Fusarium verticillioides and fumonisin management in maize in Ethiopia

Tiltak mot *Fusarium verticillioides* og fumonisin i mais i Etiopia

Philosophiae Doctor (PhD) Thesis

Hadush Tsehaye Beyene

Department of Plant Sciences
Faculty of Veterinary Medeicine and Biosciences
Norwegian University of Life Sciences

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PhD Supervisors:

Professor Anne Marte Tronsmo

Norwegian University of Life Sciences, Department of Plant Sciences, P.O. Box 5003, NO-1432 Ås, Norway

Professor May Bente Brurberg

Norwegian Institute for Bioeconomy Research, Biotechnology and Plant Health Division, P.O. Box 115, NO-1431 Ås, Norway

Professor Leif Sundheim

Norwegian Institute for Bioeconomy Research, Biotechnology and Plant Health Division, P.O. Box 115, NO-1431 Ås, Norway

Professor Arne Tronsmo

Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science, P.O. Box 5003, NO-1432 Ås, Norway

Dr. Dereje Assefa

Mekelle University, Department of Dryland Crop and Horticultural Sciences, P.O.Box 231, Mekelle, Tigray, Ethiopia

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Summary

Fusarium species are major threats to maize production worldwide, including in Ethiopia. Little has been known about the species composition and prevalence of *Fusarium* spp. as well as fumonisin contamination level of maize grains produced in different agroeclogical zones in Ethiopia. Some limited studies indicated that *F. verticillioides* is the most common pathogen in maize kernels in Ethiopia. However, there are no effective control strategies yet. Development of maize genotypes with adequate level of resistance and the use of biological control agents could be appropriate control measures to tackle the problem. Different sets of experiments were designed aiming at addressing the knowledge gaps related to *Fusarium* and fumonisin in maize in Ethiopia. To investigate the *Fusarium* species complex and fumonisin contamination associated with maize kernels, 200 samples were collected from 20 different maize growing areas in Ethiopia. *Fusarium* isolates were identified to species level, primarily based on morphological characters. Sequencing of the partial region of the translation elongation factor 1-alpha gene (*EF-1α*) was performed on representative isolates to support the morphological identification. Fumonisin contamination was investigated using Enzyme Linked Immunosorbent Assay (ELISA). Fumonisin production ability of 80 randomly selected *F. verticillioides* isolates, were tested on autoclaved maize cultures and Amplified Fragment Length Polymorphism (AFLP) analysis was used to study the genetic variation in these *F. verticillioides* isolates. A two years field experiment was conducted to evaluate Ethiopian maize cultivars for resistance to *F. verticillioides* and fumonisin conamination. The biocontrol potential of native *Trichoderma* species isolates were evaluated for ability to control *F. verticillioides* and fumonisin contamination both *in vitro* and under field condition.

The results showed that several fungi affect maize produced in Ethiopia. Eleven *Fusarium* spp. were identified to be associated with maize kernels, among these *F. verticillioides* was the predominant species, followed by the *F. graminearum* species complex. *Fusarium* species composition and relative prevalence differed greatly among the maize growing areas and agroecological zones. Fumonisin was detected on a large proportion (77 %) of the samples analysed, with concentrations ranging from 25 μg kg^{-1} to 4500 μg kg^{-1}. Proportion of kernels contaminated with fungi and fumonisin contamination varied among samples collected from the same areas as well as between maize growing areas and agroecological zones of Ethiopia. Highest fungal and fumonisin contamination were mainly recorded in samples collected from the areas with higher temperature and lower elevation. All *F. verticillioides* isolates examined

in this study produced detectