Average percent of relative standard deviation ("Ave %RSD") for all detectable qPCR standards
3. Melting temperature ("Tm") determined with accuracy of 0.5 °C

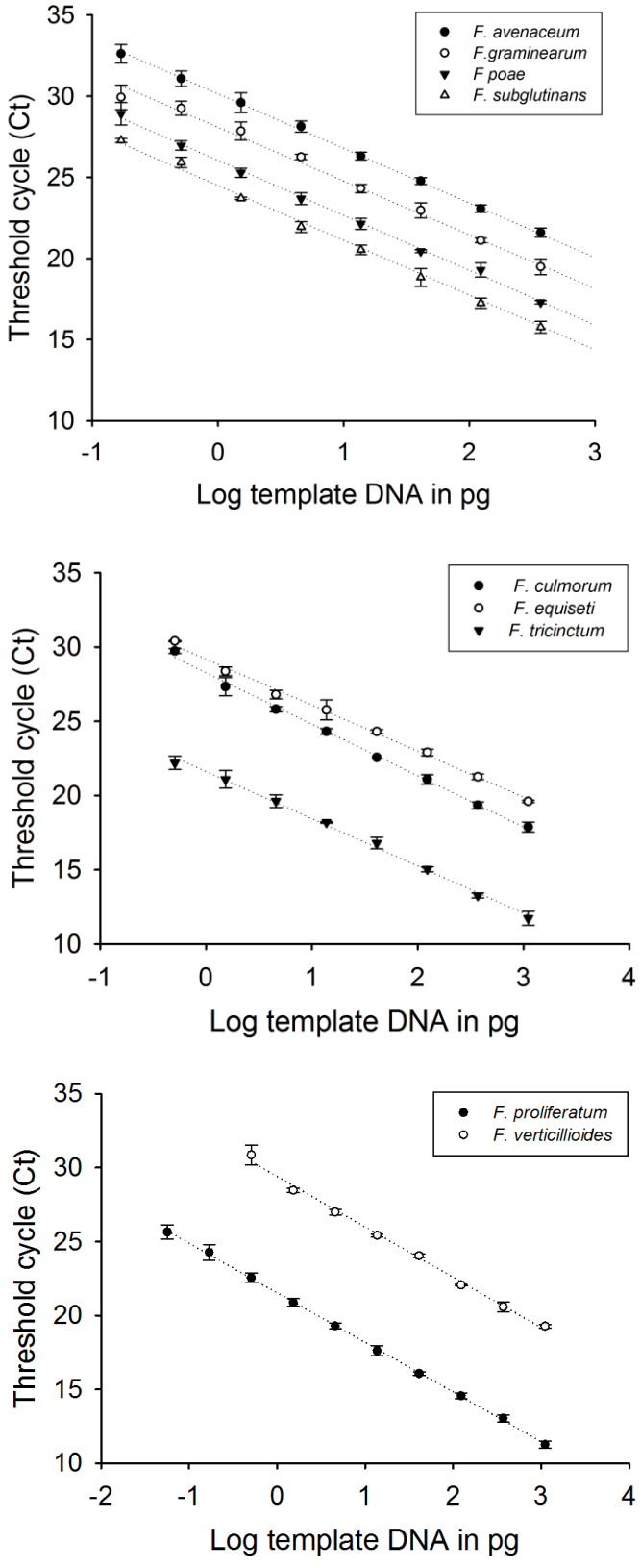

Fig. 4. Standard curves for *Fusarium* spp. qPCR assays

Discussion

Cereals are contaminated with a wide range of *Fusarium* spp., most of which produce mycotoxins (Jurado et al., 2005). Quantitative determination of fungal biomass in plant tissue is invaluable in studies of plant diseases. Furthermore, quantitative data on fungal biomass can be used for the prediction of mycotoxin content; the biomass of a *Fusarium* species and the concentration of its mycotoxin in the infected tissue often tightly correlate (Waalwijk et al., 2004; Schnerr et al., 2002; Yli-Mattila et al., 2008). Therefore, quantitative diagnosis of pathogenic Fusaria in plant tissues is an essential and powerful tool in both research and diagnostic laboratories. Sensitive and fast diagnostic methods are also important in assaying crop propagules for quarantine pathogens (Waalwijk et al., 2004). Since the range of *Fusarium* species associated with cereals is quite extensive, to facilitate fusarium diseases investigation while testing some samples for more than one species of *Fusarium*, we developed a multi-species qPCR assay in 4 µl volume reactions by using a 384-well thermocycler. The qPCR assays for all nine species, studied in this work, can be carried out in the same microplate, because they utilize the same thermocycle profile. Multi-species qPCR protocol is particularly useful when user have a few samples, but wants to check them for more than one *Fusarium* spp. In this case, we will likely have less labor intensive if we can run the samples in 384-well qPCR plate and the time of qPCR analysis could be reduced intensely to a single day. Simultaneous quantification approach allows maximum amount of data to be generated from each qPCR assay. Nine species of *Fusarium* evaluated in this study, cause important diseases on maize and small grain cereals. The qPCR optimization tests which quantified *Fusarium* spp. individually, had revealed that some of the qPCR variables can work in a wide range of PCR conditions. Therefore, concerning the low Ct values and specificity of primer annealing, the most suitable conditions were selected. On the other hand, in our experiments, assessment of common domains of qPCR variables helped us for designing a multi-species qPCR assay. Eventually, owing to applicable common range in qPCR variables we concluded on specific PCR profiling for the nine species of *Fusarium*.

Sensitivity and robustness of qPCR assay are known as the crucial factors. Standard performance parameters of analytical methods are the limit of detection (LOD) and limit of quantification (LOQ). Standard methods for the determination of LOD and LOQ, based on the

noise of the background are not suitable for qPCR. Several authors suggested alternative definitions of LOD and LOQ in qPCR (Bustin et al., 2009; Mattarucchi et al., 2005; Vaerman et al., 2004; Gao et al., 2004); but none of these procedures was accepted by the community. The use of receiver operating curve analysis is the most recent suggestion for the determination of LOQ in qPCR (Nutz et al., 2011); but the method requires the analysis of large field data sets which was not available for our assays. We, therefore, used a simple sensitivity parameter defined as the lowest amount of template DNA that generated products with the expected melting temperature in at least seven out of eight replicates. The sensitivity determined in this way depends on DNA quantities of the standards; because we used a 3-fold dilution series, sensitivity value of S specifies a confidence interval for LOD as follows: S > LOD > S/3. According this definition, the real-time PCR assay was sensitive with consistent detection of 0.05 pg μl^{-1} of standard DNA for *F. proliferatum*, 0.17 pg μl^{-1} for *F. graminearum*, *F. poae*, *F. subglutinans*, 0.50 pg for *F. avenaceum*, *F. culmorum*, *F. tricinctum* and 1.52 pg μl^{-1} for *F. equiseti* and *F. verticillioides*. Lack of adequate sensitivity for quantifying of *F. equiseti* and *F. verticillioides* in multi-species qPCR protocol indicates the importance of using the single species detection method in the precise quantification researches of these two species of *Fusarium*.

The previous published qPCR conditions for *F. culmorum, F. graminearum, F. proliferatum* and *F. verticillioides* (Brandfass & Karlovsky, 2006; Nutz et al., 2011) for 25 µl volumes were not fit when used in 4 µl reactions. In most cases, especially in low concentrations of DNA, a second peak prior to specific melting point made disorders for the qPCR dissociation curve. This wide shape peaks would be generated from primer-dimmers or unspecific amplification products and should be discarded after enhancing of qPCR conditions. Therefore, for all of those species, we re-optimized the qPCR conditions to find the best options for running a single species qPCR. The main changes for the thermal cycling parameters were involved annealing and elongation steps. Considering threshold cycle and specificity of primer annealing, the optimal concentration of PCR reagents was selected for the nine species of *Fusarium*.

The single species qPCR protocol showed high sensitivity for *F. proliferatum* (0.05 pg DNA μl^{-1}), *F. avenaceum, F. graminearum, F.poae, F. subglutinans* (0.17 pg DNA μl^{-1}) and it was 0.50 pg DNA μl^{-1} for *F. culmorum, F. equiseti, F. tricinctum* and *F. verticillioides*. Further

experiments indicated a constant higher sensitivity for *F. proliferatum* (2 fg DNA μl^{-1}) while the average of the Ct value was 29.8 (data not shown). Using the hot start *Taq* DNA polymerase could enhance the amplification of *F. equiseti* strains. Due to the results, the correspondence among single-species and multi-species qPCR assays demonstrated in this work, was optimal and the Ct values did not change by more than approximately 1 Ct in two reported protocols. The DNA samples of plant tissues often need to be diluted prior to PCR; because of the presence of different inhibitors in DNA extracts which can affect the target amplification. The PCR inhibitors are particularly known to attend in the DNA prepared from senescing plant tissues (Turner et al., 1998) and also the DNA extracts from the soil or plant roots (Van de Graaf et al., 2003; Gao et al., 2004).

The Ct value for 1 pg DNA μl^{-1} was between 24-30 cycles for all nine *Fusarium* spp. except *F. proliferatum* and *F. tricinctum*, which have a threshold cycle of 21 for this amount of the pure genomic DNA. The reason is that the primers used for these two *Fusarium* species were derived from the IGS (Intergenic Spacer) rDNA region which is a multi-copy domain in the genome and fungal detection is more sensitive for multi-copy IGS-based PCR assay (Jurado et al., 2006).

The set of species-specific primers used in this work allowed amplifying the considering specific fragments by qPCR assay. Some of the primer pairs indicated specificity problems and excluded from the further experiments. For *F. subglutinans*, Primer Fsub565/Fsub622A (Nicolaisen et al., 2009) produced non-specific PCR products for *F. subglutinans* DNA samples, Fg 71 and Fv Ita l isolates. Furthermore, primer 61-2 F/61-2 R published by Möller et al. (1999), generated specific melting temperature for some of the reference DNA. Zheng & Ploetz (2002), demonstrated primer pairs 61-2 F/61-2 R were not useful for identifying the *F. subglutinans*-like isolates such as *F. sacchari* and *F. circinatum*. As a result in our work the primer pair SUB 1/SUB 2, designed based on the calmodulin partial gene (Mulè et al., 2004) considered as the species-specific primer in real-time PCR for *F. subglutinans*. There was no cross-reaction with other *Fusarium* spp. and the fungal DNA tested. Therefore, *F. subglutinans* optimization was continued with SUB 1/SUB 2 primers. Regarding to *F. avenaceum*, Turner et al. (1998), designed primer JIAF/R based on the nuclear 5.8s rDNA ITS sequence and demonstrated to be specific for *F. avenaceum* with no cross reactivity with *F. tricinctum* or any other wheat

pathogens. Furthermore, primer MGBF/R which is a set of novel oligonucleotides including "Minor Groove Binder" (MGB) ligands has been designed by Waalwijk et al. (2004). These scientists produced this primer based on the sequencing of a 920 bp amplified DNA fragment generated by Fa F/R primer. The primer Fa F/R was reported to be specific to *F. avenaceum* (Doohan et al., 1998). In our experiments both of these primers (JIAF/R and *avenaceum* MGBF/R) produced specific peak only in *F. avenaceum* wells; but more sensitivity of primer MGBF/R with field samples led to its selection as the specific primer for *F. avenaceum*. Higher specificity of MGB ligands primers compared to the classical primers is for the nature of ligands (Afonia et al., 2002). Primer set Fp82F/R for *F. poae* was created from clone T161 by Parry and Nicholson (1996). They had presented this primer as a monomorphic marker which is able to detect *F. poae* in wheat samples; but another primer pair designed for *F. poae* (Fp8F/R) had shown polymorphic bands and, therefore, did not develop further. In our experiments primer Fp82F/R, amplified the target DNA while no amplification occurred in the reference fungal DNA nor for the negative control. Consequently, the ability of primer pairs SUB 1/SUB 2, MGBF/R and Fp82F/R was confirmed to detect corresponding *Fusarium* species in the extracts from maize plant tissue samples by qPCR. Primer pair 198F2/198R1 designed by Wilson et al. (2004) for *F. equiseti* was tested against isolates of the target species and a variety of other *Fusarium* species and other fungi associated with diseases of cereals (Wilson et al., 2004; Nicholson et al., 2004). Kulik (2008) evaluated the specificity of Tri1/Tri2 primers on genomic DNA extracted from 56 isolates representing 20 *Fusarium* species and also 12 *F. tricinctum* isolates. He found unexpected amplicons which were amplified from one isolate of *F. acuminatum* and one from *F. nurragi*. Further sequencing experiments indicated 100% identity of *F. acuminatum* isolate to *F. tricinctum* while *F. nurragi* had 99% of similarity to CBS 261.51 (*F. tricinctum*).

In conclusion it is believed that the real-time PCR protocols described in this study are specific, reliable and sensitive for singly detection and quantification of nine *Fusarium* spp. genomic DNA. Furthermore, the established multi-species qPCR method is also robust, precise, cost effective, quick and applicable for monitoring and quantifying of nine *Fusarium* spp. These protocols are widely used in our department for detection and quantification of *Fusarium* spp. in cereal crops including maize, wheat, sorghum, rice, and different other plants such as weeds

which naturally or artificially have single or multiple infection of *Fusarium* spp. It is anticipated that the reported protocols would be applicable for other crops, as well.

Author's Contributions

In this work, the specificity test for *F. subglutinans* primer sets was performed by Dr. Eva-Maria Becker.

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Supplemental Table 1. Standard curves and determination of LOQ of qPCR for the nine *Fusarium* species with a common thermocycler program

F. avenaceum			***F. culmorum***			***F. equiseti***		
DNA (pg)	Ct	Tm (°C)[1]	DNA (pg)	Ct	Tm (°C)	DNA (pg)	Ct	Tm (°C)
1111	18.99	82	1111	17.42	87	1111	19.54	86.5
1111	19.05	82	1111	17.73	87.5	1111	20.04	86.5
1111	19.34	82	1111	18.15	87	1111	19.77	86.5
370.37	21.57	81.5	370.37	18.99	87	370.37	21.10	86.5
370.37	21.39	82	370.37	19.26	87	370.37	21.50	86.5
370.37	21.25	82	370.37	19.38	87	370.37	21.20	86.5
123.45	22.60	82	123.45	21.07	87	123.45	22.98	86.5
123.45	23.37	81.5	123.45	21.17	87	123.45	23.16	86.5
123.45	Failed		123.45	21.08	87	123.45	22.87	86.5
41.15	23.28	82	41.15	22.44	87	41.15	24.75	86.5
41.15	25.38	81.5	41.15	22.77	87	41.15	24.42	86.5
41.15	25.11	81.5	41.15	22.68	87	41.15	24.25	86.5
13.71	26.74	81.5	13.71	24.54	87	13.71	26.44	86
13.71	26.33	82	13.71	24.13	87	13.71	25.75	86
13.71	25.82	82	13.71	24.32	87	13.71	Failed	
4.57	28.33	81.5	4.57	25.69	87	4.57	25.89	86
4.57	28.12	81.5	4.57	26.68	87	4.57	Failed	
4.57	28.15	82	4.57	26.02	87	4.57	27.63	86
1.52	29.37	81.5	1.52	27.29	87	1.52	28.15	86
1.52	29.94	81.5	1.52	27.51	87	1.52	27.30	86
1.52	29.26	81.5	1.52	Failed		1.52	27.72	86
0.508	31.41	81.5	0.508	30.45	87	0.508	29.89	86
0.508	31.29	81.5	0.508	28.96	87	0.508	30.01	86
0.508	31.79	82	0.508	28.35	87	0.508	Failed	
0.169	32.02	81.5	1111	17.89	87	1111	19.51	86.5
0.169	33.30	81.5	1111	18.18	87	1111	19.63	86.5
0.169	32.61	81.5	370.37	20.08	87	1111	19.65	86.5
1111	18.97	82	370.37	19.68	87.5	370.37	21.05	86.5
1111	19.73	82	123.45	21.23	87	370.37	21.42	86.5
370.37	21.62	82	123.45	21.97	87	370.37	21.30	86.5
370.37	21.26	82	41.15	23.01	87	123.45	22.96	86.5
123.45	23.15	82	41.15	22.84	87	123.45	23.10	86.5
123.45	22.91	82	13.71	24.91	87	123.45	22.66	86.5
41.15	24.80	82	13.71	24.98	87.5	41.15	24.28	86.5
41.15	24.36	82	4.57	26.24	87.5	41.15	24.44	86.5
13.71	26.64	81.5	4.57	26.13	87	41.15	24.19	86
13.71	26.35	82	1.52	27.41	87	13.71	26.44	86
4.57	29.40	81.5	1.52	26.91	87.5	13.71	25.75	86
4.57	28.07	81.5	0.508	29.31	87.5	13.71	25.10	86
1.52	29.97	81	0.508	Failed		4.57	27.03	86
1.52	29.10	81.5	1111	17.49	87	4.57	26.89	86
0.508	31.85	81.5	1111	17.98	87	4.57	26.47	86
0.508	31.35	81.5	1111	18.14	87	1.52	28.48	86
0.169	32.34	81.5	370.37	19.06	87	1.52	28.59	86
0.169	Failed		370.37	19.46	87	1.52	28.03	86
1111	19.24	82	370.37	19.48	87	0.508	30.43	86
1111	20.03	82	123.45	21.13	87.5	0.508	29.85	86
1111	19.96	82	123.45	21.37	87.5	0.508	30.37	86.5

Supplemental Table 1: continued

F. avenaceum		
DNA (pg)	Ct	Tm (°C)[1]
370.37	21.80	82
370.37	21.71	82
370.37	21.29	82
123.45	22.87	82
123.45	23.32	82
123.45	23.03	81.5
41.15	24.76	81.5
41.15	25.00	82
41.15	24.59	82
13.71	26.39	82
13.71	26.49	82
13.71	26.04	81.5
4.57	28.09	81.5
4.57	28.51	82
4.57	27.82	82
1.52	29.18	82
1.52	29.33	82
1.52	30.31	82
0.508	31.01	81.5
0.508	31.59	82
0.508	30.65	82
0.169	33.06	82
0.169	31.97	81.5
0.169	32.83	81.5
1111	19.14	81.5
1111	19.74	81.5
370.37	20.63	81.5
370.37	20.97	81.5
123.45	22.56	81.5
123.45	22.61	81.5
41.15	24.08	81.5
41.15	23.96	81.5
13.71	26.02	81.5
13.71	25.57	81.5
4.57	28.18	81.5
4.57	27.48	81.5
1.52	29.31	81.5
1.52	30.04	81.5
0.508	Failed	
0.508	30.91	81.5
0.169	31.43	81
0.169	31.25	81

10 Replications

F. culmorum		
DNA (pg)	Ct	Tm (°C)
123.45	20.74	87
41.15	22.56	87
41.15	22.56	87
41.15	22.56	87
13.71	24.14	87
13.71	24.52	87
13.71	24.28	87
4.57	25.69	87
4.57	25.74	87
4.57	26.01	87
1.52	26.75	87.5
1.52	27.96	87
1.52	27.28	87.5
0.508	29.75	87.5
0.508	29.55	87
0.508	29.89	87
1111	18.26	87.5
1111	18.20	87.5
370.37	20.21	87.5
370.37	19.61	87.5
123.45	21.48	87.5
123.45	21.32	87.5
41.15	23.21	87.5
41.15	23.33	87.5
13.71	25.24	87.5
13.71	25.05	87.5
4.57	27.23	87.5
4.57	27.08	87.5
1.52	30.12	87.5
1.52	29.05	87
0.508	Failed	87.5
0.508	31.88	87

10 Replications

F. equiseti		
DNA (pg)	Ct	Tm (°C)
1111	18.80	86
1111	19.71	86
370.37	20.42	86
370.37	20.81	86
123.45	22.18	86
123.45	22.59	86
41.15	24.75	86
41.15	24.97	86
13.71	26.58	86
13.71	26.42	86
4.57	26.91	86
4.57	27.64	86
1.52	29.26	86
1.52	29.35	86
0.508	30.14	86
0.508	30.91	86

8 Replications

Supplemental Table 1: continued

F. graminearum			***F. poae***			***F. proliferatum***		
DNA (pg)	Ct	Tm (°C)[1]	DNA (pg)	Ct	Tm (°C)	DNA (pg)	Ct	Tm (°C)
1111	17.76	82.5	1111	15.56	83	1111	12.02	89
1111	17.91	82.5	1111	16.16	83	1111	11.89	89
1111	17.89	82.5	1111	15.53	83	370.37	12.34	89
370.37	19.40	82.5	370.37	17.30	83	370.37	12.92	89
370.37	19.87	82.5	370.37	17.40	83	123.45	14.04	89
370.37	19.63	82.5	370.37	17.19	83	123.45	14.52	89
123.45	21.31	82.5	123.45	18.80	83	41.15	15.85	89
123.45	21.32	82.5	123.45	19.67	83	41.15	16.07	89
123.45	21.49	82.5	123.45	19.40	83	13.71	17.26	88.5
41.15	22.91	82.5	41.15	20.43	83	13.71	17.81	89
41.15	23.39	82.5	41.15	20.37	83	4.57	19.25	89
41.15	23.24	82	41.15	20.55	83	4.57	19.3	89
13.71	24.00	82.5	13.71	22.06	83	1.52	21.49	89
13.71	23.94	82.5	13.71	22.51	83	1.52	22.21	89
13.71	24.75	82.5	13.71	21.85	83	0.508	22.93	88.5
4.57	26.46	82.5	4.57	23.41	83	0.508	23.05	89
4.57	25.76	82.5	4.57	24.10	83	0.169	23.8	89
4.57	25.90	82.5	4.57	23.55	83	0.169	24.5	89
1.52	27.86	82.5	1.52	24.89	83	0.056	26.00	88.5
1.52	26.89	82.5	1.52	25.52	83	0.056	25.83	88.5
1.52	26.62	82.5	1.52	25.37	83	1111	11.91	89
0.508	28.72	82.5	0.508	27.14	83	1111	12.04	89
0.508	28.75	82.5	0.508	27.13	83	1111	11.68	89
0.508	29.18	82.5	0.508	26.62	82.5	370.37	12.39	89
0.169	30.17	82.5	0.169	28.58	82.5	370.37	13.49	89
0.169	Failed		0.169	28.90	83	370.37	12.97	89
0.169	29.89	82.5	0.169	28.6	82.5	123.45	14.75	89
1111	17.55	82.5	1111	16.28	83	123.45	13.82	89
1111	17.93	83	1111	16.15	83	123.45	14.25	89
370.37	19.66	82.5	1111	15.81	83	41.15	15.69	89
370.37	19.76	82.5	370.37	17.17	83	41.15	15.93	89
123.45	21.61	82.5	370.37	17.97	83	41.15	15.57	89
123.45	22.20	82.5	370.37	17.37	82.5	13.71	17.34	89
41.15	22.96	82.5	123.45	19.41	82.5	13.71	17.20	89
41.15	23.81	82.5	123.45	19.75	82.5	13.71	18.02	89
13.71	24.09	82.5	123.45	19.33	82.5	4.57	19.19	89
13.71	25.97	82.5	41.15	21.08	83	4.57	18.62	89
4.57	Failed		41.15	21.69	82.5	4.57	19.13	89
4.57	26.59	82.5	41.15	21.27	83	1.52	20.75	89
1.52	27.17	82.5	13.71	22.88	83	1.52	20.59	89
1.52	28.41	82.5	13.71	23.48	83	1.52	20.95	89
0.508	29.85	82.5	13.71	22.63	82.5	0.508	22.06	89
0.508	29.36	82.5	4.57	24.96	82.5	0.508	21.79	89
0.169	Failed		4.57	25.39	82.5	0.508	22.21	89
0.169	30.66	82.5	4.57	24.87	82.5	0.169	23.90	88.5
1111	16.85	82.5	1.52	26.97	83	0.169	24.08	89
1111	17.30	82.5	1.52	26.04	83	0.169	24.06	89
370.37	20.01	82	1.52	26.16	83	0.056	26.00	89
370.37	19.11	82.5	0.508	28.20	83	0.056	25.03	89
123.45	21.28	82	0.508	29.11	83	0.056	Failed	

Supplemental Table 1: continued

F. graminearum			***F. poae***			***F. proliferatum***		
DNA (pg)	Ct	Tm (°C)[1]	DNA (pg)	Ct	Tm (°C)	DNA (pg)	Ct	Tm (°C)
123.45	21.45	82	0.508	27.97	83	1111	12.27	89
41.15	23.61	82	0.169	27.61	83	1111	12.20	89
41.15	23.19	82	0.169	29.30	82.5	370.37	13.52	89
13.71	24.06	82	0.169	27.14	82.5	370.37	13.73	89.5
13.71	24.88	82	1111	14.56	83	123.45	15.06	89
4.57	25.64	82	1111	14.93	83	123.45	14.93	89
4.57	26.11	82	370.37	16.39	83	41.15	16.71	89
1.52	27.20	82	370.37	16.65	83	41.15	16.87	89
1.52	27.62	82	123.45	19.28	83	13.71	18.05	89
0.508	29.53	82.5	123.45	18.69	83	13.71	17.93	89
0.508	28.73	82	41.15	20.42	83	4.57	20.21	89
0.169	30.48	82.5	41.15	19.99	83	4.57	19.31	89
0.169	30.74	82.5	13.71	22.11	83	1.52	21.66	89
1111	17.45	82	13.71	21.51	83	1.52	21.96	89.5
1111	17.34	82	4.57	23.47	83	0.508	23.54	89
1111	18.38	82	4.57	23.81	83	0.508	22.56	89
370.37	19.22	82	1.52	24.78	83	0.169	24.44	89
370.37	19.20	82	1.52	25.10	83	0.169	23.89	89
370.37	20.05	82	0.508	26.49	83.00	0.056	25.93	89
123.45	21.07	82	0.508	26.52	83	0.056	25.09	89
123.45	21.00	82	0.169	27.71	83	1111	11.03	89
123.45	21.27	82	0.169	28.01	83	1111	11.25	89
41.15	22.67	82	1111	15.62	83.5	1111	11.5	89
41.15	22.73	82	1111	16.13	83.5	370.37	12.82	89
41.15	23.51	82	370.37	18.05	83.5	370.37	13.29	89
13.71	24.01	82	370.37	17.74	83.5	370.37	13.01	89
13.71	24.47	82.5	123.45	19.72	83.5	123.45	14.34	89
13.71	24.45	82	123.45	19.20	83.5	123.45	14.61	89
4.57	26.14	82	41.15	21.11	83.5	123.45	14.72	89
4.57	26.18	82	41.15	20.74	83.5	41.15	16.02	89
4.57	26.45	82	13.71	21.90	83.5	41.15	16.20	89
1.52	27.21	82	13.71	23.40	83.5	41.15	15.98	89
1.52	28.18	82	4.57	25.45	83.5	13.71	17.45	89
1.52	28.17	82.5	4.57	24.27	83.5	13.71	18.00	89
0.508	28.87	82.5	1.52	25.63	83.5	13.71	17.38	89
0.508	29.18	82	1.52	Failed		4.57	19.19	89
0.508	29.72	82	0.508	26.60	83.5	4.57	19.49	89
0.169	29.12	82	0.508	27.01	83.5	4.57	19.16	89
0.169	30.06	82	0.169	28.01	83.50	1.52	20.76	89
0.169	30.63	82.5	0.169	Failed		1.52	21.18	89
1111	16.30	82	1111	15.65	83	1.52	20.69	89
1111	16.46	82	1111	15.50	83	0.508	22.33	89
370.37	17.99	82.5	370.37	16.79	83	0.508	22.91	89
370.37	18.31	82.5	370.37	17.25	83	0.508	22.41	89
123.45	19.11	82.5	123.45	19.10	83	0.169	23.68	89
123.45	20.31	82.5	123.45	19.11	83	0.169	24.72	89
41.15	21.42	82.5	41.15	21.04	83	0.169	24.43	89
41.15	21.60	82.5	41.15	20.91	83	0.056	25.17	89.5
13.71	23.29	82.5	13.71	22.17	83	0.056	26.14	89
13.71	23.02	82.5	13.71	22.52	83	0.056	25.64	89
4.57	25.14	82.5	4.57	23.95	83	1111	10.59	89

Supplemental Table 1: continued

F. graminearum			***F. poae***			***F. proliferatum***		
DNA (pg)	Ct	Tm (°C)[1]	DNA (pg)	Ct	Tm (°C)	DNA (pg)	Ct	Tm (°C)
4.57	25.13	82.5	4.57	24.29	83	1111	10.35	89
1.52	27.10	82.5	1.52	25.75	83	370.37	12.09	89
1.52	26.72	82.5	1.52	26.17	83	370.37	12.02	89
0.508	28.99	82.5	0.508	26.76	83	123.45	12.90	89
0.508	27.92	82.5	0.508	27.39	83	123.45	13.43	89
0.169	31.27	82	0.169	28.99	83	41.15	15.10	89
0.169	30.52	82	0.169	29.73	83	41.15	15.79	89
						13.71	16.52	89
12 Replications			12 Replications			13.71	16.59	89
						4.57	19.34	89
						4.57	18.76	89
						1.52	19.94	89
						1.52	20.25	89
						0.508	23.31	89
						0.508	21.90	89
						0.169	23.83	89
						0.169	23.95	89
						0.056	Failed	
						0.056	24.99	89.5
						12 Replications		

Supplemental Table 1: continued

F. subglutinans			***F. tricinctum***			***F. verticillioides***		
DNA (pg)	Ct	Tm (°C)[1]	DNA (pg)	Ct	Tm (°C)	DNA (pg)	Ct	Tm (°C)
1111	14.92	88.5	1111	11.19	90.5	1111	20.28	88
1111	14.89	88.5	1111	11.90	90.5	1111	19.49	88
1111	14.38	88.5	1111	12.08	91	370.37	21.08	88
370.37	15.37	88.5	370.37	13.45	90.5	370.37	20.70	88
370.37	16.09	88.5	370.37	13.18	90.5	123.45	22.94	88
370.37	15.81	88.5	370.37	13.15	90.5	123.45	22.66	88
123.45	16.94	88.5	123.45	14.87	90.5	41.15	24.44	88
123.45	17.56	88.5	123.45	15.19	90.5	41.15	24.67	88
123.45	17.22	88.5	123.45	15.03	91	13.71	24.98	88
41.15	18.27	88.5	41.15	17.08	90.5	13.71	25.60	88
41.15	18.84	88.5	41.15	16.95	90.5	4.57	27.12	88
41.15	19.37	88.5	41.15	16.35	90.5	4.57	Failed	
13.71	20.85	88.5	13.71	18.17	90.5	1.52	28.67	88
13.71	20.27	88.5	13.71	18.15	90.5	1.52	29.24	88
13.71	20.47	88.5	13.71	18.24	91.5	0.508	30.60	88
4.57	22.20	88.5	4.57	19.26	90.5	0.508	29.24	88
4.57	22.06	88.5	4.57	20.09	90.5	1111	18.79	88
4.57	21.58	88.5	4.57	19.50	90.5	1111	19.02	88
1.52	23.76	88.5	1.52	20.47	90.5	370.37	19.88	88.5
1.52	23.73	88.5	1.52	21.12	91	370.37	19.97	88.5
1.52	23.62	88.5	1.52	21.68	91.5	123.45	22.98	88.5
0.508	25.60	88.5	0.508	21.70	90.5	123.45	22.59	88
0.508	25.91	88.5	0.508	Failed		41.15	24.00	88
0.508	26.26	88.5	0.508	Failed		41.15	24.18	88.5
0.169	27.19	88.5	1111	11.20	91	13.71	24.79	88
0.169	27.20	88.5	1111	11.83	91	13.71	25.63	88
0.169	27.41	88.5	370.37	13.20	91	4.57	Failed	
1111	15.20	88.5	370.37	13.53	91.5	4.57	27.24	88.5
1111	15.18	88.5	123.45	14.93	91	1.52	28.45	88
1111	14.89	88.5	123.45	15.31	91	1.52	29.49	88
370.37	15.74	88.5	41.15	16.29	91	0.508	Failed	
370.37	16.44	88.5	41.15	17.42	91	0.508	30.46	88
370.37	15.87	88.5	13.71	17.75	91	1111	18.84	88.5
123.45	19.69	88.5	13.71	18.37	91	1111	18.63	88
123.45	18.47	88.5	4.57	19.43	91	370.37	20.46	88.5
123.45	16.79	88.5	4.57	20.01	91	370.37	20.77	88.5
41.15	19.33	88.5	1.52	20.70	91	123.45	22.29	88.5
41.15	18.33	88.5	1.52	20.99	91	123.45	21.82	88.5
41.15	19.48	88.5	0.508	22.34	90.5	41.15	23.93	88
13.71	20.29	88.5	0.508	22.57	91	41.15	23.82	88.5
13.71	20.33	88.5	1111	11.54	91	13.71	25.87	88
13.71	20.61	88.5	1111	11.84	91	13.71	25.69	88
4.57	23.28	88.5	1111	11.03	91	4.57	27.29	88
4.57	21.99	88.5	1111	11.00	91	4.57	28.09	88.5
4.57	Failed		1111	11.61	91	1.52	28.55	88.5
1.52	21.69	88.5	370.37	13.0	91	1.52	29.14	88
1.52	25.20	88.5	370.37	13.79	91	0.508	30.55	88.5
1.52	24.41	88.5	370.37	13.4	91	0.508	29.74	88
0.508	25.71	88.5	370.37	13.05	91	1111	18.61	88.5
0.508	26.13	88.5	370.37	13.36	91.5	1111	18.28	88.5

Supplemental Table 1: continued

F. subglutinans			***F. tricinctum***			***F. verticillioides***		
DNA (pg)	Ct	Tm (°C)[1]	DNA (pg)	Ct	Tm (°C)	DNA (pg)	Ct	Tm (°C)
0.508	26.41	88.5	123.45	14.75	91	370.37	20.33	88.5
0.169	29.54	88.5	123.45	15.09	91	370.37	20.16	88
0.169	27.74	88.5	123.45	14.84	91	123.45	22.12	88
0.169	27.60	88.5	123.45	14.69	91	123.45	22.28	88
1111	14.01	88.5	123.45	15.17	91	41.15	23.69	88
1111	15.32	88.5	41.15	16.47	91	41.15	23.56	88
370.37	16.74	88.5	41.15	16.63	91	13.71	25.90	88
370.37	15.93	88.5	41.15	16.30	91	13.71	25.50	88
123.45	17.01	88.5	41.15	16.11	91	4.57	27.50	88.5
123.45	17.39	88.5	41.15	17.27	91	4.57	27.44	88.5
41.15	18.22	88.5	13.71	18.06	91	1.52	28.72	88
41.15	18.00	88.5	13.71	18.04	91	1.52	29.20	88
13.71	19.81	88.5	13.71	18.11	91	0.508	30.56	88
13.71	20.01	88.5	13.71	17.55	91	0.508	30.38	88
4.57	21.51	88.5	13.71	18.21	91	1111	18.95	88.5
4.57	21.68	88.5	4.57	19.45	91	1111	18.19	88.5
1.52	23.04	88.5	4.57	20.00	91	370.37	19.84	88.5
1.52	Failed		4.57	19.47	91	370.37	19.65	88
0.508	24.63	88.5	4.57	19.24	91	123.45	22.24	88
0.508	25.21	88	4.57	19.79	91	123.45	22.20	88
0.169	26.66	88.5	1.52	20.77	91	41.15	23.98	88
0.169	26.93	88.5	1.52	21.09	91	41.15	23.62	88
			1.52	20.82	91	13.71	25.02	88.5
8 Replications			1.52	20.51	91	13.71	24.81	88
			1.52	20.76	91	4.57	27.66	88
			0.508	21.70	90.5	4.57	27.14	88
			0.508	21.72	91	1.52	27.93	88
			0.508	21.41	90.5	1.52	Failed	
			0.508	22.19	90.5	0.508	29.62	88.5
			0.508	22.35	91	0.508	30.07	88
			1111	11.92	91.5	1111	18.85	88.5
			1111	12.48	91.5	1111	18.78	88.5
			370.37	13.47	91.5	1111	18.68	88
			370.37	13.41	91.5	370.37	20.82	88.5
			123.45	15.29	91.5	370.37	20.51	88
			123.45	15.39	91.5	370.37	20.52	88.5
			41.15	17.08	91.5	123.45	22.32	88.5
			41.15	17.24	91.5	123.45	22.14	88.5
			13.71	18.67	91.5	123.45	22.15	88
			13.71	18.98	91.5	41.15	24.16	88.5
			4.57	20.38	91.5	41.15	23.96	88.5
			4.57	20.58	91.5	41.15	24.00	88
			1.52	21.97	91.5	13.71	27.17	88.5
			1.52	21.95	91.5	13.71	25.20	88.5
			0.508	22.26	91.5	13.71	26.22	82
			0.508	22.47	91.5	4.57	28.07	88.5
						4.57	Failed	
			12 Replications			4.57	27.11	88.5
						1.52	28.96	88.5
						1.52	27.93	88.5
						1.52	29.04	88

Supplemental Table 1: continued

F. subglutinans			***F. tricinctum***			***F. verticillioides***		
DNA (pg)	Ct	Tm (°C)[1]	DNA (pg)	Ct	Tm (°C)	DNA (pg)	Ct	Tm (°C)
						0.508	30.73	88.5
						0.508	31.08	88.5
						0.508	31.15	88.5
						1111	19.26	88.5
						1111	19.16	88.5
						1111	19.38	88.5
						370.37	20.88	88.5
						370.37	20.21	88.5
						370.37	20.66	88.5
						123.45	22.04	88.5
						123.45	22.01	88.5
						123.45	22.14	88.5
						41.15	24.15	88.5
						41.15	24.04	88.5
						41.15	23.95	88.5
						13.71	25.33	88.5
						13.71	25.43	88.5
						13.71	25.51	88.5
						4.57	26.78	88.5
						4.57	27.12	88
						4.57	27.09	88.5
						1.52	28.49	88.5
						1.52	28.31	88.5
						1.52	28.61	88
						0.508	31.44	88.5
						0.508	31.00	88.5
						0.508	30.14	88
						1111	18.05	88
						1111	18.87	88
						370.37	19.64	88
						370.37	19.89	88
						123.45	21.31	88
						123.45	22.00	88
						41.15	23.47	88
						41.15	23.77	88
						13.71	26.05	88
						13.71	25.54	88
						4.57	26.68	88
						4.57	27.83	88
						1.52	Failed	
						1.52	29.02	88
						0.508	29.93	88
						0.508	29.99	88
							18 Replications	

For each species, the standard curves generated on different days are separated by a horizontal bar.
LOQ was defined as the lowest standard concentration which generated specific products on at least seven out of eight replicates.
[1.] Tm: melting temperature

Chapter 3

Colonization of Weed Species with *Fusarium* spp. in Maize Fields

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Abstract

The qPCR assay detected eight *Fusarium* species in 201 weed samples representing 36 weed species collected from maize fields. The highest frequency was observed for *F. equiseti* (49%) and *F. avenaceum* (34.7%). It was followed by *F. tricinctum*, *F. culmorum* (each 18%), *F. proliferatum* (11%) and *F. graminearum* (8%). The diversity of *Fusarium* spp. in comparison between organic and conventional farming was similar. Isolations were made from 66 plants belonging to 12 common weed species. Strains were identified based on morphology and translation elongation factor 1-alpha (TEF-1α) gene sequence. The recovery rate was high for *F. equiseti* (32.7%) and *F. avenaceum* (21%). We could not isolate *F. poae* and *F. subglutinans* which were detected in low incidence rates (3% and 1%) in qPCR assessments. In contrast, *F. oxysporum* (16%) and *F. venenatum* (8.5%) were obtained from 10 weed species except *Matricaria inodora* and *Galium aparine*. None of the field samples as well as weed plants tested in inoculation studies show obvious symptoms of *Fusarium* infection. Re-isolation of the strains confirmed endophytic infection of weeds by *Fusarium* spp. The present study identified six new alternative hosts for *Fusarium* species in maize fields. High incidence rates of beauvericin and enniatins contamination were obtained in weed samples while trichothecenes, fumonisins and zearalenone were not detected in any of the weeds studied.

Additional keywords: weed hosts, *Fusarium* spp., mycotoxins, maize

Introduction

Genus *Fusarium* comprises phytopathogenic species causing important diseases of cereals wherever corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) or other small-grain cereals are grown. The most commonly reported *Fusarium* spp. associated with maize plants include *Fusarium avenaceum* (Fr.) Sacc., *F. culmorum* (W.G. Smith) Sacc., *F. graminearum* Schwabe, *F. proliferatum* (Matsushima) Nirenberg, *F. subglutinans* (Wollenweber & Reinking) P. E. Nelson, Toussoun & Marasas and *F. verticillioides* (Sacc.) Nirenberg (Dornet al., 2009; Logrieco et al., 2002). Other *Fusarium* species also encounter maize tissues and have a distinct effect on the level of infection (Munkvold, 2003). Since *Fusarium* spp. have the potential to produce different mycotoxins, they had always been a serious concern for human and animal health. The quality and quantity of crops are affected when the *Fusarium* species colonize the ears and the kernels of wheat or corn produced for food or feed. Main *Fusarium* mycotoxins usually reported from infected maize tissues include trichothecenes, fumonisins, zearalenone, enniatins and moniliformin (Glenn, 2007; Placinta et al., 1999).

Although several sources of inoculum are considered for the survival of *Fusarium* pathogens, the primary source of inoculum in most fields is a remnant of plant residues. All cereal-related *Fusarium* spp. can survive saprophytically on the crop residues that remain in the field after harvesting (Parry et al., 1995). In maize fields, *Fusarium* species survive as mycelium or other survival structures on maize crop debris (Sutton, 1982; Cotten & Munkvold, 1998; Naef & De´fago, 2006). This crop debris is then the major reservoir for the infection of kernels of the subsequent maize crop (reviewed in Munkvold, 2003). More recent studies in Europe and North America have approved that in comparison with wheat residue, the maize residue has much more effect as a source of inoculum for the infection of wheat to fusarium head blight (Dill-Macky & Jones, 2000; Schaafsma et al., 2001). In Uruguayan cultivation systems, a lower contribution of *F. graminearum* inoculum is allocated to corn (Pereyra & Dill-Macky, 2008).

In spite of many research on biology, pathology and toxicology of *Fusarium* species, there is still need for improvement of the effective management of *Fusarium* diseases occurred in maize crop. Current management protocols for maize *Fusarium* diseases mostly involve the

avoidance of crop residues (Maiorano et al., 2008; Naef & De´fago, 2006) to reduce fungal inoculum; but rarely consider weed hosts as a source for crop infection. Weed plants are mostly considered as organisms which can provide favorable conditions such as humidity for developing of *Fusarium* maize diseases. However, the role of weeds could be likely more. Weed and wild plants can be hosts which provide an important source of inoculum (Parry et al., 1995). Although the importance of weeds as a reservoir of fungal pathogens in fields was identified by Garrett in 1960, but still little literary work has been carried out on weeds as alternative hosts for fungal pathogens and mostly has been concentrated on *F. oxysporum*. There are contradictory reports for the interaction between weed plants density and fusarium head blight severity in different experimental investigations (Teich & Nelson, 1984). It has been demonstrated that *Fusarium* populations in the soil can colonize senescent tissues of weed plants as well as maize tissues and produce different types of infectious propagules such as perithecia and ascospores or asexual spores of microconidia and macroconidia (Parry et al., 1995; Munkvold, 2003). Recovery of *Fusarium* spp. from 19 species of cereals and grasses and 24 species of common weed plants is one of the first reports that introduce weeds as a harbor for *Fusarium* species (Gordon, 1959). The reports from sugar beet, tomato, soybean and eggplant fields also show that *F. oxysporum* pathogenic on these crops has survival capability on several common weed plants (McDonald & Leach, 1976; Haware & Nene, 1982; Helbig & Carroll, 1984; Altinok, 2013).

Some *Fusarium* strains are weed pathogens and, therefore, have been used as biocontrol agents. They are also used in the development of mycoherbicides and fungal phytotoxins for control of weeds (Boyette & Walker, 1985; Abbas et al., 1991; Abbas & Boyette, 1992; Roy et al., 1994). On the other hand, there are *Fusarium* strains that are not pathogenic to weeds. One reason for the absence of pathogenicity would be the ectosymbiotic bacteria complexes that are able to modulate the expression of pathogenicity genes in interaction with *Fusarium* strains (Minerdi et al., 2008). The pathogenic *Fusarium* strains show protective behavior against other antagonists while they harbor on non-host plants such as weeds. Subsequently, weed plants occupied by such endophytic *Fusarium* strains act as symptomless hosts to increase inoculum potential (Altinok, 2013). There is growing evidence that reveal weed plants are colonized by pathogenic fungi as symptomless hosts (Roy, 1982; Cerkauskas et al., 1983; Helbig & Carrol, 1984; Roy et al., 1994; Jenkinson & Parry, 1994; Postic et al., 2012; Altinok, 2013). Exhibition

no symptoms on weed plants by *Fusarium* species has always been a question. Some reports assigned that only less aggressive strains can invade weeds and produce infection without symptoms (Helbig & Carroll, 1984). Furthermore, lack of strong adaption performance of *Fusarium* on alternative hosts could also explain the symptomless infection of weeds. The reports show passage through alternative host causes reduction of the pathogenic fitness (aggressiveness) but increment of the overall fungal reproductivity (saprophytic behavior). As a result of transition through an alternative host, a conversion of pathogen behavior may be occurred and colonization of primary host would be improved (Akinsanmi et al., 2007). All these studies conclude that effective weed management can be a useful approach for reduction of *Fusarium* diseases inoculum in maize fields (reviewed in Fandohan et al., 2003).

On the average, organically grown maize is to the same extent or even less contaminated with mycotoxins than conventionally grown maize; but extremely high mycotoxin levels have been occasionally reported for organic maize products over the years. Low frequency but regular occurrence of unusually high mycotoxin values and fungal colonization has been observed in well-controlled field trials too which is difficult to explain (Nutz & Karlovsky, unpublished). There is a hypothesis that particular species of weed plants that host *Fusarium* spp. maybe increase the infection pressure locally and account for high levels of *Fusarium* mycotoxins in organic maize fields. International developments in mycotoxin regulation make high pressure for finding novel strategies to manage the contamination of maize products by *Fusarium* mycotoxins. Therefore, understanding of *Fusarium* spp. survival and the sources of *Fusarium* inoculum potential as well as the factors influencing this potential is a prerequisite for plant pathologists to prevent the build-up of inoculum sources and, subsequently, management of the *Fusarium* diseases in maize fields.

This study was carried out to investigate the role of weed plants in the survival and inoculum production of maize *Fusarium* pathogens and to assess their ability for producing of the main mycotoxins. The question is if weed plants play a role as alternative hosts for *Fusarium* species pathogenic to maize and if they can provide a significant source of inoculum that help to increase maize mycotoxin contamination?

Material and Methods

Collection and Processing of Weed Plants from Maize Fields

In 2010 and 2011, weed plants were sampled from 11 maize fields located in Germany (Table 1). Five fields were managed organically while the remaining six were conventionally cultured. In latter farming system chemical herbicides were used for the weed control. The plants were collected at random from five areas in each field during they were growing, fully developed but prior to senescence. All weed plants were identified and dried for around four days at 30°C (Westerman & Gerowitt, 2012). Samples were inspected for visible symptoms and no obvious symptoms of *Fusarium* infection was observed on any of the plants sampled. The experiments, therefore, were continued with anticipation of endophytic growth of *Fusarium* spp. in weed plants. For this purpose, the whole dried plant sample including root, stem, leaf and flowers or seeds were initially crushed by a mixer. Then a subsample was taken and ground more by using a reciprocal mill (Retsch, Haan, Germany).

Total genomic DNA was extracted by using 40 mg of the fine powder plant material based on the CTAB method according to Brandfass & Karlovsky (2008). DNA quality and concentration was estimated by electrophoresis in 0.8% (w/v) agarose gel (Cambrex, Rockland, ME, USA), prepared in TAE buffer (40 mM Tris, 1 mM EDTA (ethylene diamine tetra acetic acid), pH set to 8.5) (both substances were obtained from Carl Roth, Karlsruhe, Germany). DNA was stained with ethidium bromide (0.5 µg ml^{-1}) (Applichem, Darmstadt, Germany) and visualized using a digital imaging system (VilberLourmat, Marne La Vallee, France). Prior to PCR, genomic DNA was diluted hundred or fifty times (v/v) with double distilled water for inhibitors reduction.

Table 1. Maize fields sampled for weed plants

Field	Location	Farming type	No. of assessed plants
1	Kremlin	Organic	17
2	Püggen	Organic	49
3	Sallahn	Organic	28
4	Rusch-Raduhn	Organic	30
5	Koblentz	Organic	23
6	Kritzmow	Conventional	11
7	Niendorf	Conventional	19

Table 1: continued

Field	Location	Farming type	No. of assessed plants
8	Dummers-torf	Conventional	39
9	Niex	Conventional	34
10	Göttingen	Conventional	27
11	Göttingen	Conventional	17

Detection of *Fusarium* spp. in Weeds by Real-time PCR

Fusarium species were detected in weed samples based on a previously developed low-volume qPCR assay (see **Chapter 2**). The qPCR primers, specific conditions and thermal parameters for single or simultaneous detection of the nine *Fusarium* species are presented in Table 2, 3a and 3b of Chapter 2.

Fungal Isolation and Morphological Identification of *Fusarium* spp.

Fusarium spp. were isolated from some of the symptom-free weed plant materials of mostly three weed species including *Chenopodium album* L., *Echinochloa crus-galli* (L.) Beauv and *Polygonum convolvulus* L. Other weed species used for *Fusarium* isolation are listed in Table 5. Plant tissue pieces (5-10 mm^2) were surface disinfested in 1% (v/v) sodium hypochlorite (NaClO) solution for 1 min. After rinsing three times with sterile distilled water, the samples were dried on filter paper for a few minutes. Five to six small plant segments were placed in 9-cm petri dishes containing Pepton PCNB Agar (PPA) (15 g Pepton, 1 g KH_2PO_4, 0.5 g $MgSO_4$ $7H_2O$, 750 mg PCNB, 15 g agar/liter distilled water; pH: 5.5-6.5) also called Nash Snyder as a selective medium with streptomycin sulphate (100 mg ml^{-1}) and neomycin sulphate (12 mg ml^{-1}) antibiotics (Nelson et al., 1983). Plates were incubated at 25°C for 7-10 days. Distinct fungal colonies temporarily identified as *Fusarium* were transferred on Potato Dextrose Agar (PDA) (Roth, Karlsruhe, Germany) and recorded for colony morphology and pigmentation based on the visual inspection of the plates. Since *Fusarium* isolates produce uniform spores on SNA (Synthetic Nutrient Agar) medium (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4$ $7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose and 15 g agar/liter distilled water) (Nirenberg, 1976), all single spores isolates were transferred to SNA plates and incubated at 25°C under near UV light for 4-7 days. Small pieces of sterile filter paper were placed on the surface of cooled agar to induce fungal sporulation. Identification was carried out at species level based on the morphological

descriptions of spores and conidiogenous cells according to Leslie & Summerell (2006). *Fusarium* strains were stored as spore suspension in 15% (v/v) glycerol (Carl Roth, Karlsruhe, Germany) at -70°C.

Fusarium DNA Sequencing and Taxonomic Analysis

Identification of recovered *Fusarium* isolates was performed according to the combination of morphological and molecular characters. Typical *Fusarium* structures were employed for morphological identification. Afterward, identification at species level was confirmed by the results of DNA sequence of the "Translaton Elongation Factor 1-alpha" (TEF-1α) gene, which is taxonomically most informative part in this fungal genus. For this purpose, representatives of each *Fusarium* group (totally 61 isolates) have been employed. Single spore cultures were cultivated in Potato Dextrose Broth (PDB) (Carl Roth, Karlsruhe, Germany) for 5 to 7 days at 25°C. The mycelia were collected by filtration on sterile filter paper and freeze-dried. Lyophilized mycelium was ground to a fine powder by using a reciprocal mill in 2 ml Eppendorf tubes with 4-5 wolfram carbide spheres (diameter 3 mm, Retsch, Haan, Germany). DNA was extracted as described by Brandfass & Karlovsky (2008).

Hot start PCR protocol was used to amplify the TEF-1α gene region using the fungal specific primer set: ef1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and ef2 (5'-GA(G/A)GTACCAGT(G/C)ATCATGTT-3') described by O'Donnell et al. (1998). The reaction mixture consisted of reaction buffer (16 mM $(NH4)_2SO_4$; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH: 8.8 at 25°C), 0.1 mM concentration of each of the four deoxynucleoside triphosphates (Bioline, Lükenwalde, Germany), 2 mM of $MgCl_2$, 1.75 U of hot-start DNA polymerase (Immolase DNA Pol, Lükenwalde, Germany), 0.3 µM concentration of each primer. Every reaction contained 1 µl template DNA or 1 µl sterile water as negative control. The PCR reactions were performed in a total volume of 25 µl reactions with a peQ STAR Thermocycler (96 Universal Gradient). The cycling conditions were as follows: 1 cycle of 10 min at 95°C, 30 cycles of 60 s at 94°C (denaturalization), 45 s at 58.5°C (annealing), 60 s at 72°C (extension) and followed by a final extension cycle at 72°C for 5 min. Fungal amplified DNA fragments were sequenced (LGC Genomics, Berlin, Germany) by Sanger method in one direction using ef1 primer. The chromatogram of TEF-1α sequence for each *Fusarium* species has been inspected

visually and sequence reads edited when necessary. The sequences were, then, used as queries for Fusarium-ID v. 1.0 database (Geiser et al., 2004). In order to construct a phylogenetic tree sequences with the highest similarity to the query, together with some sequences retrieved from the GenBank database (Supplemental Table 1) were aligned. The distance matrix was constructed from pairwise similarities expressed as fractions of identical nucleotides with Jukes-Cantor nucleotide distance measure model for multiple substitutions, and an UPGMA (Un-weighted Pair Group Method with Averages) dendrogram was generated by using the program CLC Main Workbench 6.9. The reliability of analysis was estimated by bootstrap method with 1000 replications.

Inoculation Studies

Inoculation tests were conducted with six arbitrary selected *Fusarium* isolates belonging to four species (RD22, RD98 for *F. avenaceum*; RD100, RD102 for *F. culmorum*; RD92 for *F. graminearum,* and RD94 for *F. proliferatum*). The *Fusarium* strains had previously been recovered from weed plants. The isolates were tested to young seedlings of 11 weed species (Table 4) and also maize plants. The seeds of selected weed species were prepared from the Herbiseed Company at UK and also from the Botanical Garden Department, Göttingen, Germany. Mini maize cultivar (Gaspe Flint landrace, originally collected in Quebec, Canada, provided by Prof. Dr. J. Schirawski, Department of Molecular Biology of Plant-Microbe Interaction, University of Göttingen, Germany) was also used in this study. The seeds were sown in plastic multi-pots containing sterile fine sand (< 2 mm granularity) and irrigated as required. Seven to ten seeds of each weed species were planted per cavity and thinned to five plants when seedlings appeared. For some of the weed species including *Galium aparine* L., *Polygonum convolvulus* L., *Polygonum persicaria* L. and *Amaranthus retroflexus* L. vernalization at 4°C under dark conditions for 3-4 weeks was necessary.

Two or three weeks after germination, the seedlings were inoculated with conidial spore suspension of each species of *Fusarium*. Conidial inoculum was produced in liquid mung bean media (Bai & Shaner, 1996) and has been stored in 15% (v/v) glycerol at -70°C till inoculation time. Prior to plant inoculation, spore germination test was carried out on PDA plates to assess the viability and rate of spore germination. The concentration of each conidial suspension was,

then, determined using a Thoma Chamber (0.0025 mm²) and adjusted to 1×10^6 spore ml^{-1} of sterile tap water. Inoculum was contained 0.1% (v/v) Tween®20 (Applichem, Darmstadt, Germany) as a surfactant. Spore suspensions were kept on ice during inoculation and homogenized well before using.

Root-dip inoculation method was employed for artificial inoculation in the greenhouse by soaking the roots of individual plant in spore suspension for 30 min. Inoculations were carried out in early morning. After inoculation the seedlings were transferred to new pots (9 × 9 × 9.5cm) including mixed soil consisting of commercial plant substrate (Fruhstorfer Erde, Typ T25, HAWITA Group, Vechta, Germany) and sand (1:1). All pots were arranged according to a completely randomized design on the greenhouse benches. The plants were maintained at 25 ± 5°C with alternating 12-h light (mercury vapour lamps, 6600 lux at ear height) and dark periods. During the experiment, relative humidity was 34-86%. Five replicate pots were set up for each species of *Fusarium* and each pot included two plants. Control treatments were inoculated with autoclaved tap water mixed with adhesion detergent of Tween®20. Visual symptoms were determined on a weekly basis and six weeks after inoculation the plants were harvested. In order to verify the presence of fungus in artificial inoculated plants, subsequent detection of *Fusarium* spp. in plant tissue was performed by qPCR assay. For a few inoculated samples, fungal re-isolation was carried out from surface sterilized stem tissues.

Mycotoxin Analysis

The field weed samples which indicated a positive signal in qPCR experiments were analyzed for a number of *Fusarium* related mycotoxins including deoxynivalenol (DON), 3ADON, 15ADON, nivalenol (NIV), zearalenone (ZEN), fumonisin B1 (FB1), beauvericin (BEA), enniatins (B, B1, A, A1) by HPLC-MS/MS. Mycotoxin extraction was performed by adding 1 ml acetonitrile-water (84:16) to 100 mg fine powder of weed plant tissues. The extract (600 µl) was dried in a rotational-Vacuum-Concentrator RVC 2-25 (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 40°C. The raw organic extract was resuspended in 600 µl of methanol-water (85:15) and defatted with the same volume of cyclohexane. After centrigfugation the lower phase was taken for analysis.

HPLC separation was carried out on a reverse-phase Polaris C18-ether column (100 × 2 mm, 3 µm particle size; Agilent, Darmstadt, Germany) at 40°C and trichothecenes B and zearalenone were detected by tandem mass spectrometry using triple quadrupol 1200 L (Varian, Darmstadt, Germany) according to Adejumo et al. (2007a) and Adejumo et al. (2007b), but two mass transitions were used for each mycotoxin. Beauvericin, fumonisin B1 and enniatins were separated on a reverse phase Kinetex C18 column **(**50.0 × 2.1 mm, 2.6 µm particle size; Phenomenex (Aschaffenburg, Germany) and detected using ion trap 500MS (Varian, Darmstadt, Germany) as described by Nutz et al. (2011). For these mycotoxins three mass transitions were used. Pure analytical standards in methanol/water (1:1) were used to construct calibration curves.

Data Processing and Statistical Analysis

SigmaPlot 12.3 Notebook was used for statistical analysis. Non-parametric tests were performed while data distribution was not normal. The qPCR data were assessed for finding the positive samples based on the melting temperature (Tm), starting quantity (SQ) and threshold cycle (Ct) value. The lowest standard DNA for each *Fusarium* spp. employed in the qPCR assays was considered as the limit of quantification (LOQ). According to the qPCR data, the quantity of fungal biomass (pg mg^{-1}) for the positive samples was calculated. DNA quantity for the samples containing values lower than LOQ was substituted as LOQ/2 (Hornunga & Reeda, 1990). These samples indicated the species-specific melting temperature in the PCR cycles lower than 30.

Results

A total of 294 plants (147 samples from each farming type) representing 55 weed species were collected from 11 maize fields in Germany. The qPCR *Fusarium* spp. monitoring indicated that 201 weed samples belonging to 36 weed species (3 monocotyledonous and 33 dicotyledonous) were infected with 8 species of *Fusarium* (Fig. 1). In total, 421 *Fusarium* infections were detected by species-specific real time PCR assay. In most cases, individual weed species appeared to be harbor of several species of *Fusarium,* although not necessarily in the same plant (see Table 3). Sometimes one sample was infected with two, three or more *Fusarium* species at the same time. The final results showed that 144 weed samples, which are 49% of total assessed weeds, were infected with *F. equiseti*. *F. avenaceum* had the second grade of infection with around 34.7%. Rate of infection for *F. culmorum* and *F. tricinctum* (18%) was equal across the experiments. For *F. proliferatum*, around 11% of total assessed samples were infected while the infection rate for *F. graminearum* was 8%. Further assessment revealed insignificant rate of infection for *F. poae* and *F. subglutinans* (3% and 1.0%, respectively) in different weed samples.

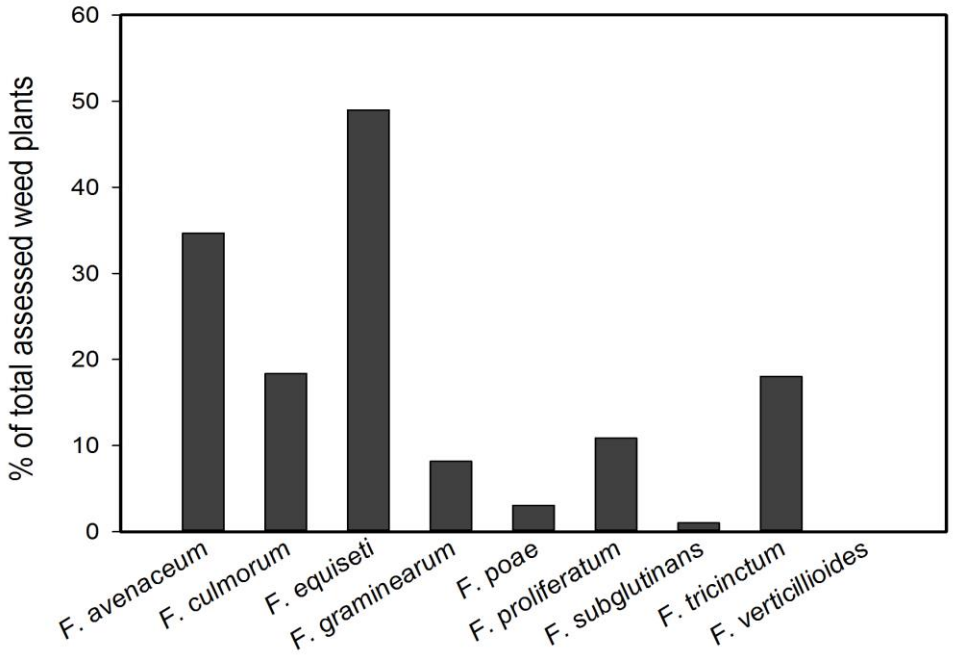

Fig. 1. Colonization of weed plants in maize fields by *Fusarium* spp.

The incidence of *Fusarium* species in *Echinochloa crus-galli*, *Polygonum convolvulus*, and *Chenopodium album* was higher than in other weed plants, respectively. *F. equiseti* and

F. avenaceum were detected in almost all common studied weed species (Table 2). All species of weeds displayed in Table 2, except *Polygonum persicaria*, were infected with *F. tricinctum* and *F. culmorum*. *Arabidopsis thaliana* L. which is also a popular model organism in plant biology indicated infection with four *Fusarium* spp. In this assessment, *F. verticillioides* was the only species that failed to be detected.

No discrepancy for infection frequency appeared in the study of *Fusarium* spp. population in organic and conventional maize farming concerning to *F. poae* and also *F. proliferatum* (Fig. 2). *F. subglutinans* was not detected in the conventional farming, while only three infected weed samples were discovered in the maize fields under organic system. In ecological farms the frequency of *F. equiseti* and *F. graminearum* was higher than in the conventional ones (62.6% and 11.6% comparing to 35.4% and 4.8%, respectively). In contrast, the conventional fields indicated a higher rate of infection for *F. avenaceum*, *F. culmorum*, and *F. tricinctum*. Comparisons between the amounts of fungal biomass in two cultivation systems revealed a significant difference for *F. equiseti* and also for *F. tricinctum* ($P = 0.05$). No statistical difference in fungal biomass was found for other *Fusarium* spp. between organic and conventional fields (Fig. 3).

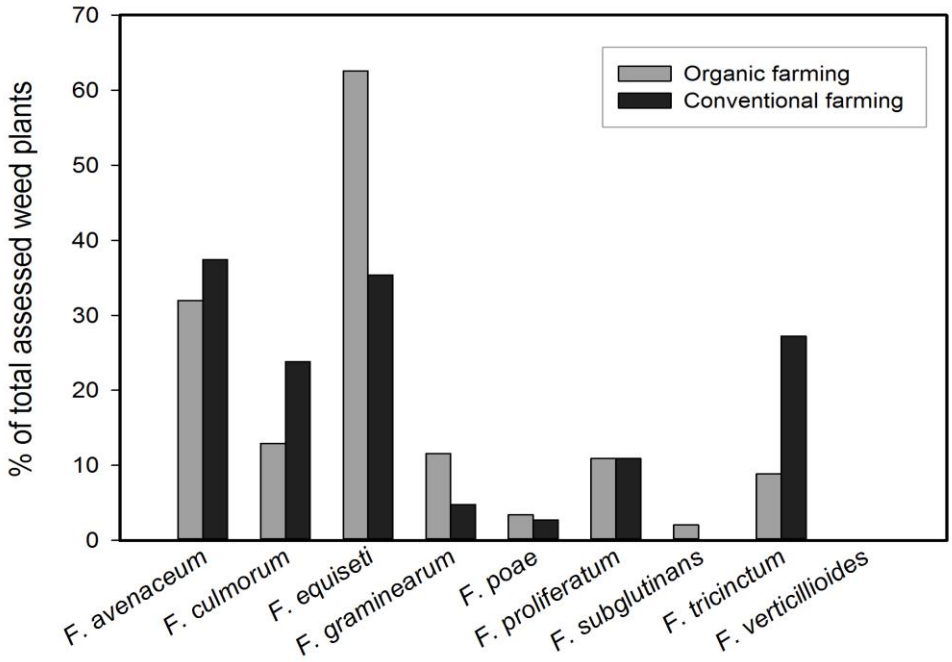

Fig. 2. Infection rate of weed plants by *Fusarium* spp. in organic and conventional maize fields

Table 2. Overview of the incidence of *Fusarium* spp. detected in 294 weeds by qPCR assay

Weed species	No. of assessed plants	% of infected plants						
		F. avenaceum	*F. culmorum*	*F. equiseti*	*F. graminearum*	*F. proliferatum*	*F. tricinctum*	Other *Fusarium*
Agropyron repens	7	29	71	71	0	58	29	14
Amaranthus retroflexus	7	57	43	43	29	0	100	0
Arabidopsis thaliana	4	25	25	25	0	0	25	0
Capselle bursa-pastoris	9	45	11	56	0	11	22	0
Chenopodium album	51	22	6	59	14	4	14	0
Echinochloa crus-galli	27	33	22	74	26	33	7	11
Galium aparine	11	9	9	73	0	0	9	0
Matricaria inodora	18	50	39	28	0	22	28	0
Polygonum aviculare	14	57	21	21	0	21	14	36
Polygonum convolvulus	30	70	20	67	10	7	37	3
Polygonum persicaria	5	40	0	40	20	0	0	20
Stellaria media	13	31	8	46	0	8	8	8
Veronica spp.	7	57	14	86	0	29	14	0
Viola arvensis	20	35	25	25	0	10	20	0
Other weed species	71	21	16	35	6	3	10	0

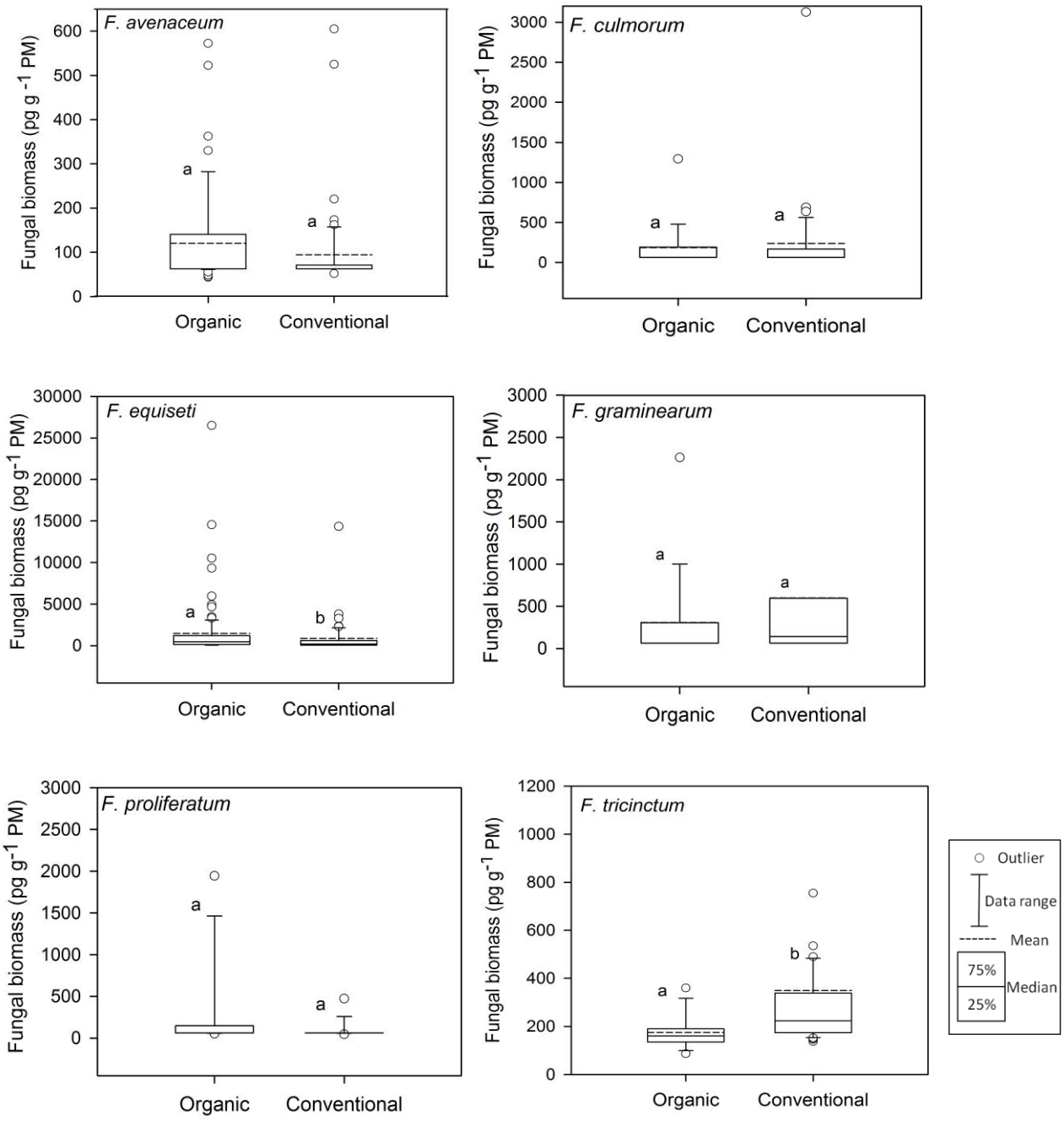

Fig. 3. Fungal biomass in weeds sampled from organic and conventional maize fields. Statistical differences within two cropping systems (Mann-Whitney U test, P = 0.05) are labeled with letters ("a" and "b"). PM= Plant material. *F. poae* and *F. subglutinans* are not constructed; because of low numbers of infected weed plants.

Fungal Isolation and Identification

146 isolates recovered from different weed species were distributed to nine species of *Fusarium*, based on the morphological and molecular identification. Seven isolates were only identified to genus *Fusarium* level and excluded from further experiments. Distribution of isolated *Fusarium* spp. from common weed species of maize farms is presented in Table 3. Occasionally, more than one species were isolated from an identical weed sample. 50 (32.7%) isolates of 153 *Fusarium* isolates were identified as *F. equiseti*. *F. avenaceum* included 31 isolates which was about 21% of total isolation. This was followed by *F. oxysporum* (16.3%), *F. venenatum* (8.5%), *F. culmorum* (7.8%) and *F. tricinctum* (5.2%). Less prevalently recovered species comprise *F. graminearum* (3.3%) and *F. proliferatum* (1.3%). We could not obtain *F. poae* and *F. subglutinans* from the few infected weed plants. Among recovered isolates, the strains of *F. avenaceum* RD22 and RD98, *F. culmorum* RD100 and RD102, *F. graminearum* RD92 and *F. proliferatum* RD94 were selected for further experiments in the greenhouse.

Table 3. Distribution of *Fusarium* spp. isolated from common weeds of maize fields studied

Weed species	Plant	Detected by qPCR	Additional recovered species
Amaranthus retroflexus	1	**ave**, **cul**, tri, **equ**	oxy
	2	**tri, equ**	
	3	**ave**, tri	
Capsella bursa-pastoris	1	equ	ven
	2	**ave**	
	3	**equ**	oxy
Chenopodium album	1	**cul**	
	2	**ave, tri, equ**	oxy
	3	**pro, ave, equ**	
	4	**ave, equ**	oxy
	5	**equ**	oxy
	6	**gra, equ**	oxy, ven
	7	**boothii, equ**	oxy
	8	**ave, equ**	
	9	**ave**	oxy, ven
	10	**equ**	
	11	**equ**	oxy
	12	gra, **equ**	ven
	13	**equ**	oxy, ven
	14	**equ**	
	15	gra, **equ**	
	16	ave, **tri, equ**	oxy
	17	**ave, tri**	
Echinochloa crus-galli	1	**gra**, **equ**	
	2	**ave, equ**	oxy
	3	**ave,** gra, poa**, equ,** tri	ven

Table 3: continued

Weed species	Plant	Detected by qPCR	Additional recovered species
	4	**gra**, cul, poa, **equ**	
	5	cul, **equ**	oxy
	6	pro, **equ**	
	7	**ave, equ**	
	8	**ave, equ**	oxy, ven
	9	**cul**, pro	ven
Elymus repens	1	pro, **cul, ave**	oxy
	2	pro, **equ**	
	3	pro, cul, **equ**	ven
	4	**cul, equ**	
	5	cul, **ave**, poa, **equ, tri**	ven
Galium aparinae	1	**ave, equ**	
	2	**cul**, tri, **equ**	
Matricaria inodora	1	**cul, ave, tri**	
	2	**ave**	
	3	cul, **ave**	
	4	**ave, equ**	
	5	**cul**	
Polygonum convolvulus	1	**cul, ave, tri, equ**	ven
	2	**cul, ave**, tri, **equ**	oxy, ven
	3	**ave**	oxy
	4	**ave, equ**	
	5	**ave, equ**	
	6	**gra, equ**	
	7	**ave**, equ	
	8	**pro**, equ	
	9	**cul,** ave, **equ**	oxy
	10	**cul**, ave, **tri, equ**	oxy
Polygonum persicaria	1	ave, **equ**	oxy
	2	ave, **equ**	oxy
Stellaria media	1	**ave, equ,** sub, tri	oxy
	2	pro, **equ**	
	3	ave, **equ**	
Veronica spp.	1	**ave, equ**	oxy
	2	**equ**	
	3	**ave, equ**	oxy
Viola arvensis	1	ave, **equ**	oxy, ven
	2	**ave, equ**	
	3	**ave**	
	4	**equ**	

Bold letters indicate the recovered *Fusarium* species
The plant column shows number of plant samples employed for *Fusarium* isolation. It is not equal with the total number of plants sampled in this study
Abbreviations: ave: *F. avenaceum*; cul: *F. culmorum*; gra: *F. graminearum*; equ: *F. equiseti*; oxy: *F. oxysporum*; poa*: F. poae;* pro: *F. proliferatum*; sub: *F. subglutinans*; tri: *F. tricinctum*; ven: *F. venenatum*

Fusarium DNA Sequencing and Phylogenetic Analysis

To confirm morphological identification, 62 representative isolates were sequenced based on the TEF-1α gene which has been widely used for species identification. Thermal PCR conditions suggested by Geiser et al. (2004), lead to a mixture of specific and non-specific products. The situations were achieved by running a gradient PCR in the range of 53-63°C under "Hot start PCR". At the annealing temperature of 58.5°C, a single band in the size range of 610 to 653 bp was successfully amplified from all isolates. After DNA sequencing, each unknown *Fusarium* strain was identified according to the deposited sequences in Fusarium-ID database which had a high homology (≥ 99%) with them. The first three hits for the sequence of each isolate have been assigned in Supplemental Table 2. The studied sequences were, then, aligned with TEF-1α sequences of *F. acuminatum*, *F. arthrosporioides*, *F. avenaceum*, *F. culmorum*, *F. flocciferum*, *F. graminearum*, *F. torulosum*, *F. tricinctum*, and *F. venenatum* retrieved from sequence database (Supplemental Table 1) and exposed to phylogenetic analysis (Fig. 4, 5, 6). The UPGMA dendrogram clearly clustered distinct clades regarding to *Fusarium* spp. studied. Identification was well supported with bootstrap values. The studied strains, therefore, were placed into groups matching with those determined by Fusarium-ID database. The strain RD99 which was identified morphologically as *F. graminearum*, made a distinct branch close to *F. graminearum*. Fusarium-ID database identified this strain as *F. boothii*. Furthermore, the isolates morphologically identified as *F. equiseti* were grouped in three different haplotypes of *Fusarium incarnatum-equiseti* species complex (FIESC) based on the molecular identification. Similarly, isolates identified morphologically as *F. oxysporum* placed in three different sub-clades of *Fusarium oxysporum* species complex (FOSC).

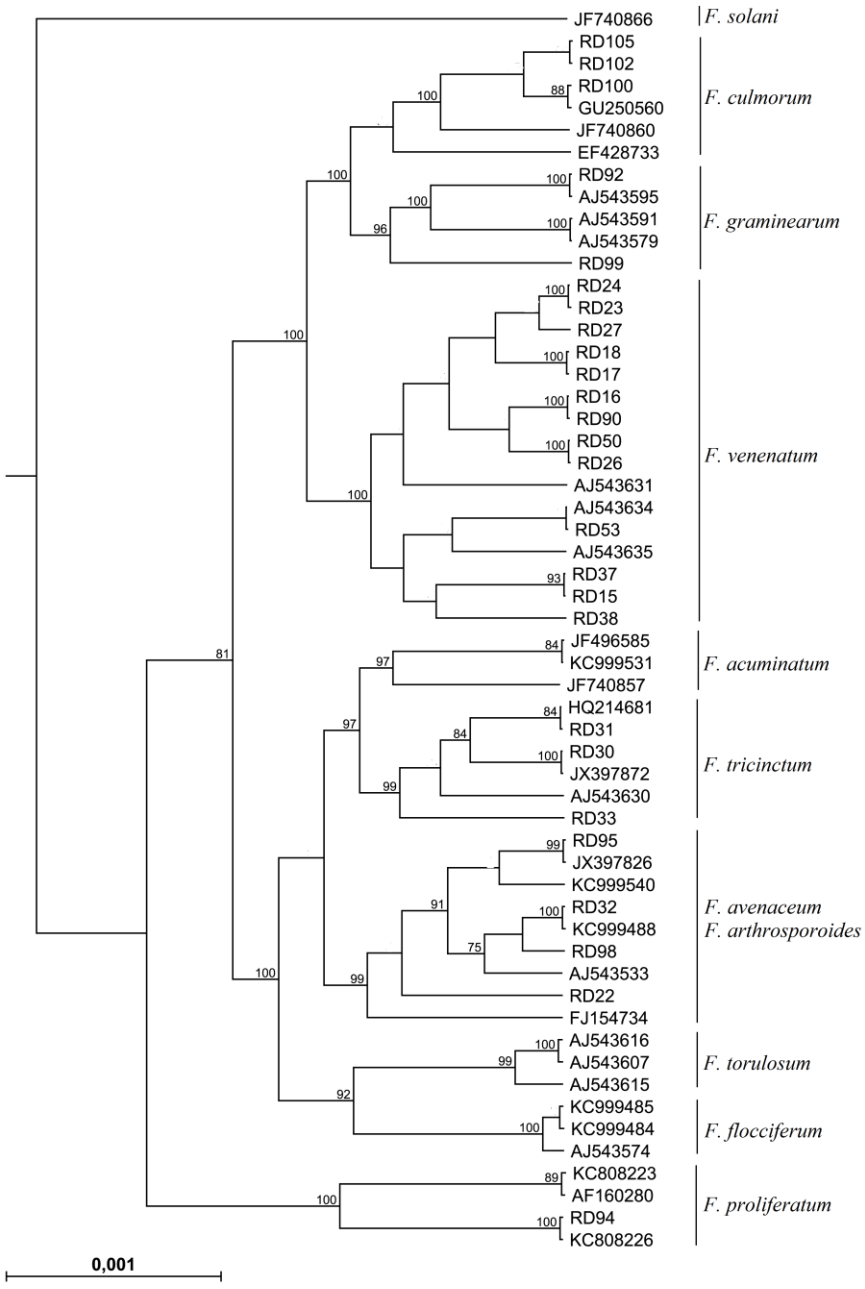

Fig. 4. UPGMA tree showing the relationship of *Fusarium* isolates inferred from TEF-1α gene using Juckes-Cantor method. Numbers associated with each node indicate the proportion of 1000 bootstrap samples in which the certain clade was found. Only percentages ≥75% are shown. *F. solani* JF740866 is used as outgroup to root the tree. Isolates with RD label have been identified in this study and the rest have been retrieved from the GenBank.

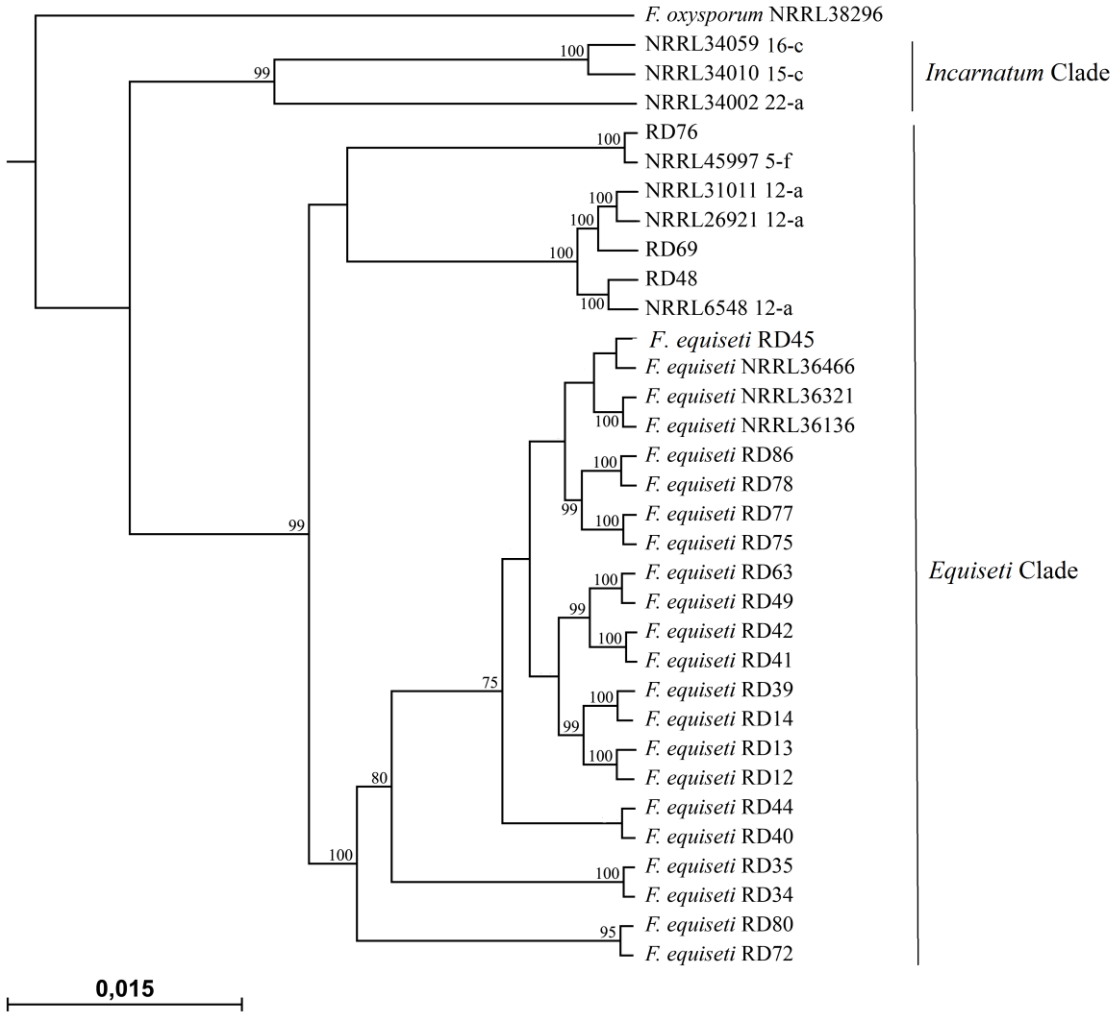

Fig. 5. UPGMA dendrogram for *Fusarium incarnatum-equiseti* species complex (FIESC) based on the nucleotide sequence of TEF-1α gene. Only bootstrap values ≥75% are presented. *F. oxysporum* is used as outgroup. Isolates with RD label have been identified in this study and the rest have been retrieved from the Fusarium-ID database.

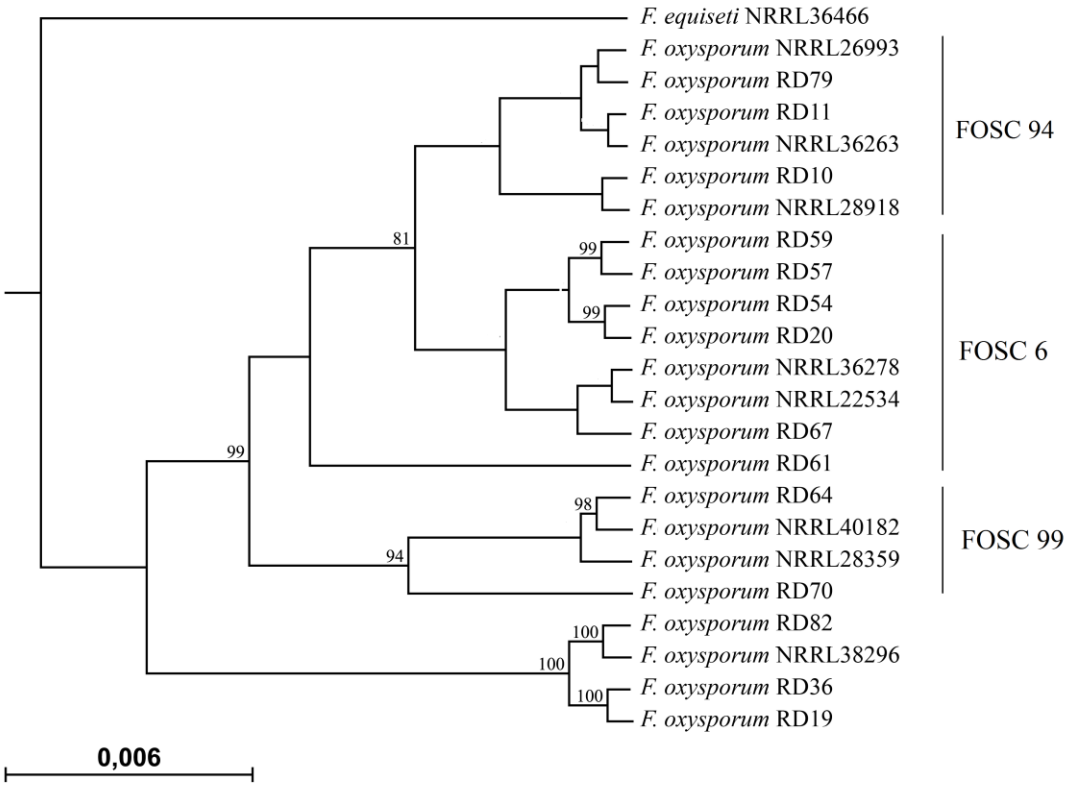

Fig. 6. Phylogenetic tree for isolates identified as *Fusarium oxysporum* species complex (FOSC) based on the nucleotide sequence of TEF-1α gene. The bootstrap values ≥75% are indicated next to the branches. *F. equiseti* is used as outgroup. Isolates with RD label have been identified in this study and the rest have been retrieved from the Fusarium-ID database.

Greenhouse Experiments

Inoculation of recovered *Fusarium* spp. on common weed species and also maize plant was performed under greenhouse conditions. No obvious symptom was observed on any of the weeds or maize plants. Therefore, quantity of fungal DNA in plant tissues was considered as a marker for finding of *Fusarium* presence in samples tested. Attendance of *Fusarium* in tissues was checked by real time PCR. The results of fungal biomass measurement in inoculated plant materials are demonstrated in Table 4. The *Fusarium* spp. were not detected in any of the control plants. Routine pathogen re-isolation from different parts of some samples confirmed the plant systemic infection. Table 4 shows the weeds that were identified as the host plants for *Fusarium* spp. in maize fields.

Table 4. Determination of fungal biomass (pg mg^{-1}) of inoculated weeds and maize plants

Weed species	*F. avenaceum*	P/R	*F. culmorum*	P/R	*F. graminearum*	P/R	*F. proliferatum*	P/R
Amaranthus retroflexus	nd	0/5	nd	0/5	189 ± 88	5/5	nd	0/5
Beta vulgaris	nd	0/5	181 ± 120	3/4	171 ± 50	4/5	nd	0/5
Capselle bursa pastoris	70 ± 62	5/5	182 ± 124	4/5	nd	0/5	<LOQ	3/5
Chenopodium album	290 ± 542	5/5	183 ± 126	4/5	157 ± 102	5/5	55 ± 31	4/5
Echinochloa crus-galli	90 ± 78 *	4/5	190 ± 45 *	4/5	151 ± 58	4/5	51 ± 20	4/5
Galium aparine	160 ± 31 *	3/3	<LOQ	3/3	nd	0/3	nd	0/3
Matricaria inodora	108 ± 59	4/5	371 ± 40	3/5	nd	0/5	17 ± 4 *	4/5
Polygonum convolvulus	86 ± 83	3/3	nd	0/1	161 ± 74	3/3	nd	0/1
Polygonum persicaria	135 ± 61	3/3	nd	0/3	171 ± 25	3/3	nd	0/1
Veronica persica	301 ± 58 *	4/5	196 ± 55	4/5	nd	0/5	52 ± 32	3/5
Viola arvensis	<LOQ	4/5	185 ± 132	3/3	nd	0/1	24 ± 22 *	3/5
Zea mays	112 ± 87	4/4	171 ± 6	4/4	165 ± 17	3/3	61 ± 27	5/5

Samples with values less than LOQ were considered as positive with the quantity equal LOQ/2
LOQ for *F. avenaceum*, *F. culmorum*, *F. graminearum* and *F. proliferatum*, respectively: 50 pg mg^{-1}, 150 pg mg^{-1}, 150 pg mg^{-1} and 16.7 pg mg^{-1}
Abbreviations: P: number of positive samples; R: number of replications; nd: not detected; *: new hosts for the relevant *Fusarium* species

Determination of Mycotoxins Content

The content of mycotoxins was measured for the common weeds of maize fields. The results are summarized in Table 5. Trichothecenes, fumonisins and also zearalenone were not detected in any of the weeds sampled, whereas occurrence of beauvericin and enniatins was common. High incidence rates of contamination for toxins, beauvericin and enniatins were observed in some of the weed plant samples, while others contained low or no toxin. For example, regarding to *Chenopodium album*, only one plant sample showed high level (>5 mg kg^{-1}) of enniatins and four samples out of 30 were contaminated with high amount of beauvericin (Fig. 7). As it is presented in Table 4, high values (up to 66 mg kg^{-1}) of beauvericin were recorded in some of the plant samples belonging to *Matricaria inodora* and *Chenopodium album*. Similarly, potentially harmful levels (up to 24 mg kg^{-1}) of enniatins were observed in *Chenopodium album* and *Elymus repens*. The relationship between fungal biomass and mycotoxin content was investigated. There was no apparent correlation between enniatins content and fungal biomass determined with all enniatins-producing species tested (*F. avenaceum*, *F. poae*, *F. proliferatum*, and *F. tricinctum*). No clear trend was found for beauvericin, as well.

Tabel 5. An overview of mycotoxins concentration in common weeds of maize fields

Weed species	% of positive samples		Median of positive samples (ng g^{-1})		Max value (ng g^{-1})	
	BEA	Enns	BEA	Enns	BEA	Enns
Chenopodium album	93	100	174	67	45,000	24,000
Echinochloa crus-galli	91	100	55	116	5,700	4,900
Galium aparine	87	88	79	16	190	230
Matricaria inodora	93	71	5	15	66,000	68
Polygonum aviculare	100	89	12	68	1,400	1,500
Polygonum convolvulus	100	92	54	67	750	4,200
Viola arvensis	64	93	27	158	530	4,500

Only the weed species which had more than 8 plant samples are shown.
Half of the LOQ was considered as value for the samples containing toxin lower than LOQ.
For all mycotoxins, the amount of LOQ and LOD were 10 ng g^{-1} and 3 ng g^{-1}, respectively.
Abbreviations: BEA: beauvericin; Enns: enniatins

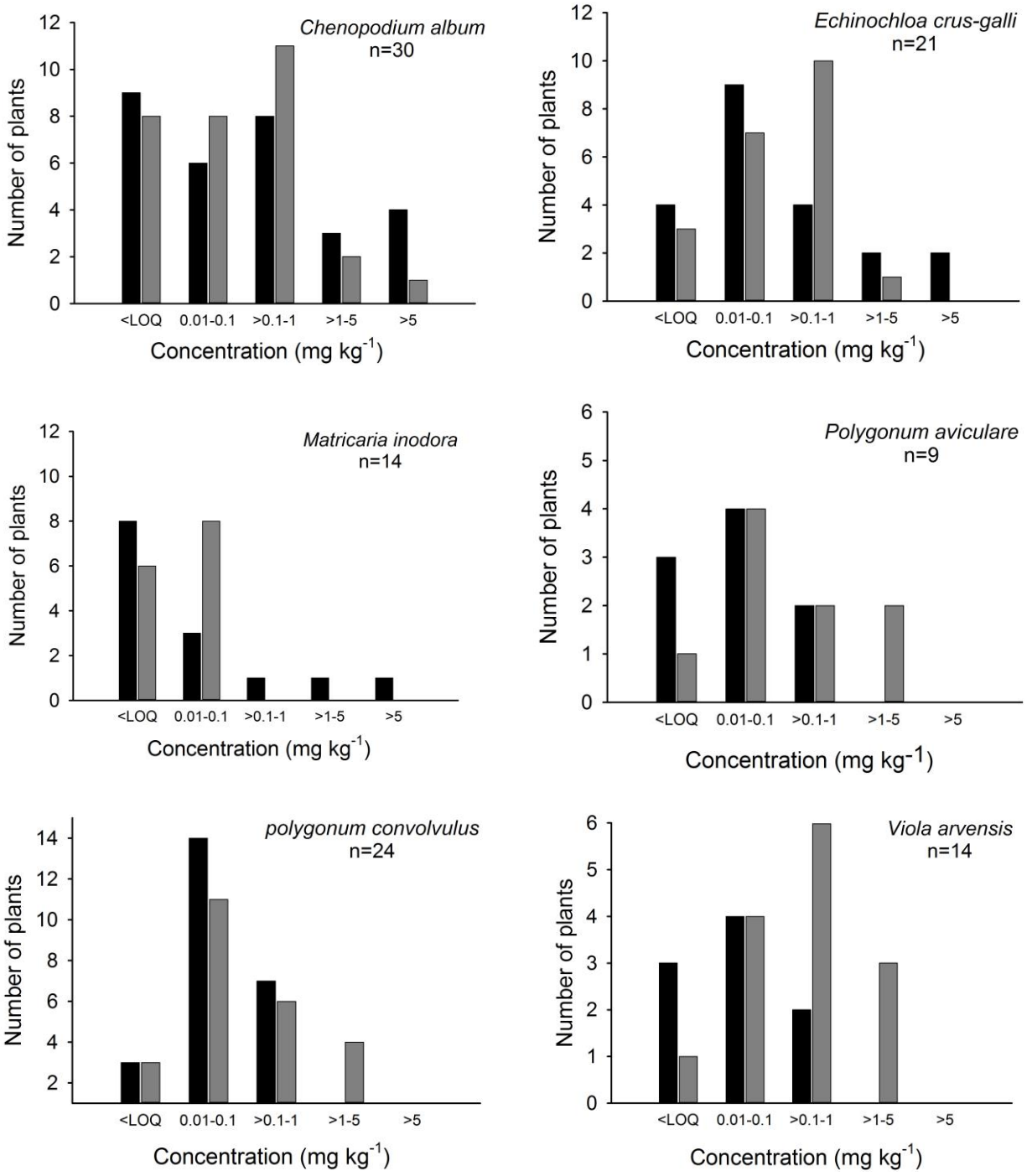

Fig. 7. Concentration of beauvericin and enniatins in common weed species of maize fields. Black bars and gray bars show beauvericin and enniatins concentrations, respectively. n: total number of plant samples tested.

Discussion

A high biodiversity of *Fusarium* species associated with maize diseases are demonstrated. In this research, the qPCR assay successfully detected eight species of *Fusarium* in weeds sampled from maize farms. Morphological and molecular characteristics also confirmed the presence of eight different species, whereas *F. equiseti* and *F. avenaceum* were predominant and most frequently found in common weeds of the maize fields. This finding triggers the preliminary assumption that these two species are the most abundant *Fusarium* in the northern parts of Germany which may help to incidence of maize *Fusarium* diseases. Different reports confirm *F. avenaceum* as a common species associated with cereals in Europe while *F. equiseti* has less frequently (Logrieco et al., 2002; Yli-Mattila, 2010; Kosiak et al., 2003). Environmental and agronomic factors affect the occurrence of *F. avenaceum* on cereals (Gäfenhan et al., 2013). *F. tricinctum* which is another important species in cereals of Northern Europe (Yli-Mattila, 2010) was detected in 18% of total assessed weed samples. Regarding to *F. graminearum* described as the most important *Fusarium* in Central and Northern Europe, although the reports show replacement of this species with *F. culmorum* (Yli-Mattila, 2010), our study revealed a higher incidence of *F. culmorum* (18%) in weeds than *F. graminearum* (8%). We could not recover *F. poae* and *F. subglutinans* which were detected in very low frequency (3% and 1%, respectively) by qPCR detection. *F. oxysporum* has been demonstrated from maize and other cereal crops as a less common species (Logrieco et al., 2002; Kosiak et al., 2003). In our sampling this species was isolated from most common weed plants. *F. venenatum* which has been sporadically recovered from maize tissues (Logrieco et al., 2002) was isolated from six different weed species. Therefore, the biodiversity of *Fusarium* of weeds sampled was as high as reported in maize fields. In addition, prevalence of *F. avenaceum* and *F. equiseti* on almost all common weeds studied indicates that these two species are more competitive *Fusarium* for weed colonization compared to the main *Fusarium* of cereals such as *F. graminearum*. As we expected, *F. verticillioides* was not detected in any of the weeds sampled. *F. verticillioides* is a serious problem of maize production in southern parts of Germany, while our samples had been collected from the north and eastern-north. Further work, thus, is needed to find the role of weeds in survival of this species as well as in fumonisin production. The frequent detection of *Fusarium* spp. on weed plants raised the importance of their role in the survival of *Fusarium* fungus.

The diversity of *Fusarium* species was similar in two farming types. Comparsion of *Fusarium* population in two different cultivation systems indicated a higher incidence of *Fusarium* spp., not necessarily significant, among organic fields except *F. avenaceum*, *F. culmorum* and *F. tricinctum*. The comparison of fungal biomass for six *Fusarium* species (out of eight) revealed no significant difference in aggressiveness of the fungi in two corn cropping systems. However, there was a statistical difference in fungal biomass of *F. equiseti* and *F. tricinctum* among two different farming types. *F. equiseti* had a higher level of biomass in ecological fields while *F. tricinctum* was more aggressive in conventional farms. Lack of fungicide application for the control management of *Fusarium* maize pathogens in both cropping systems have been likely leaded to the similar *Fusarium* diversity in addition to equal fungal biomass. According to Nutz & Karlovsky (unpublished) there is no significant difference between conventional and organic farming in occurrence of fusarium ear rot and level of fungal biomass. They demonstrated variety levels of infection in two cultivation methods.

Weed plants sampled from maize fields did not show any disease symptoms what was obviously evidenced in inoculation experiments in the greenhouse. Furthermore, the *Fusarium* strains were recovered from surface sterilized weed segments in both field and greenhouse samples. This issue confirmed that the isolates have originated from inner parts of the contaminated weeds. Symptomless colonization of weed plants has been observed by several *Fusarium* spp. (Haware & Nene, 1982; Clark and Watson 1983; Jenkinson & Parry, 1994; Postic et al., 2012; Altinok, 2013). Lack of visible symptoms on the weed plants and their re-isolation would suggest that the infection of these hosts by *Fusarium* species could be endophytic.

In this study, weeds were clearly shown to harbor different species of *Fusarium*. To our knowledge, some of the weed species demonstrated in this study (Table 4) are new alternative hosts and have not been reported for the relevant *Fusarium* species. Our results increased the possible role of weeds in the survival of fungus and production of primary inoculum in maize fields. In the absence of susceptible host plants, *Fusarium* spp. can survive in weeds as an alternative hosts and increase the inoculum potential for infecting the main crop during the growing season. This is much more expected for the species such as *F. poae* or *F. subglutinans* which are not able to survive saprotrophically on crop debris by producing resting spores (Jenkinson & Parry, 1994). Although plant residues produce the main source of

inoculum in maize fields (Cotten & Munkvold, 1998), different studies also show survival of *Fusarium* species on non-host plants such as wild plants and weeds (Jenkinson & Parry, 1994; Inch & Gilbert, 2003; Pereyra & Dill-Macky, 2008; Postic et al., 2012; Altinok, 2013). As a result, for determination of the relative importance of weeds as a source of inoculum, the pathogenicity of isolated *Fusarium* should be compared to that isolates obtained from maize.

High frequency of *Fusarium* spp. was detected in *Echinochloa crus-galli, Polygonum convolvulus,* and *Chenopodium album* based on the qPCR assay. These plants were the main weed species reported from maize fields sampled (Westerman & Gerowitt, 2012). The study also pointed out very low incidence of *Fusarium* infection in some weed species. For example, the only *Fusarium* species detected in *Cirsium arvense* L., *Sinapis arvensis* L., *Alopecurus myosuroides* (L.) Huds. and *Euphorbia helioscopia* L. were *F. proliferatum, F, equiseti, F. culmorum* and *F. equiseti*, respectively. Furthermore, in several weed species such as *Sonchus asper* L., *Anchusa arvensis* L., *Papaver rhoeas* L., *Solanum nigrum* L., *Geranium dissectum* L., *Lolium* sp. and *Erysimum cheirathoides* L. no *Fusarium* infection was observed. Number of relevant weed samples was not enough for resulting. Thus, the relative importance of the same above mentioned weeds as *Fusarium* hosts or inoculum source in maize fields cannot be determined from this work.

DNA sequences of genes such as TEF-1α have been widely used for supporting morphological traits of *Fusarium* species (Yli-Mattila et al., 2002; Harrow et al., 2010; Geiser et al., 2004; Hsuan et al., 2011). In the present study, molecular investigation based on the TEF-1α gene leaded to more precise identification of *Fusarium* isolates. The isolates that were grouped morphologically as *F. equiseti* were placed in FIESC according to the closest match of BLAST search analysis using Fusarium-ID database. A high level of genetic diversity has been observed in FIESC (O'Donnell et al., 2009a). This species complex with two clades (*Incarnatum* and *Equiseti*) has been included 62 sub-clades in each one to eight isolates are introduced. Currently reports have revealed 28 different genetic lineages in FIESC while Latin binomials were applied with confidence to only three of the species and others were identified with the English numerals (O'Donnell et al., 2009a). In our work, 22 isolates which have morphologically been identified as *F. equiseti* showed a high identity to the sub-clades of FIESC 14-a, FIESC 12-a, and FIESC 5-f (Supplemental Table 2). FIESC 14-a has been named *F. equiseti* while the next two

sub-clades have still no Latin name (O'Donnell et al., 2009a). The percentage of similarity of the isolates belonged to FIESC ranged from 99.06-99.84%. The possible reasons for not perfect matching can be the occurrence of allelic variant, the existence of a new species, no sequence representative in the database or poorly defining of the query sequence. In some cases, all known isolates of a pathogen have identical TEF-1α sequence; but variation among TEF-1α alleles within some species may vary (Geiser et al., 2004). Similarly, the isolates morphologically identified as *F. oxysporum* placed in three different sub-clades of FOSC. Fusarium-ID database introduce 256 sub-caldes in this *Fusarium* complex. Based on the sequencing homology, strain RD61 was identified as a member of FOSC6; but molecular phylogenetic analysis grouped it in a distinct branch close to FOSC6. More investigation with other DNA sequences or mycotoxin profile is necessary for finding the exact place of this strain in FOSC.

Furthermore, based on the morphological assessment strain RD99 was identified as *F. graminearum*; but it was placed in a distinct branch close to *F. graminearum*. The TEF-1α sequence of this strain indicated 99.68% identity with *F. boothii* NRRL29105. *F. boothii* (= *F. graminearum*, lineage 3) was formerly described as one of the nine species lineages within the *F. graminearum* clade. These two species are morphologically similar, but have slightly different conidia. Recently, thirteen species lineage of *F. graminearum* clade have been supported through the genealogical concordance and phylogenetic analysis of DNA sequences of nuclear genes (O'Donnell et al., 2000, 2004, 2008; Starkey et al., 2007).

According to the morphological characters (orange color sporodochia and pyriform microconidia) and the results of species-specific qPCR assay the strains RD22, RD32, RD95 and RD98 were identified as *F. avenaceum*. In UPGMA dendrogram, constructed with TEF-1α sequences from *F. avenaceum* and closely related species, the reference strains of *F. arthrosporioides* did not form completely separated group (Fig. 4). They were nested within *F. avenaceum* strains. The previous studies with the combined IGS and ß-tubulin sequences data could not distinguish *F. avenaceum* and *F. arthrosporioides* strains to form separate groups (Yli-Mattila et al., 2002). However, investigations based on the ATP Citrate Lyase (acl1) and TEF-1α gene sequences were able to form a distinct lineage for *F. arthrosporioides* in the *F. avenaceum* main clade (Gräfenhan et al., 2013).

Common weeds were assessed for the main toxin contents by HPLC-MS/MS. Lack of trichothecenes, zearalenone and fumonisin contamination in weed samples containing their producers, made the assumption that these fungi are not active for toxin production in wild host plants. We suppose that the production of toxins by some *Fusarium* strains may be affected by inappropriate environmental factors. Uhlig et al. (2007) demonstrated that field situations in Northern Europe are not favor for moniliformin production while enniatins are frequently detected in cereal grains. On the other hand, poor performance of *Fusarium* pathogens such as *F. graminearum* on weeds (Akinsanmi et al., 2007), may explain the lack of symptoms and mycotoxin production on these alternative hosts. In this study, DON and NIV were not detected in weed samples highly contaminated with *F. equiseti*. There are controversial reports regarding DON and NIV production by *F. equiseti* strains (Kosiak et al., 2005; Marín et al., 2012). Therefore, based on our knowledge of weeds, this question is still remained that whether *F. equiseti* strains harbored in weeds have potential contribution for rising of contamination risk of the DON and NIV in cereal grains.

Whereas, *F. equiseti* and *F. avenaceum* were found in 49% and 35% of all samples, respectively, beauvericin and enniatins content were also determined in weed samples. The results indicated that weeds were contaminated with beauvericin at the levels ranging from 65621 ng g^{-1} to 5 ng g^{-1} plant tissues. Beauvericin as a toxic contaminant has been reported from maize ears infected with *Fusarium* pathogen (Logrieco et al., 1993; Bottalico et al., 1995; Ritieni et al., 1997; Logrieco et al., 1995; Yli-Mattila, 2010). The values for enniatins contamination were in the range of 24236 ng g^{-1} to 5 ng g^{-1}. Previous analytical surveys have indicated the contamination of cereal grains by enniatins resulting of *F. avenaceum*. Single weed samples showed extremely high level of toxins. There was no significant correlation between mycotoxin level and *Fusarium* DNA. High level of fungal DNA in some beauvericin/enniatin-free samples or beauvericin/enniatin-low samples revealed that some strains of fungal producers were not able to yield relevant toxins. On the other hand, high contamination of both toxins with low levels of fungal genomic DNA can be explained by the presence of other *Fusarium* species such as *F. acuminatum, F. sambucinum, F. venenatum* which are able to produce related toxins.

In summary, the present results demonstrat the frequent occurrence of *Fusarium* species in weed plants. Large number of recovered *Fusarium* spp. can support the role of weeds as

symptomless hosts for *Fusarim* species. The possible importance of weeds in increasing of the pressure of primary inoculum in maize fields raises the risk of toxin contamination in cereal grains. Although the main toxins were not detected in weed samples, the significance of *Fusarium* strains, harbored in weeds, for mycotoxin production in primary host plants should not be disregarded. These findings indicate that weed management in maize fileds would aim to reduce *Fusarium* inoculum and assist in the control of *Fusarium* maize diseases.

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Authors' Contributions

In this study, the weed samples were collected and identified by Prof. Dr. Bärbel Gerowitt and Dr. Horst-Henning Steinmann. The mycotoxin experiments were carried out by Dr. Katharina Döll.

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Supplemental Table 1. Discription of *Fusarium* accessions for TEF-1α sequence used in this study

Accession No.	Strain	Species	Reference
JF496585	H2RA.033	*F. acuminatum*	Marín et al., 2012
JF740857	NRRL52789	*F. acuminatum*	O'Donnell et al., 2012
KC999531	S4B-10-15	*F. acuminatum*	Gräfenhan et al., 2013
JX397826	BBA:67701	*F. arthrosporioides*	Niessen et al., 2012
KC999488	BBA:64134	*F. arthrosporioides*	Gräfenhan et al., 2013
AJ543533	VI01199	*F. avenaceum*	Kristensen et al., 2005
FJ154734	R-3053	*F. avenaceum*	Nalim et al., 2009
KC999540	S7A-10-15	*F. avenaceum*	Gräfenhan et al., 2013
EF428733	NRRL34698	*F. culmorum*	Ward et al., 2008
GU250560	NRRL46661	*F. culmorum*	Balmas et al., 2010
JF740860	NRRL52792	*F. culmorum*	O'Donnell et al., 2012
	NRRL6548	FIESC[a] 12-a[b]	O'Donnell et al., 2009a
	NRRL26921	FIESC 12-a	O'Donnell et al., 2009a
	NRRL31011	FIESC 12-a	O'Donnell et al., 2009a
	NRRL34002	FIESC 22-a	O'Donnell et al., 2009a
	NRRL34010	FIESC 15-c	O'Donnell et al., 2009a
	NRRL34059	FIESC 16-c	O'Donnell et al., 2009a
	NRRL36136	FIESC 14-a	O'Donnell et al., 2009a
	NRRL36321	FIESC 14-a	O'Donnell et al., 2009a
	NRRL36466	FIESC 14-a	O'Donnell et al., 2009a
	NRRL45997	FIESC 5-f	O'Donnell et al., 2009a
AJ543574	VI02440	*F. flocciferum*	Kristensen et al., 2005
KC999484	F-424	*F. flocciferum*	Gräfenhan et al., 2013
KC999485	F5-89	*F. flocciferum*	Gräfenhan et al., 2013
AJ543579	VI01020	*F. graminearum*	Kristensen et al., 2005
AJ543591	VI01177	*F. graminearum*	Kristensen et al., 2005
AJ543595	VI01182	*F. graminearum*	Kristensen et al., 2005
FJ985267	NRRL22534	*F. oxysporum* f.sp. *apii*	O'Donnell et al., 2009b
AF246846	NRRL26993	*F. oxysporum* f.sp. *gladioli* race lit	Baayen et al., 2000
AF246856	NRRL28359	*F. oxysporum*	Baayen et al., 2000
AF246874	NRRL28918	*F. oxysporum* f.sp. *gladioli*	Baayen et al., 2000
	NRRL36263	*F. oxysporum* f.sp. *gladioli*	
	NRRL36278	*F. oxysporum*	
FJ985372	NRRL38296	*F. oxysporum* f.sp. *betae*	O'Donnell et al., 2009b
	NRRL40182	*F. oxysporum*	
KC808223	NRRL62546	*F. proliferatum*	O'Donnell et al., 2013, unpublished
KC808226	NRRL54994	*F. proliferatum*	O'Donnell et al., 2013, unpublished
AF160280	NRRL22944	*F. proliferatum*	O'Donnell et al., 2000
FJ740866[c]	NRRL52798	*F. solani*	O'Donnell et al., 2012
AJ543607	VI01184	*F. torulosum*	Kristensen et al., 2005
AJ543615	VI01424	*F. torulosum*	Kristensen et al., 2005
AJ543616	VI01194	*F. torulosum*	Kristensen et al., 2005
HQ214681	TTAz1a	*F. tricinctum*	Castanares et al., 2011
JX397872	DAOM:235659	*F. tricinctum*	Niessen et al., 2012
AJ543630	VI01423	*F. tricinctum*	Kristensen et al., 2005
AJ543631	VI01412	*F. venenatum*	Kristensen et al., 2005
AJ543634	VI01176	*F. venenatum*	Kristensen et al., 2005
AJ543635	VI01179	*F. venenatum*	Kristensen et al., 2005

[a.] *Fusarium incarnatum-equiseti* species complex
[b.] English numeral identify species and small letters identify unique haplotypes within each species
[c.] Sequence of *F. solani* FJ740866 was used to root the phylogeny (Fig. 4)

Supplemental Table 2. Species identity of 62 *Fusarium* isolates based on morphological characters and TEF-1α sequences

Isolate	GenBank accession No.	*Fusarium* spp.	Host	Location in Germany	Year	Accessions for the first three matched hits in Fusarium-ID database	Related *Fusarium* spp.	Similarity (%)[a]
RD22	KJ652275	*F. avenaceum*	*Polygonum convolvulus*	Koblentz	2010	NRRL31101	*F. avenaceum*	98.16
						NRRL25472	*Fusarium* sp cf. *acuminatum*	97.23
						NRRL20682	*F. negundis*	95.14
RD32	KJ652276	*F. avenaceum*	*Chenopodium album*	Dummers-torf	2010	NRRL31101	*F. avenaceum*	99.23
						NRRL25472	*Fusarium* sp cf. *acuminatum*	97.38
						NRRL20682	*F. negundis*	95.88
RD95	KJ652277	*F. avenaceum*	*Chenopodium album*	Niex	2010	NRRL31101	*F. avenaceum*	99.38
						NRRL25472	*Fusarium* sp cf. *acuminatum*	97.53
						NRRL20682	*F. negundis*	95.72
RD98	KJ652278	*F. avenaceum*	*Chenopodium album*	Kremlin	2010	NRRL31101	*F. avenaceum*	99.07
						NRRL25472	*Fusarium* sp cf. *acuminatum*	97.23
						NRRL20682	*F. negundis*	95.72
RD100	KJ652269	*F. culmorum*	*Chenopodium album*	Niex	2010	NRRL3288	*F. culmorum*	99.83
						NRRL25475	*F. culmorum*	99.83
						NRRL25475	*F. culmorum*	99.83
RD102	KJ652270	*F. culmorum*	*Polygonum convolvulus*	Püggen	2010	NRRL3288	*F. culmorum*	99.83
						NRRL25475	*F. culmorum*	99.83
						NRRL25475	*F. culmorum*	99.83
RD105	KJ652271	*F. culmorum*	*Polygonum convolvulus*	Niex	2010	NRRL3288	*F. culmorum*	99.83
						NRRL25475	*F. culmorum*	99.83
						NRRL25475	*F. culmorum*	99.83
RD12	KJ652292	*F. equiseti*	*Chenopodium album*	Niex	2010	NRRL36466	FIESC[b]14-a[c]	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD13	KJ652293	*F. equiseti*	*Chenopodium album*	Püggen	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD14	KJ652294	*F. equiseti*	*Chenopodium album*	Püggen	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD34	KJ652295	*F. equiseti*	*Elymus repens*	Göttingen	2011	NRRL36466	FIESC 14-a	99.53
						NRRL36321	FIESC 14-a	99.53

Supplemental Table 2: continued

Isolate	GenBank accession No.	*Fusarium* spp.	Host	Location in Germany	Year	Accessions for the first three matched hits in Fusarium-ID database	Related *Fusarium* spp.	Similarity (%)[a]
						NRRL36136	FIESC 14-a	99.53
RD35	KJ652296	*F. equiseti*	*Elymus repens*	Rusch-Raduhn	2010	NRRL36466	FIESC 14-a	99.53
						NRRL36321	FIESC 14-a	99.53
						NRRL36136	FIESC 14-a	99.53
RD39	KJ652297	*F. equiseti*	*Chenopodium album*	Dummers-torf	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD40	KJ652298	*F. equiseti*	*Amaranthus retroflexus*	Göttingen	2011	NRRL36466	FIESC 14-a	99.68
						NRRL36321	FIESC 14-a	99.68
						NRRL36136	FIESC 14-a	99.68
RD41	KJ652299	*F. equiseti*	*Polygonum convolvulus*	Koblentz	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD42	KJ652300	*F. equiseti*	*Chenopodium album*	Sallahn	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD44	KJ652301	*F. equiseti*	*Amaranthus retroflexus*	Göttingen	2011	NRRL36466	FIESC 14-a	99.68
						NRRL36321	FIESC 14-a	99.68
						NRRL36136	FIESC 14-a	99.68
RD45	KJ652302	*F. equiseti*	*Chenopodium album*	Sallahn	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD48	KJ652303	*Fusarium* sp.	*Echinochloa crus-galli*	Püggen	2010	NRRL31011	FIESC 12-a	99.84
						NRRL26921	FIESC 12-a	99.84
						NRRL6548	FIESC 12-a	99.84
RD49	KJ652304	*F. equiseti*	*Echinochloa crus-galli*	Püggen	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD63	KJ652305	*F. equiseti*	*Polygonum convolvulus*	Püggen	2010	NRRL36466	FIESC 14-a	99.68
						NRRL36321	FIESC 14-a	99.68
						NRRL36136	FIESC 14-a	99.68
RD69	KJ652306	*Fusarium* sp.	*Polygonum persicaria*	Rusch-Raduhn	2010	NRRL31011	FIESC 12-a	99.84
						NRRL26921	FIESC 12-a	99.84
						NRRL6548	FIESC 12-a	99.84

Supplemental Table 2: continued

Isolate	GenBank accession No.	*Fusarium* spp.	Host	Location in Germany	Year	Accessions for the first three matched hits in Fusarium-ID database	Related *Fusarium* spp.	Similarity (%)[a]
RD72	KJ652307	*F. equiseti*	*Veronica* sp.	Niex	2010	NRRL36466	FIESC 14-a	99.06
						NRRL36321	FIESC 14-a	99.06
						NRRL36136	FIESC 14-a	99.06
RD75	KJ652308	*F. equiseti*	*Veronica* sp.	Niex	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD76	KJ652309	*Fusarium* sp.	*Veronica* sp.	Sallahn	2010	NRRL45997	FIESC 5-f	99.68
						NRRL45995	FIESC 5-b	99.37
						NRRL34037	FIESC 5-b	99.37
RD77	KJ652310	*F. equiseti*	*Veronica* sp.	Sallahn	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD78	KJ652311	*F. equiseti*	*Veronica* sp.	Püggen	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD80	KJ652312	*F. equiseti*	*Polygonum convolvulus*	Niex	2010	NRRL36466	FIESC 14-a	99.21
						NRRL36321	FIESC 14-a	99.21
						NRRL36136	FIESC 14-a	99.21
RD86	KJ652313	*F. equiseti*	*Polygonum convolvulus*	Püggen	2010	NRRL36466	FIESC 14-a	99.84
						NRRL36321	FIESC 14-a	99.84
						NRRL36136	FIESC 14-a	99.84
RD92	KJ652267	*F. graminearum*	*Chenopodium album*	Kremlin	2010	NRRL28336	*F. graminearum*	99.36
						NRRL5883	*F. graminearum*	99.36
						NRRL6394	*F. graminearum*	99.36
RD99	KJ652268	*F. graminearum*	*Chenopodium album*	Kremlin	2010	NRRL29105	*F. boothii*	99.68
						NRRL26916	*F. boothii*	99.68
						NRRL26916	*F. boothii*	99.68
RD10	KJ652252	*F. oxysporum*	*Chenopodium album*	Niex	2010	NRRL36263	FOSC[d] 94	99.84
						NRRL36227	FOSC 94	99.84
						NRRL28918	*F. oxysporum*	99.84
RD11	KJ652253	*F. oxysporum*	*Chenopodium album*	Sallahn	2010	NRRL36263	FOSC 94	100
						NRRL36227	FOSC 94	100
						NRRL28918	*F. oxysporum*	100
RD19	KJ652254	*F. oxysporum*	*Chenopodium album*	Dummers-torf	2010	NRRL38296	*F. oxysporum*	99.84

Supplemental Table 2: continued

Isolate	GenBank accession No.	*Fusarium* spp.	Host	Location in Germany	Year	Accessions for the first three matched hits in Fusarium-ID database	Related *Fusarium* spp.	Similarity (%)[a]
						NRRL38291	FOSC 180	99.84
						NRRL36408	FOSC 164	99.84
RD20	KJ652255	*F. oxysporum*	*Polygonum convolvulus*	Niex	2010	NRRL36278	FOSC 6	99.84
						NRRL22534	FOSC 6	99.84
						NRRL38474	FOSC 209	99.84
RD36	KJ652256	*F. oxysporum*	*Chenopodium album*	Göttingen	2011	NRRL38296	*F. oxysporum*	99.84
						NRRL38291	FOSC 180	99.84
						NRRL36408	FOSC 164	99.84
RD54	KJ652257	*F. oxysporum*	*Stellaria media*	Kremlin	2010	NRRL36278	FOSC 6	99.84
						NRRL22534	FOSC 6	99.84
						NRRL38474	FOSC 209	99.84
RD57	KJ652258	*F. oxysporum*	*Echinochloa crus-galli*	Kremlin	2010	NRRL36278	FOSC 6	99.84
						NRRL22534	FOSC 6	99.84
						NRRL38474	FOSC 209	99.84
RD59	KJ652259	*F. oxysporum*	*Elymus repens*	Niendorf	2010	NRRL36278	FOSC 6	99.84
						NRRL22534	FOSC 6	99.84
						NRRL38474	FOSC 209	99.84
RD61	KJ652260	*F. oxysporum*	*Echinochloa crus-galli*	Sallahn	2010	NRRL36278	FOSC 6	99.84
						NRRL22534	FOSC 6	99.84
						NRRL38474	FOSC 209	99.84
RD64	KJ652261	*F. oxysporum*	*Echinochloa crus-galli*	Dummers-torf	2010	NRRL40182	FOSC 99	99.84
						NRRL38476	FOSC 160	99.84
						NRRL36364	FOSC 160	99.84
RD67	KJ652262	*F. oxysporum*	*Polygonum convolvulus*	Püggen	2010	NRRL36278	FOSC 6	99.84
						NRRL22534	FOSC 6	99.84
						NRRL38474	FOSC 209	99.84
RD70	KJ652263	*F. oxysporum*	*Polygonum persicaria*	Rusch-Raduhn	2010	NRRL40182	FOSC 99	99.69
						NRRL38476	FOSC 160	99.69
						NRRL36364	FOSC 160	99.69
RD79	KJ652264	*F. oxysporum*	*Polygonum convolvulus*	Püggen	2010	NRRL36263	FOSC 94	99.84
						NRRL36227	FOSC 94	99.84
						NRRL28918	*F. oxysporum*	99.84
RD82	KJ652265	*F. oxysporum*	*Chenopodium album*	Kremlin	2010	NRRL38296	*F. oxysporum*	99.84
						NRRL38291	FOSC 180	99.84

Supplemental Table 2: continued

Isolate	GenBank accession No.	*Fusarium* spp.	Host	Location in Germany	Year	Accessions for the first three matched hits in Fusarium-ID database	Related *Fusarium* spp.	Similarity (%)[a]
						NRRL36408	FOSC 164	99.84
RD94	KJ652266	*F. proliferatum*	*Polygonum convolvulus*	Püggen	2010	Zm16 (CSIRO Entomology)	*F. proliferatum*	99.84
						Zm62 (CSIRO Entomology)	*F. proliferatum*	99.84
						Zm54 (CSIRO Entomology)	*F. proliferatum*	99.69
RD30	KJ652272	*F. tricinctum*	*Amaranthus retroflexus*	Göttingen	2011	NRRL25481	*Fusarium* sp cf. *tricinctum*	99.68
						NRRL20682	*Fusarium negundis*	97.72
						NRRL36147	FTSC[e] 2-a	97.65
RD31	KJ652273	*F. tricinctum*	*Chenopodium album*	Göttingen	2011	NRRL25481	*Fusarium* sp cf. *tricinctum*	99.68
						NRRL20682	*Fusarium negundis*	97.70
						NRRL36147	FTSC 2-a	97.65
RD33	KJ652274	*F. tricinctum*	*Chenopodium album*	Göttingen	2011	NRRL25481	*Fusarium* sp cf. *tricinctum*	99.52
						NRRL20682	*Fusarium negundis*	95.56
						NRRL36147	FTSC 2-a	97.50
RD15	KJ652279	*F. venenatum*	*Amaranthus retroflexus*	Dummers-torf	2010	NRRL22196	*F. venenatum*	98.86
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.79
						NRRL13392	*Fusarium* sp cf. *robustum*	92.38
RD16	KJ652280	*F. venenatum*	*Echinochloa crus-galli*	Sallahn	2010	NRRL22196	*F. venenatum*	99.83
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.09
						NRRL13392	*Fusarium* sp cf. *robustum*	92.89
RD17	KJ652281	*F. venenatum*	*Echinochloa crus-galli*	Püggen	2010	NRRL22196	*F. venenatum*	99.83
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.09
						NRRL13392	*Fusarium* sp cf. *robustum*	92.89
RD18	KJ652282	*F. venenatum*	*Amaranthus retroflexus*	Göttingen	2011	NRRL22196	*F. venenatum*	100
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.23
						NRRL13392	*Fusarium* sp cf. *robustum*	93.05
RD23	KJ652283	*F. venenatum*	*Amaranthus retroflexus*	Niex	2010	NRRL22196	*F. venenatum*	100
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.28
						NRRL13392	*Fusarium* sp cf. *robustum*	93.06
RD24	KJ652284	*F. venenatum*	*Chenopodium album*	Kremlin	2010	NRRL22196	*F. venenatum*	99.83
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.12
						NRRL13392	*Fusarium* sp cf. *robustum*	92.89
RD26	KJ652285	*F. venenatum*	*Agropyron repens*	Rusch-Raduhn	2010	NRRL22196	*F. venenatum*	99.83
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.12
						NRRL13392	*Fusarium* sp cf. *robustum*	92.89

Supplemental Table 2: continued

Isolate	GenBank accession No.	*Fusarium* spp.	Host	Location in Germany	Year	Accessions for the first three matched hits in Fusarium-ID database	Related *Fusarium* spp.	Similarity (%)[a]
RD27	KJ652286	*F. venenatum*	*Chenopodium album*	Dummers-torf	2010	NRRL22196	*F. venenatum*	100
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.24
						NRRL13392	*Fusarium* sp cf. *robustum*	93.05
RD37	KJ652287	*F. venenatum*	*Amaranthus retroflexus*	Niex	2010	NRRL22196	*F. venenatum*	98.87
						NRRL13392	*Fusarium* sp cf. *robustum*	92.43
						NRRL13394	*Fusarium* sp cf. *tumidum*	92.62
RD38	KJ652288	*F. venenatum*	*Amaranthus retroflexus*	Niex	2010	NRRL22196	*F. venenatum*	98.87
						NRRL13392	*Fusarium* sp cf. *robustum*	92.44
						NRRL13394	*Fusarium* sp cf. *tumidum*	92.64
RD50	KJ652289	*F. venenatum*	*Echinochloa crus-galli*	Göttingen	2011	NRRL22196	*F. venenatum*	99.83
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.11
						NRRL13392	*Fusarium* sp cf. *robustum*	92.89
RD53	KJ652290	*F. venenatum*	*Chenopodium album*	Püggen	2010	NRRL22196	*F. venenatum*	99.19
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.00
						NRRL13392	*Fusarium* sp cf. *robustum*	92.91
RD90	KJ652291	*F. venenatum*	*Chenopodium album*	Püggen	2010	NRRL22196	*F. venenatum*	99.83
						NRRL13394	*Fusarium* sp cf. *tumidum*	93.11
						NRRL13392	*Fusarium* sp cf. *robustum*	92.89

[a.] Nucleotide sequences of the gene encoding TEF-1α was compared with homologous sequences retrieved from Fusarium-ID database
[b.] *Fusarium incarnatum-equiseti* species complex
[c.] English numeral identify species and small letters identify unique haplotypes within each species
[d.] *Fusarium oxysporum* species complex
[e.] *Fusarium tricinctum* species complex

Chapter 4

Aggressiveness of *Fusarium verticillioides* Strains Differing in Ability to Produce Fumonisin in Maize, Sorghum, Rice, and Beetroot Seedlings

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Abstract

Two fumonisin-nonproducing strains of *Fusarium verticillioides* (*fum 1-3* and *fum 1-4*) and their progenitors (*FUM 1-1*) were tested for aggressiveness toward different plant species including maize, sorghum, rice and beetroot seedlings grown under greenhouse conditions. None of the plants showed obvious disease symptoms following root inoculation. The quantity of species-specific fungal biomass was measured by real-time PCR. No significant (P = 0.05) differences in colonization of tissues were detected between each wild type and the relevant mutant. *F. verticillioides* was not detected in any of the non-inoculated control plants. The fungus could transfer from the roots to the first two internodes/leaves of maize, rice/beetroot, regardless of fumonisin production. The symptomless systemic transmission rate, however, was low for sorghum. This phenomenon could be explained by different host preferences of biological species of *Gibberella fujikuroi*. The results indicate that in our system, fumonisin production was not required as a virulence factor for *F. verticillioides* to colonize the maize, rice and beetroot tissues.

Additional keywords: maize, *Fusarium verticillioides*, fumonisins

Introduction

Fumonisins are polyketide mycotoxins that contaminate commercial maize-based human foodstuffs and animal feeds worldwide. Although number species of *Fusarium*, including *F. anthophilum*, *F. fujikuroi*, *F. nygamai*, *F. oxysporum* and *F. proliferatum* produce fumonisins, one species of particular concern is *F. verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* Scheld.), teleomorph *Gibberella fujikuroi* (syn. *G. moniliformis* Wineland) (Munkvold & Desjardins, 1997). This fungus is a common ear rot pathogen of maize (*Zea mays*) and can also infect maize stalks and roots without any visible symptoms of disease (Nelson et al., 1993; Munkvold & Desjardins, 1997). Latent infections of maize stalk for extended periods of time have been demonstrated (Munkvold et al., 1997). *F. verticillioides* reduces yield and quality of contaminated kernels and also produces mycotoxins such as fumonisins. Since the discovery of fumonisins (Bezuidenhout et al., 1988), these toxins have been studied due to the presence of sphingolipid metabolism at the cellular level (Wang et al., 1991) and subsequently the impairment of animal health (Nelson et al., 1993). Fumonisin-contaminated maize causes various animal mycotoxicoses including leucoencephalomalacia in horses, pulmonary edema and hepatic syndrome in pigs, poor performance in poultry, liver cancer in rats and alteration in hepatic and immune function in cattle (reviewed in Logrieco et al., 2002). Studies have also indicated an epidemiological correlation between human esophageal cancer and consumption of fumonisin-contaminated maize (Marasas, 1995). In plants, toxicity of fumonisins is associated with inhibition of Acyl CoA-dependent ceramide synthase (Williams et al., 2006). More than 10 fumonisins have been characterized, of which fumonisin B1 (FB1) is the most abundant in naturally contaminated maize. Other B-series fumonisin homologues including FB2, FB3 and FB4 make up 10-20% of the total fumonisins in infected maize (Nelson et al., 1993). The fumonisin biosynthetic (*FUM*) gene cluster includes 16 genes (Brown et al., 2007). According genetic studies, four closely linked loci, *fum1*, *fum2*, *fum3* and *fum4* have been identified as being responsible in fumonisin biosynthesis (Desjardins et al., 1992, 1996). The lack of the correct gene at the *fum1* locus blocks fumonisin production, while strains defective at *fum2* produce FB2, but not FB1 or FB3. Similarly, changes at *fum3* could stop FB1 and FB2, but not FB3 (Desjardins et al., 1992, 1996).

There is growing evidence of the occurrence of FB1 in maize all over the world, including Europe (reviewed in Logrieco et al., 2002). In southern European countries such as Italy, *F. verticillioides* is the most frequently isolated fungus from infected maize and high levels of FB1 have been reported (reviewed in Logrieco et al., 2002). *F. proliferatum* as another source of FB1 production has been found with *F. verticillioides* in southern Europe (Logrieco et al., 1995; Bottalico et al., 1995). In contrast, the levels of FB1 in central and north-eastern European areas such as Germany had been significantly lower (Usleber et al., 1994). Furthermore, *F. verticillioides* is well-known pathogen on a variety of plants such as wheat, barley, soybean (Castella et al., 1999), sorghum (da Silva et al., 2000; dos Reis et al., 2010), rice (Bhargava et al., 1979; Cartwright et al., 1995; Kushiro et al., 2008; Maheshwar et al., 2009; Tansakul et al., 2012) and banana (Glenn et al., 2008).

Toxicity of fumonisins to plants and animals has been clearly demonstrated (Lamprecht et al., 1994), but there are controversial reports regarding the potential function of fumonisins in virulence on maize. Some indirect evidence indicates that fumonisins may play a role in maize seedling diseases caused by *F. verticillioides*. Fumonisins have similar structure to AAL toxins, which are responsible for pathogenicity of *Alternaria alternata* on tomato cultivars (Gilchrist, 1998). AAL toxin-nonproducing strains were not able to provide leaf necrosis on susceptible tomato cultivars (Akamatsu et al., 1997). Other studies have been shown decreasing shoot and root length as well as leaf necrosis symptoms of maize and tomato seedlings while exposed to low concentrations of purified fumonisins (Gilchrist et al., 1992; Lamprecht et al., 1994). Although Desjardins et al. (1995) reported the importance of fumonisin production in virulence on maize seedlings; they mentioned that fumonisin production is not necessary or sufficient to cause maize blight disease. On the other hand, the greenhouse results of Jardine & Leslie (1999) showed a fumonisin-nonproducing natural variant could infect the mature maize plants with the same aggressiveness as the fumonisin producing strain, and caused stalk rot. Fumonisin bioavailability to maize roots has been shown to cause a reduction in stalk weight and root mass and increased leaf lesions (Williams et al., 2006). These findings have supported the importance of fumonisins in plant pathogenesis. Recently, studies have shown that the expression of foliar maize diseases is associated with fumonisin production, and this toxin can contribute to all aspects of *F. verticillioides* maize seedling diseases (Williams et al., 2007; Glenn et al., 2008).

In relation to maize ear rot, Desjardins & Plattner (2000) employed three different natural FB1-nonproducing strains and compared them with fumonisin-producing (FB1, FB2, FB3) strains under field conditions. Both groups of the strains had the same capability to produce maize ear rot. The studies conducted with natural variants were improved by generating isogenic fumonisin non-producing mutants. Using these identical strains, which differ only in a fumonisin production gene, helped to demonstrate the importance of fumonisins as a virulence factor for *F. verticillioides* on maize (Desjardins et al., 2002). The results with two independent fumonisin non-producing (*fum1-3* and *fum1-4*) mutants in field tests indicated that fumonisins are not necessary to cause maize ear infection by *F. verticillioides* (Desjardins et al., 2002).

On the basis of these observations, it has been proposed that the biological function of fumonisins regarding their possible role in maize ear rot is still not completely clear (Munkvold, 2003). Fumonisins may have no real function in virulence, which is a complex physiological process; however, it is possible to affect other host plants and enhance the pathogenicity (Proctor et al., 2002). This is what has been demonstrated for trichothecenes. These toxins are the virulence factor of *Gibberella pulicaris* (anamorph: *F. sambucinum*) and *F. sporotrichioides* on parsnip roots; but there is no known function for trichothecenes in the virulence on potato tubers (Desjardins et al., 1993). On the other hand, fumonisins could be responsible for other aspects of the ecology of *F. verticillioides* (Proctor et al., 2002).

In this study, we examined the biological function of fumonisins in the growth of *F. verticillioides* in maize and other possible hosts. We believed that maize silks/cobs may be a very special environment that only became available for *F. verticillioides* a couple of thousands years ago, which is too short to develop fumonisin biosynthesis. This means that fumonisin synthesis would be originated on hosts other than maize and in tissues other than silks/cobs. Therefore, we employed fumonisin-nonproducing strains as well as wild type strains to determine whether fumonisin production is a potential and general virulence factor for *F. verticillioides* on different plants such as sorghum, rice and beetroot as well as maize. Understanding the role of fumonisin in pathogenicity can help pathologists in finding a strategy to control cereal crop diseases and subsequently reduction of mycotoxin contamination.

Material and Methods

Fungal Strains and Inoculum Preparation

Two mutants of *F. verticillioides* and their progenitor strains were obtained from Mycotoxin Research, National Center for Agricultural Utilization Research, United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Peoria, USA. GFA2364 (Proctor et al., 1999) is a fumonisin non-producing mutant derived from virulent wild-type strain M-3125 (Proctor et al., 2002), which is representative for FB1, FB2, and FB3 producing strains. Another mutant used in this study was GFA2556, a fumonisin non-producing mutant (Desjardins et al., 2002) and its progenitor 109-R-7 (Desjardins et al., 1996) as a FB2-producing strain. Both M-3125 and 109-R-7 were designated as wild type *FUM 1-1* alleles. This wild type functional allele was replaced with the mutant *fum 1-3* allele to generate GFA2364. Mutant GFA2556 with mutant allele *fum 1-4* was generated by gene disruption in 109-R-7.

Conidia from all four strains were produced for inoculum by growing the fungus on liquid mung bean media (Bai & Shaner, 1996) at 25°C for 5-7 days. The cultures were filtered through sterile cheesecloth and centrifuged at 7300 g for 10 min. The spores were re-dissolved in 15% (v/v) glycerol (Carl Roth, Karlsruhe, Germany) and the number of spores was counted with a Thoma Chamber (0.0025 mm²). The spore suspensions were stored at -70°C. Prior to inoculation, spores were checked for viability on PDA plates. The concentration of each conidial suspension was then determined and diluted to 1×10^6 spore ml^{-1} with sterile tap water. The inoculum contained 0.1% (v/v) Tween®20 (Applichem, Darmstadt, Germany) as a surfactant. Spore suspensions were kept on ice during inoculation and homogenized well before using.

Greenhouse Tests

Mini maize cultivar ‘Gaspe Flint landrace’ (originally collected in Quebec, Canada, provided by Prof. Dr. J. Schirawski, Albrecht-von-Haller Institute for Plant Sciences, University of Göttingen, Germany), Rice genotype ‘Taichung Sen 10 (TCS 10)’ obtained from Africa Rice Center, Cotonou, Benin, Sorghum bicolor ‘Tall Polish’ cultivar (originating from Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben, provided by Prof. Dr. J. Schirawski, Albrecht-von-Haller Institute for Plant Sciences, University of Göttingen, Germany),

and Weiße Bete (Beetroot) cultivar 'Weißer Kegel' (Bio-Saatgut Gaby Krautkrämer, Armsheim) were used in this study. The seeds of beetroot, maize and sorghum were surface disinfected in 1% (v/v) sodium hypochlorite (NaClO) solution for 1 min and absence of *Fusaium* spp. propagules in or on the seeds was verified by plating a representative sample on potato dextrose agar (PDA) (Roth, Karlsruhe, Germany) prior to the greenhouse experiments. The seeds were then sown in plastic multi-pots containing sterile fine sand (< 2 mm granularity). Two to three seeds of each cultivar were planted per cavity and thinned to one plant when seedlings appeared. The multi-pots were maintained at 22°C and a relative humidity of 60% with alternating 12-h light (mercury vapour lamps, 6600 lux at ear height) and dark periods. The rice seeds were placed on wet paper towel and incubated at 25°C at darkness. Germinated rice seeds were transferred to pots containing a mixture of sterile commercial plant substrate (Fruhstorfer Erde, Typ T25, HAWITA Group, Vechta, Germany), sand and compost (1: 1/2: 1) and placed at 30°C with 70-75% humidity. Two or three weeks after germination, all seedlings were inoculated with conidial spore suspension.

Root-dip inoculation method was employed by soaking the roots of individual plants in spore suspension for 30 min. After inoculation, the seedlings were transferred to new pots (9 × 9 × 9.5cm) with sterile mixed soil consisting of commercial plant substrate and sand (1:1). The plants were maintained under the same conditions as described above for each cultivar. Plants were irrigated with tap water as required. After one week of inoculation, the plants were supplemented (twice during the growth) with a 15-10-15 (N- P- K) soluble fertilizer. Rice seedlings were irrigated weekly with fertilizer containing N-P-K and Fe. Ten replicate pots were set up for each treatment. For rice, number of replications was ten with two plants per pot and the experiment was conducted two times. Control treatments were inoculated with autoclaved tap water mixed with adhesion detergent Tween®20. The plants were inspected at weekly intervals for symptoms. Seven to nine (for sorghum) weeks after inoculation, the plants were harvested and the roots were washed carefully under tap water. The sorghum, maize and rice plants were cut into three parts including roots, the first two or three internodes above the soil line and upper plant part and then placed separately in plastic bags. In the case of beetroot plants, they were divided into two parts: root and shoot. In order to quantify the fungal biomass in artificial

inoculated plants and determine the function of fumonisins, the harvested plant materials were freeze dried and prepared for further experiments by real-time PCR (qPCR) assay.

DNA Extraction and Determination of Matrix Effects

Total genomic DNA was extracted by using 30-60 mg of fine powder plant material based on the CTAB method according Brandfass & Karlovsky (2008). DNA quality and concentration was estimated by electrophoresis in 0.8% (w/v) agarose gel (Cambrex, Rockland, ME, USA), prepared in TAE buffer (40 mM Tris, 1mM EDTA (ethylene diamine tetra acetic acid), pH set to 8.5) (both substances were obtained from Carl Roth, Karlsruhe, Germany). DNA was stained with ethidium bromide (0.5 µg ml^{-1}) (Applichem, Darmstadt, Germany) and visualized using a digital imaging system (VilberLourmat, Marne La Vallee, France).

For accurate quantification of fungal DNA in inoculated samples, DNA standards of *F. verticillioides* and DNA from plant samples should be amplified under identical conditions. Therefore, to perform the qPCR without matrix effects, the inhibitors were tested under a common technique. DNA from an uncontaminated sample of each plant cultivar was mixed in varying dilutions (1:25, 1:50, 1:100) with defined concentration of standard DNA, and amplification was then carried out by qPCR. In this way, we also run the uncontaminated DNA sample as well as DNA standard individually to be able to compare the results in different treatments. According to the threshold value (Ct), the best dilution factor that could imitate matrix effects was chosen.

Molecular Quantification of Fungal DNA

A thermocycler (CFX384™ Real-Time System, C1000™ Thermal Cycler, BioRad, USA) with 384-well microplates (Kisker Biotech GmbH, Steinfurt, Germany) was used for the qPCR analysis. *F. verticillioides* was assessed in inoculated samples based on a previously developed low-volume qPCR protocol (Dastjerdi et al., 2013, Chapter 2, unpublished). The Primer pairs VER 1 (CTTCCTGCGATGTTTCTCC) and VER 2 (AATTGGCCATTGGTATTATATATCTA) designed by Mul`e et al. (2004) were used to amplify a 578 bp fragment, specific for *F. verticillioides.* The reaction mixture consisted of reaction buffer (16 mM $(NH4)_2SO_4$; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH 8.8 at 25°C,

Bioline, Lükenwalde, Germany), 0.1 mM of each of the four deoxynucleoside triphosphates (dNTPs; Bioline, Lükenwalde, Germany), 2.5 mM $MgCl_2$, 0.1U of *Taq* DNA polymerase (BIOTaq, Bioline, Lükenwalde, Germany), 0.3 µM of each primer, 0.1x SYBR Green I (Invitrogen, Karlsruhe, Germany). The cycling conditions were as follows: 1 cycle of 2 min at 95°C, 34 cycles of 30 s at 94°C (denaturalization), 30 s at 62.5°C (annealing), 40 s at 72°C (extension) and followed by a final extension cycle at 72°C for 5 min. Fluorescent data were obtained during the annealing phase to construct a melting curve at the end of assay. The qPCR was completed by running a melting curve analysis. Quantity of fungal biomass was assessed as a parameter of fungal development.

Data Processing and Statistical Analysis

The software SigmaPlot 12.3 Notebook was used for statistical analysis. Non-parametric tests were performed when data distribution was not normal. The qPCR data were assessed to find the positive samples based on the melting temperature (Tm), starting quantity (SQ) and Ct value. The lowest DNA standard employed in qPCR assays was considered as the limit of quantification (LOQ). According to the qPCR data, the quantity of fungal biomass (pg mg^{-1}) for the positive samples was calculated. DNA quantity for the samples containing values lower than LOQ was substituted as LOQ/2 (Hornunga & Reeda, 1990). These samples had the right melting temperature and the logical Ct values.

Results

A common method was used to evaluate the influence of plant inhibitors on successful qPCR performance. The results indicated a significant effect for all plant extracts tested. The difference in Ct values between the standard reference DNA and the DNA from uncontaminated plant material was considered as an indicator to find the matrix effects. The results are presented in Table 1. High qPCR inhibition was seen in the root extracts; so that 100 dilution for maize and sorghum and 50 for beetroot samples were necessary to imitate matrix effects. Diluting to 1:25 (v/v) (for aboveground tissues of sorghum and beetroot) or 1:50 (v/v) (for aboveground maize parts) with double distilled water was preferred to reduce the Ct value to the same level as the standard-DNA. In the case of rice plant material, DNA dilution, even 100-fold, was insufficient for reduction of plant inhibitor effects. Adding 1 mg ml^{-1} bovine serum albumin (BSA) to the

reaction mixture together with 1:50 dilution (v/v), could improve the qPCR amplification in DNA extracts of healthy plants mixed with standard-DNA and made the results comparable. Based on these results, all DNA samples were diluted with a suitable dilution factor and employed, then, for further real-time PCR assays.

Table 1. Determination of matrix effects in different plants studied

Plant DNA sample	Ct values derived from qPCR assay for :				
	Maize	Sorghum	Beetroot	Rice (without BSA)	Rice (with BSA)
R (undiluted)	NA	NA	NA	NA	NA
R 1:25	NA	NA	NA	NA	NA
R 1:50	NA	NA	NA	NA	NA
R 1:100	NA	NA	NA	NA	NA
LS (undiluted)	NA	NA	NA	NA	NA
LS 1:25	NA	NA	NA	NA	NA
LS 1:50	NA	NA	NA	NA	NA
LS 1:100	NA	NA	NA	NA	NA
STD	28.53	28.53	25.62	25.92	25.79
STD + DNA R	NA	NA	NA	NA	28.33
STD + DNA R 1:25	NA	NA	27.93	28.80	26.80
STD + DNA R 1:50	30.05	29.98	**25.27**	28.95	**26.03**
STD + DNA R 1: 100	**28.84**	**28.07**	25.41	28.78	26.09
STD + DNA LS	NA	29.63	28.08	30.11	27.79
STD + DNA LS 1:25	29.06	**28.63**	**25.98**	28.18	26.06
STD + DNA LS 1:50	**28.30**	27.91	25.44	28.38	**25.90**
STD + DNA LS 1:100	28.19	27.99	---	28.12	26.08

Bold letters are the Ct values related to the suitable dilution factor
Abbreviations: R: root; LS: leaf and stem (aboveground plant tissues); STD: standard DNA (pg μl^{-1}) which was 4.5 for maize, sorghum; 16.6 for beetroot and rice; BSA: bovine serum albumin; NA: not amplified

We compared the ability of two *FUM 1-1* strains and the mutants derived from them (*fum 1-3* and *fum 1-4*) to cause disease in maize, grain sorghum, beetroot and rice seedlings. None of the plants studied show any visible disease symptoms. Some necrosis spots were observed only on rice leaves, regardless of fumonisin or non-fumionisin production, which were not related to the fungus (Supplemental Fig. 1). *F. verticillioides* was not detected in any of these leaf samples. Lack of symptoms suggests that plant and fungus may coexist without obvious disease symptoms. Therefore, total fungal biomass was quantified by real-time PCR and it was used as an indicator of fungal aggressiveness. In all plants tested, no significant (P = 0.05) difference in aggressiveness was detected between each wild type and the relevant mutant. *F. verticillioides* was not detected in any of the control plants. The incidence of fungus for 10

plant replicates of maize, sorghum, and beetroot also for 40 replicates of rice plants are presented in Fig. 1, 2, 4 and 3, respectively. Fungal transmission from roots to the first two internodes/leaves in maize and beetroot occurred following the root inoculation, but a low systemic infection rate was observed for the rice and sorghum cultivars. Lack of significant difference between fumonisin-producing strains and non-fumunisin producing strains in tissue colonization, therefore, supported the idea that fumonisin was not a virulent factor in our system.

Discussion

F. verticillioides is the most prevalent fungus associated with maize seedling diseases, ear rot and stalk rot (Logrieco et al., 2002; Srobarova et al., 2002). Fungus can colonize stalks without any visible disease symptoms (Kedera et al., 1992; Munkvold & Carlton. 1997; Munkvold et al., 1997). The function of fumonisins, as the most widespread toxins reported from *F. verticillioides* strains, in virulence is still not completely identified. In this study, two fumonisin non-producing strains (*fum 1-3* and *fum 1-4*) and their progenitors (fumonisin-producing) *FUM 1-1* strains were employed to examine the possible role of toxin in the growth of the fungus in maize, grain sorghum, rice and beetroot seedlings. There was no relationship between fungal aggressiveness and fumonisin production in the plants studied.

Regarding the maize plants, movement of the fungus from the inoculated roots to the remainder of the plant parts occurred, regardless of whether the strain produced fumonisin or not. The rate of symptomless systemic colonization was lower above the second internodes in stalks. Munkvold et al. (1997) showed that restricted movement of fungus from maize seed or crown to higher internodes in stalk can limit systemic colonization of kernels. However, other studies provide evidence that systemic infection may contribute to the contamination of kernels by mycotoxins (Desjardins et al., 2002; Desjardins and Plattner, 2000). The possibility of systemic development of infection by *F. verticillioides* will be greater at higher temperatures (Williams & Munkvold, 2008). There was no significant difference between fungal biomass of each wild type *FUM 1-1* strain and the relevant mutant. Although high level of fungal biomass was observed in the first two stalk internodes, it was independent from fumonisin production. The GFA2364 and GFA2556 strains make relatively little, or no fumonisins in vitro (Desjardins et al., 2002), but in our experiments these two strains caused significant colonization of tissues at the same level of

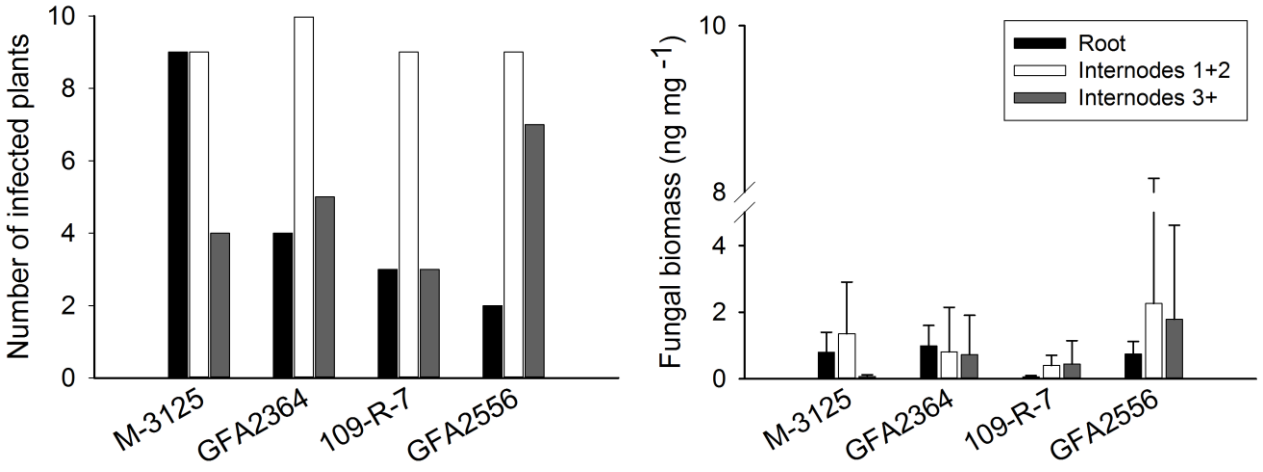

Fig. 1. Incidence of indicated strains of *F. verticillioides* on maize seedlings (left); mean amounts of fungal biomass in positive samples (right) inoculated in the greenhouse. Limit of quantification (LOQ) was set at 84.7 pg mg^{-1} for root samples and 50.8 pg mg^{-1} for upper parts of the plants. M-3125: FB1, FB2 & FB3 producing; GFA2364: fumonisin non-producing; 109-R-7: FB2 producing; GFA2556: fumonisin non-producing.

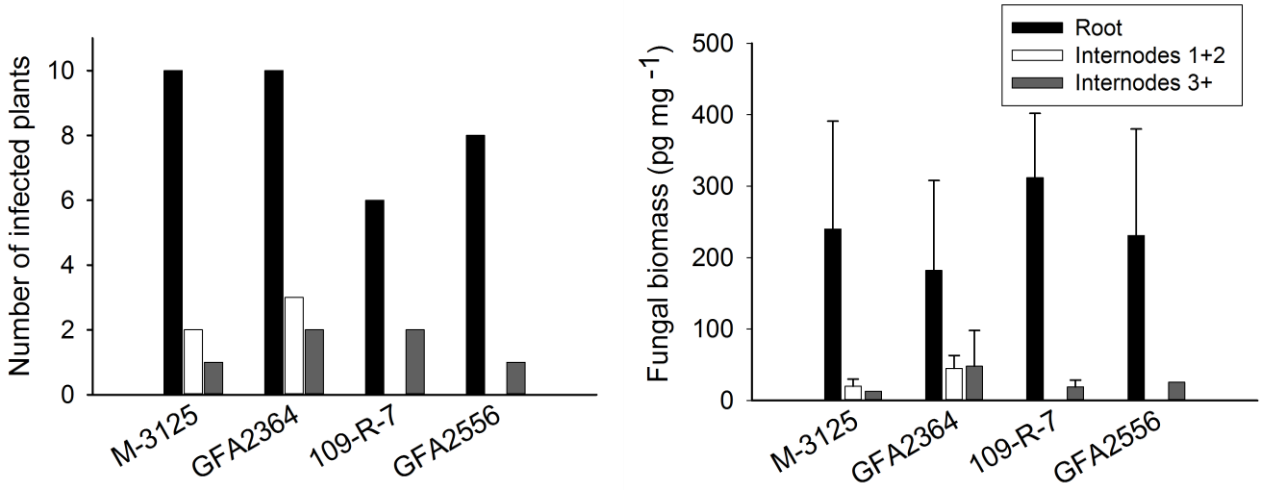

Fig. 2. Incidence of indicated strains of *F. verticillioides* on sorghum seedlings (left); mean amounts of fungal biomass in positive samples (right) inoculated in the greenhouse. Limit of quantification (LOQ) was set at 84.7 pg mg^{-1} for root samples and 25.4 pg mg^{-1} for upper parts of the plants. M-3125: FB1, FB2 & FB3 producing; GFA2364: fumonisin non-producing; 109-R-7: FB2 producing; GFA2556: fumonisin non-producing.

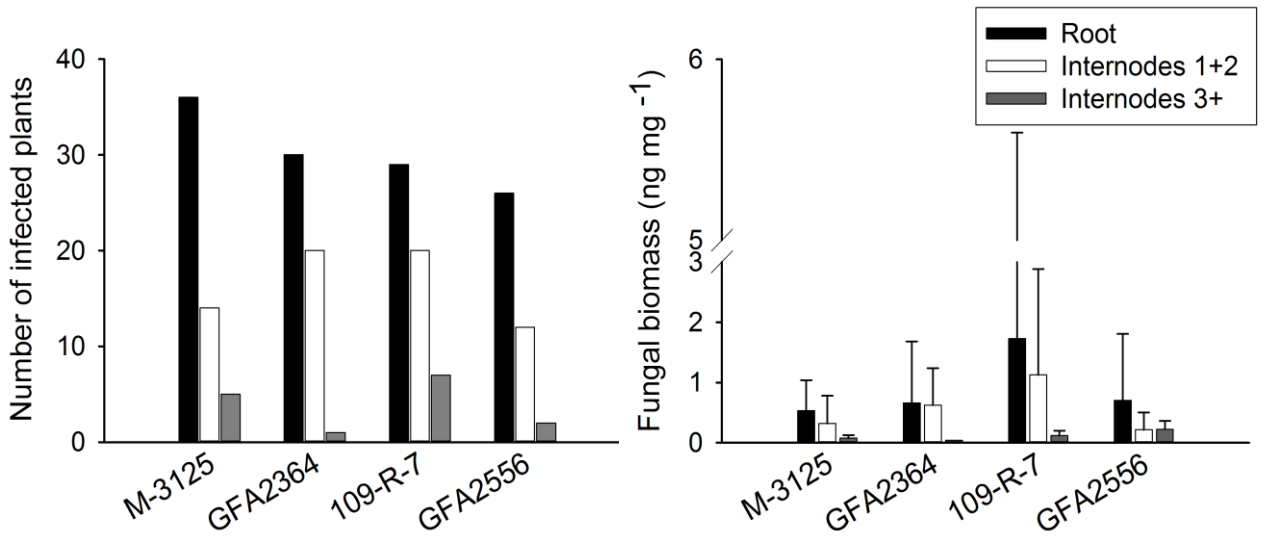

Fig. 3. Incidence of indicated strains of *F. verticillioides* on rice seedlings (left); mean amounts of fungal biomass in positive samples (right) inoculated in the greenhouse. Limit of quantification (LOQ) was set at 84.7 pg mg^{-1} for root samples and 42.3 pg mg^{-1} for upper parts of the plants. M-3125: FB1, FB2 & FB3 producing; GFA2364: fumonisin non-producing; 109-R-7: FB2 producing; GFA2556: fumonisin non-producing.

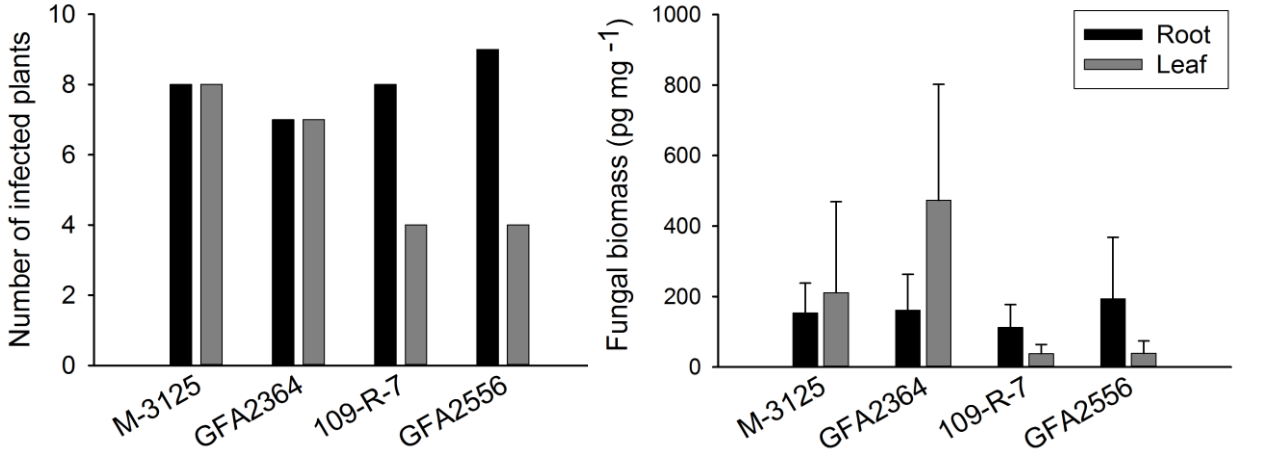

Fig. 4. Incidence of indicated strains of *F. verticillioides* on beetroot seedlings (left); mean amounts of fungal biomass in positive samples (right) inoculated in the greenhouse. Limit of quantification (LOQ) was set at 62.5 pg mg^{-1} for root samples and 31.3 pg mg^{-1} for leaf. M-3125: FB1, FB2 & FB3 producing; GFA2364: fumonisin non-producing; 109-R-7: FB2 producing; GFA2556: fumonisin non-producing.

fumonisin-producing strains. The results indicate that fumonisin production was not required for *F. verticillioides* to colonize the maize tissues following root inoculation.

In the case of sorghum, after root inoculation the fungus remained in the below ground tissues and fungal transmission did not occur. Only 20-30% of the plants inoculated with the M-3125 strain and its mutant show the fungus in the first two internodes. This would be explained by host preference of biological species of *G. fujikuroi* in which mating population A (MPA) is predominant on maize while *G. fujikuroi* mating population F (MPF) can infect sorghum plants (Leslie et al., 1992). These differences may cause differences in the pathogenic potential of the strains (Leslie, 1991). The selection of suitable strains is, therefore, important in breeding programs (Jardine & Leslie, 1999). It is also possible that the sorghum variety used in this study reacted to the fungus with a partial resistance response, whereby the pathogen was unable to spread through the plant. However, sorghum root colonization and lack of fungal movement to the upper parts of the plant suggest that *F. verticillioides* is able to survive in the roots of non-host plants when the main host is not available. This will clearly help to maintain fumonisin synthesis. We believe infection of maize silks or cobs may be a very special environment that only became available for the fungus a couple of thousands of years ago, which is a short time period to develop fumonisin biosynthesis. For millions of years previously, the fungus had infected roots or stems, living as endophyte. This means that fumonisin synthesis would be maintained by the selection pressure exerted on hosts other than maize and in tissues other than silks or cobs. The presence of fungus in upper internodes while it is absent in lower nodes (as was the case for two plants inoculated with 109-R-7 strain and one plant inoculated with GFA2556 strain) could be explained by spore splashing during the inoculation. Based on the data obtained from this work, it is not possible to make a conclusion with confidence regarding the function of fumonisin in pathogenicity on sorghum seedlings.

Although the fungus could have developed inside the young plants of rice and beetroot, the aggressiveness of wild type strains and their mutants to the rice or beetroot seedlings was not statistically different. Occurrence of fumonisins in rice plants has not been found as frequently as in maize, but more recently contamination of rice with FB1 and FB2 toxins has been reported (Kushiro et al., 2008; Maheshwar et al., 2009; Tansakul et al., 2012). The fungus has been demonstrated to causes panicle rot of rice (Bhargava et al., 1979). *G. fujikuroi* complex including

MPA (anamorph, *F. verticillioides*), MPC (anamorph, *F. fujikuroi*) and MPD (anamorph, *F. proliferatum*) have been demonstrated in infected rice seeds (Desjardins et al., 2000). They show that some strains of *G. fujikuroi* MPD are capable of producing fumonisin as well as beauvericin and moniliformin; but not gibberellic acid, which has been reported in bakanae. The prevalence of MPD may be related to the role of this biological species in the complex symptoms of bakanae disease of rice (Desjardins et al., 2000). *F. verticillioides* causes seedling damping-off in sugar beet and has been reported as a pathogen which can produce symptoms similar to fusarium yellows (Hanson & Hill, 2004; Drycott, 2006). In the present study, 15-20 days after inoculation some necrosis spots were observed on rice leaves. The spots were irregular, small, light brown in the center and dark brown at the margins in the initial stage. The necrotic lesions usually developed, joined together, made larger spots and extended along the length of the leaf. The spots were seen regardless of fumonisin or non-fumonisin producing strain tested. However, the qPCR assay could not detect the fungus in the leaf extracts. Control plants did not show any necrotic spots. Rice experiments were conducted twice (in summer 2012 and winter 2013) and the results were identical. Further assessments are, therefore, necessary to find the agent of these necrosis spots.

Potential plant inhibitors such as proteins, polyphenols, polysaccharides, secondary metabolites (Horne et al., 2004) are released during the DNA extraction process. These compounds in DNA extracts can affect the qPCR reactions. They prevent the amplification of target DNA, increase the Ct values or lead to reduction of overall efficiency and reproducibility of PCR and eventually may contribute to inaccurate results (Demeke & Jenkins, 2010; Cankar et al., 2006). In this study, the effect of sample matrix properties on real-time PCR reactions was assessed for maize, sorghum, rice and beetroot tissues by adding standard reference DNA to the DNA of uncontaminated plant material. Initially experiments indicated a high potency of plant inhibitors, so that the qPCR assays were completely inhibited and no amplification was detected (Table 1). Dilution of DNA samples is a simple method to reduce the inhibitor concentration in plant extracts especially in samples involving complex matrices such as root extracts. On the other hand, for the samples with lower DNA concentration, a high DNA dilution factor may decrease PCR sensitivity (Demeke & Jenkins, 2010). In this study, a dilution factor of 1:25 or 1:50 for the aboveground plant tissues and 1:100 for the root samples could decrease the PCR

inhibition and the Ct values then reached to the same level of pure fungal DNA. In the case of rice plant tissues, although 100-fold dilution could generate PCR products, the Ct values were still far from the Ct expected for target molecules added to the reaction. More dilution was not possible due to decreasing qPCR sensitivity. BSA is a chemical which has been employed for deactivation of PCR inhibitors in various sample types and recently for plant materials as well (Wei et al., 2008; Plante et al., 2010). Adding this chemical directly into the PCR reactions was sufficient to enhance the qPCR results.

In this work, maize seedlings colonization with fumonisin non-producing strains and fumonisin-producing strains was similar. It is possible that such non-producing toxin strains can be used as biocontrol agents of maize fusarium diseases if they provide a substantial level of symptomless colonization in different maize plant tissues without causing any ear rot (Desjardins & Plattner, 2000). In summary, the present results demonstrate that fumonisins may have a function in pathogenicity of *F. verticillioides*, but in our system, the ability to synthesize these toxins was not related to the fungal aggressiveness and fumonisins did not determine the pathogenicity of *F. verticillioides* pathogen.

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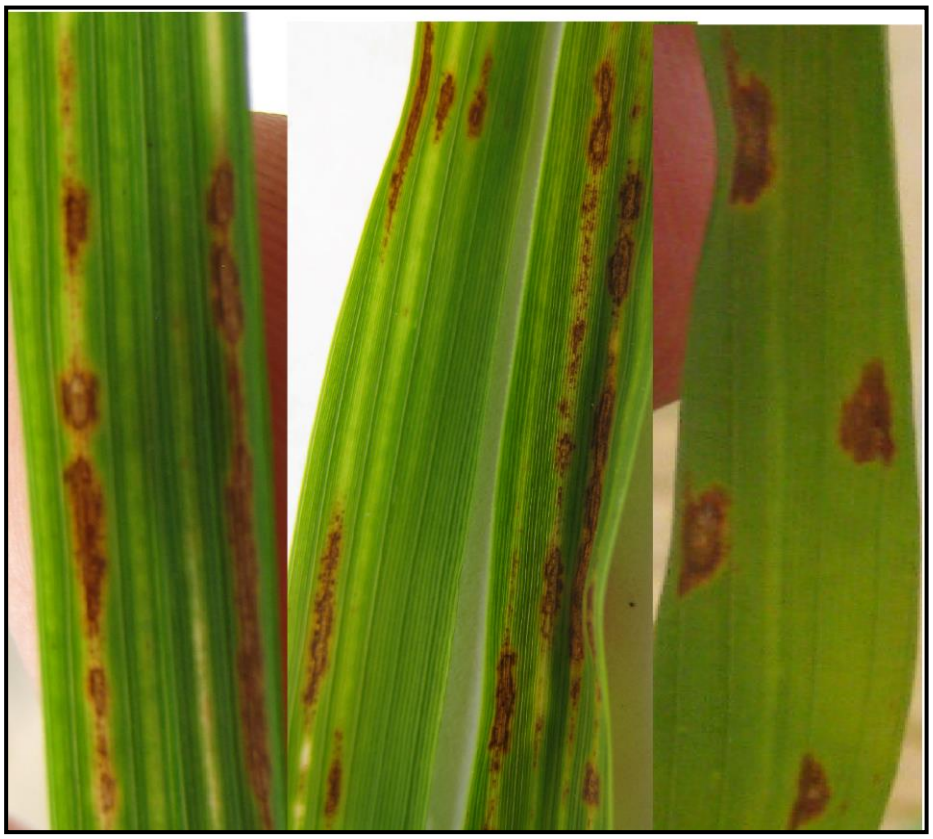

Supplemental Fig. 1. Leaf necrotic spots on inoculated rice plants in the greenhouse

Chapter 5

General Discussion

Phytopathogenic fungi of the genus *Fusarium* are distributed worldwide and cause economically serious diseases in strategic cereals (Jurado et al., 2005). Additionally, some species are able to produce significant quantities of mycotoxins such as trichothecenes, fumonisins, enniatins, zearalenone and beauvericin (Logrieco et al., 2002). Owing to the positive correlation between fungal biomass and mycotoxin content (Waalwijk et al., 2004; Schnerr et al., 2002; Yli-Mattila et al., 2008), one possible method of predicting mycotoxin contamination is the quantification of fungal biomass in infected plant tissues. Among several quantification methods, using the well-known real-time PCR (qPCR) technique is growing. In this study, we developed a real-time PCR assay for quantification of the nine most important *Fusarium* pathogens of maize and small-grain cereals in 384-well microplates and in a total volume of 4 µl. The method is suitable for fast and cost-effective analysis of a large number of samples in a short time. The qPCR assay for all nine species utilizes the same thermocycler profile and, therefore, can be performed in the same microplate.

In spite of availability of thermocyclers with 384-well blocks, most of the published qPCR assays are set for screening only one target DNA in each assay and the protocols still work in a total volume of 15 to 25 µl. Similarly, the qPCR conditions were set up in our laboratory for *F. culmorum*, *F. graminearum*, *F. proliferatum*, and *F. verticillioides* (Brandfass & Karlovsky, 2006; Nutz et al., 2011). The protocols, however, did not work well when the total volume was decreased to 4 µl reactions. A second peak prior to specific melting point resulted in issues for the qPCR melting curve, especially in low DNA concentrations. Modification of thermal parameters (mostly annealing and elongation steps) and re-optimization of PCR reagents for each *Fusarium* spp. enhanced the qPCR assay and the wide shape peaks disappeared.

The PCR efficiency, one of the most important qPCR parameters, is defined as the amount of amplified template DNA during one cycle. Correct determination of this parameter is necessary while the PCR data are used for the quantitative purposes (Rebrikov & Trofimov, 2006). In present optimized multi-species qPCR protocol, the PCR efficiency ranged from 81%

(*F. verticillioides*) to 120% (*F. equiseti*). In this work, sensitivity which is the crucial factor for interpretation of the qPCR results was defined as the lowest standard DNA that was able to be amplified at the expected melting temperature in at least seven out of eight replicates. Based on this definition, sensitivity trials showed a detection limit of 0.05 pg μl^{-1} of standard DNA for *F. proliferatum,* 0.17 pg μl^{-1} for *F. graminearum, F. poae, F. subglutinans*, 0.50 pg μl^{-1} for *F. avenaceum, F. culmorum, F. tricinctum* and 1.52 pg μl^{-1} for *F. equiseti, F. verticillioides*. The developed multi-species qPCR assay did not show a delicate sensitivity for the last two *Fusarium* species. Therefore, in order to quantify these species while low amounts of fungal biomass are present in plant materials, it is suggested to follow the single species qPCR protocol developed in the present research. For both species, thus, sensitivity will be improved to 0.50 pg μl^{-1} in a background free of plant tissues and other contaminants. In summary, both single and multi-species qPCR methods developed in this project are suitable for quick, cost-effective and high-throughput quantification of the nine *Fusarium* spp. in plant material.

Inhibitors have been reported as a challenge for the successful detection and reliable quantification of fungal biomass in plant tissues. Efficiency and sensitivity of qPCR assay will be affected by the inhibitors present in DNA extracts. Developed high sensitive qPCR protocols, therefore, maybe could not generate reliable data when a set of DNA extracts containing considerable inhibitors are used (Gao et al., 2004; Demeke & Jenkins., 2010). Potential plant inhibitors such as proteins, polyphenols, polysaccharides, secondary metabolites (Horne et al., 2004), as well as components of DNA isolation buffers (Rossen et al., 1992) influence the qPCR reaction and cause a reduction in efficiency (Gao et al., 2004). In some cases, samples with strong matrix properties are able to completely block PCR amplification and result in a false negative reaction. Significant PCR inhibition is demonstrated from soil and root DNA extracts (Braid et al., 2003; Gao et al., 2004; Van de Graaf et al., 2003) in addition to DNA from senescing plant material (Turner et al., 1998). In this investigation, the DNA extracts obtained from different parts of maize, sorghum, rice and beetroot indicated different matrix effects. Similarity between the PCR efficiency of unknown samples and standard reference material is a prerequisite to determine the accurate quantity of fungal biomass (Cankar et al., 2006). For this purpose, diluting DNA is a simple way to overcome the qPCR inhibition. A dilution factor of 25, 50 or 100 fold could decrease the threshold values (Ct) to the same level of the reference DNA

standard and enhanced the qPCR efficiency. One hundred times dilution was not sufficient for the DNA of the rice plants. Since a lower DNA concentration could decrease the PCR sensitivity, 1 mg ml^{-1} bovine serum albumin (BSA) was directly added to the PCR reaction mixture. This chemical has been successfully employed for deactivation of PCR inhibitors (Wei et al., 2008; Plante et al., 2010).

Both developed qPCR protocols were successfully applied for detecting the nine *Fusarium* spp. in weeds sampled from maize fields. These species are known as the most common *Fusarium* associated with maize diseases. The qPCR analysis revealed that 201 weed samples out of 294 were infected with at least one *Fusarium* species. Sometimes more than one species of *Fusarium* were detected in individual sample. Weeds have been demonstrated to be reservoirs of potential fungal pathogens. They serve as alternative hosts for several species of *Fusarium*, usually when the economically important host plants are not present (Helbig & Carroll, 1984; Jenkinson & Parry, 1994; Postic et al., 2012; Altinok, 2013). In this study, 36 weed species in the maize fields had been colonized with ten species of *Fusarium*. The rate of infection was high for *F. equiseti* (49%) and *F. avenaceum* (34.7%). *F. avenaceum* is well-known as a common species associated with cereals in Europe, while *F. equiseti* has less frequently (Logrieco et al., 2002; Yli-Mattila, 2010; Kosiak et al., 2003). *F. culmorum* and *F. tricinctum* indicated the same infection ratio (18%). It was followed by *F. proliferatum* (11%) and *F. graminearum* (8%). *F. poae* and *F. subglutinans* were detected at very low frequencies (3% and 1%, respectively). In isolation experiments, *F. oxysporum* and *F. venenatum* were also recovered from weed samples in addition to other species.

The present study identified some of the weed species as new hosts for each *Fusarium* spp. tested in the greenhouse (see **Chapter 3, Table 4**). The frequent screening of *Fusarium* on common weeds (by qPCR or isolation on selective medium culture) increased their possible role in the survival of fungus. The previous studies have indicated that plant residues are the main inoculum source of *Fusarium* diseases (Cotten & Munkvold, 1998). Other works have also demonstrated survival of *Fusarium* spp. on non-host plants such as weeds (Jenkinson & Parry, 1994; Inch & Gilbert, 2003; Postic et al., 2012; Altinok, 2013). Therefore, weeds as a harbor for *Fusarium* have the potential to provide an inoculum reservoir for the infection of main crop during the growing season. In this work, high incidence of *Fusarium* spp. occurred in

Echinochloa crus-galli, *Polygonum convolvulus* and *Chenopodium album* which have been reported as the main weed species from maize farms (Westerman & Gerowitt, 2012). Prevalence of *F. avenaceum* and *F. equiseti* on almost all common weed species studied (see **Chapter 3, Table 2**) may indicate that these *Fusarium* are more competitive species for colonization of weeds compared to the main cereal fusaria such as *F. graminearum*.

In this work, weeds were symptomless carriers of *Fusarium* spp. No obvious disease symptoms were observed on weeds assayed from the maize fields and also after inoculation in the greenhouse conditions. This finding is in agreement with others (Haware & Nene, 1982; Clark & Watson, 1983; Helbig & Carroll, 1984; Jenkinson & Parry, 1994; Postic et al., 2012; Altinok, 2013). Akinsanmi et al. (2007) reported that the pathogenic fitness of *Fusarium* pathogens was significantly reduced during passage through alternative hosts, while their fertility on the primary host increased. Other reports show only less aggressive isolates could attack weeds (Helbig & Carroll, 1984). The above reasons may explain the symptomless infection of the weeds. On the other hand, *Fusarium* strains were recovered from surface sterilized roots and stems of weeds in both field and artificially inoculated samples. Successful infection of weed tissues in addition to the lack of symptoms on weeds suggested that the infection of weeds by *Fusarium* spp. could be endophytic.

Common weeds were assessed for the main toxic secondary metabolites by HPLC-MS/MS. All samples tested were negative (below the LOD of 3 ng g^{-1}) for contamination of trichothecenes, zearalenone and fumonisins, even the samples containing the related producers. As it is mentioned above, poor performance of *Fusarium* strains on alternative hosts (Akinsanmi et al., 2007) may explain the lack of symptoms and mycotoxin production. Another possibility is field conditions that did not favor mycotoxin production. In contrast, high levels of beauvericin and enniatins were detected in the samples contaminated with *F. equiseti* and *F. avenaceum*. Enniatins were measured most often at high levels (up to 24 mg kg^{-1}) in *Chenopodium album*, *Elymus repens* and *Polygonum aviculare.* Concentration of beauvericin in some weed species, such as *Matricaria inodora*, ranged from 0.0 to 65.6 mg kg^{-1}. However, there was no correlation between mycotoxin content and fungal biomass. Contamination of cereal grains by enniatins, resulting from *F. avenaceum*, and the natural occurrence of beauvericin in maize ears has been reported (Logrieco et al., 1993; Bottalico et al., 1995; Ritieni

et al., 1997; Logrieco et al., 1995; Yli-Mattila, 2010). In summary, although the main cereal toxins were not found in the weed samples tested, the significance of toxin production in the main host should not be disregarded.

There are limits on the use of morphological characters for identification of *Fusarium* spp. These limitations increase when differentiation is within members belonging to a species complex with highly similar morphology. In recent years, molecular techniques based mostly on DNA sequencing of genes have supported morphological identification of *Fusarium* species (Yli-Mattila et al., 2002; Harrow et al., 2010; Geiser et al., 2004; Hsuan et al., 2011). Nucleotide sequencing of genes is performed particularly in informative parts of genome such as translation elongation factor 1-alpha (TEF-1α). In this work, typical *Fusarium* structures were utilized to identify the recovered isolates according to Leslie & Summerell (2006). Molecular information obtained from the DNA sequence of TEF-1α and phylogenetic analysis were then employed to confirm the morphological identification and probably distinguish closely related strains. The isolates morphologically identified as *F. equiseti*, were placed in the *Fusarium incarnatum-equiseti* species complex (FIESC) according to the closest match of BLAST search analysis using the Fusarium–ID database (Geiser et al., 2004). The results show they belong to three sub-clades of FIESC, only one of which has been called *F. equiseti*, and the next two sub-clades still have no latin name (O'Donnell et al., 2009a). *Fusarium oxysporum* species complex (FOSC) also comprises different sub-clades (O'Donnell et al., 2009b). Members of FOSC are ubiquitous soil borne pathogens and have been demonstrated from maize and other cereals to be a less common species (Logrieco et al., 2002; Kosiak et al., 2003). In this study, although closely related species to *F. avenaceum* such as *F. torulosum*, *F. flocciferum* and *F. accuminatum* formed different clades in the constructed UPGMA dendrogram, the reference strains of *F. arthrosporioides*, however, fell in the *F. avenaceum* clade. These two species (*F. avenaceum, F. arthrosporioides*) are morphologically very similar. They are distinguished by lack of orange sporodochia in *F. arthrosporioides* after growing on SNA (Synthetic Nutrient Agar) medium in darkness (Yli-Mattila et al., 2002). A combined DNA sequence data is necessary to be able to distinguish these two species. The ATP Citrate Lyase (acl1) sequence together with the TEF-1α gene could make a distinct lineage for *F. arthrosporioides* in the main clade of *F. avenaceum* (Gräfenhan et al., 2013).

F. verticillioides, as the most important maize pathogen, is able to cause a wide range of symptoms, from asymptomatic infection to severe rotting and wilting. The fungus can colonize maize stalks with no obvious disease symptoms (Kedera et al., 1992; Bacon & Hinton, 1996; Munkvold et al., 1997; Logrieco et al., 2002; Srobarova et al., 2002). This species was not detected in any of the weed samples. Furthermore, we could not recover it from different weed samples. Therefore, the importance of weeds as a possible source of *F. verticillioides* inoculum in maize fields remains still unknown. Additional studies need to be performed to evaluate if weed plants can serve as an alternative host for *F. verticillioides* in maize fields. Further weed sampling from maize fields of Italy and southern belt of Germany, where the fungus is a serious problem, has been carried out and the experiments are running in our group. We hope the results help us to find out if weed plants harbor *F. verticillioides* in maize fields and whether they can increase the infection pressure locally. The answer may also be helpful for finding the biological role of fumonisins in the growth of fungus in the host plants other than maize. We believe the fumonisin synthesis has been maintained by natural selection on weeds (or hosts other than maize) and in plant tissues other than silk/cobs (such as roots) for millions of years.

Fumonisins are polyketide mycotoxins produced by several species of *Fusarium* among which *F. verticillioides* is well-known as a main producer (Munkvold & Desjardins, 1997). The biological role of fumonisins in virulence of *F. verticillioides* has been an eristic issue. In this study, two fumonisin non-producing strains (*fum 1-3* and *fum 1-4*) and their progenitor (fumonisin-producing) *FUM 1-1* strains were employed to determine the potential role of toxins in the growth of the fungus in maize, grain sorghum, rice and beetroot seedlings. The results of this study confirmed the previous reports (Kedera et al., 1992; Bacon & Hinton, 1996; Munkvold et al., 1997) that *F. verticillioides* can infect maize plants without causing symptoms. Systemic movement of fungus within the root inoculated plants was detected by qPCR. Transmission of the fungus from roots to the first two internodes in maize plants occurred regardless of toxin production. Our experiments show that the infection through the roots has been an effective pathway for developing fungus within the plant. However, the rate of symptomless systemic colonization decreased after the second internode. This restricted movement of fungus may limit the infection of kernels. Williams & Munkvold (2008) indicated that the systemic development of *F. verticillioides* could be increased in high temperatures. Movement of the fungus appeared

to be limited in sorghum plants. Only in 20-30% of inoculated plants with the M-3125 strain or its mutant, GFA2364, the fungus was detectable in the first two internodes. Host preference of the biological species of *Gibberella fujikuroi* has been demonstrated (Leslie et al., 1992) and it could explain the lack of movement of fungus in sorghum plants. Another possibility would be the partial resistance of the sorghum variety which was utilized in this study. The relative distribution of the fungus in rice and beetroot seedlings was similar to maize plants. The quantified fugal biomass was considered as a marker to show the aggressiveness of the fungus. No infection was detected in control plants inoculated with sterile water. There was no significant difference ($P = 0.05$) between fumonisin-producing strains and fumonisin non-producing strains in colonization of plant tissues. The high level of fungal biomass was detected in roots and also in the first two internodes of the maize stalks; but it was independent of fumonisins production. Based on the results in our system, fumonisins have no role in fungal growth of *F. verticillioides* in maize, rice and beetroot seedlings and the aggressiveness of fungus was independent of toxin production.

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Summary

Fusarium is a large and complex genus, comprises important pathogens most of which are able to produce mycotoxins. Maize is a host of several *Fusarium* species that cause ear rot, kernel rot, stalk rot and also seedling blight. *Fusarium* contamination of kernels and maize products had always been a serious concern. Owing to the positive correlation between fungal biomass and mycotoxin content, quantification of fungal DNA in plant materials would be considered as an initial fast and cost-effective mean to evaluate the risk of grain contamination. Several quantification methods are well-known of which real-time PCR (qPCR) has been used as an effective tool for species-specific quantification of fungal biomass in plant tissues. The method mostly relies on standard thermocyclers (96-well or 384-well blocks) with a separate run for each template and usually set in total reaction volume of 15 to 25 µl. In this investigation, we developed a multi-species qPCR assay for simultaneous quantification of genomic DNA of the nine *Fusarium* species with 384-well microplates in a total volume of 4 µl. The sensitivity of the method ranged from 0.05 - 1.52 pg DNA per reaction, and the repeatability ranged from 0.81% to 1.71% RSD.

Developed low volume qPCR assay was successfully employed for the analysis of weed plants to the infection of nine *Fusarium* spp. The main objectives were determination the role of weeds in the survival of maize fusarium pathogens and assessment of their ability for producing of the main mycotoxins. The Real-time PCR detected eight *Fusarium* species in 201 weed samples representing 36 weed species, collected from maize fields. The highest frequency was observed for *F. equiseti* (49%) and *F. avenaceum* (34.7%). Similar diversity of *Fusarium* spp. was observed in both conventional and organic farming systems. Isolation of *Fusarium* strains from 12 common weed species were carried out, the strains were identified based on the morphological characters and then identification was confirmed by using the translation elongation factor 1-alpha (TEF-1α) gene sequence. The recovery rate was high for *F. equiseti* (32.7%) and *F. avenaceum* (21%). None of the field samples as well as weed plants tested in inoculation studies show obvious symptoms of *Fusarium* infection. Re-isolation of the strains from artificially inoculated plants confirmed the endophytic infection of weeds by *Fusarium* spp. The present study reports five new alternative hosts for *Fusarium* species in maize fields. High

incidence rate of beauvericin and enniatins contamination was obtained in weed samples while trichothecenes, fumonisins and zearalenone were not detected in any of the weeds studied.

Although toxicity of fumonisins to plants and field animals has been clearly demonstrated, the function of this toxin, however, in virulence of *F. verticillioides* toward maize plants is still unknown. In present study, virulence of two non-fumonisins producing strains (*fum 1-3* and *fum 1-4*) and their progenitors (*FUM 1-1*) was assessed on different plants including maize, sorghum, rice and beetroot seedlings grown under greenhouse conditions. The quantity of fungal biomass in plant tissues was considered as an indicator of fungal aggressiveness and it was measured by the developed low volume qPCR protocol. There was no significant (P = 0.05) differences between each wild type and the relevant mutant for colonization of plant tissues. In inoculated maize, rice and beetroot seedlings, systemic fungal infection was observed from roots to the aboveground parts; but rate of systemic transmission was low in sorghum plants. Although our results are not enough to make a final conclusion of fumonisin function in virulence of *F. verticillioides* on rice and sorghum seedlings, however, the results show diseases incidence was independent of fumonisins production in maize seedlings. In our system, therefore, fumonisins had no role as a pathogenicity factor.

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Curriculum Vitae

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Work Experience

Since 05/2002	Faculty member and research leader of Horticulture Crops Research Department, Seed and Plant Improvement Research Institute (SPII), Tehran-Karaj, Iran

Publications

Publications During the Ph. D. Study

- **Dastjerdi, R.,** Döll, K., Gerowitt, B., Steinmann, H. H., and Karlovsky, P. 2013. *Fusarium* species and mycotoxins in weed plants. 35th Mycotoxin Workshop. Ghent, Beljium.

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Other Publications

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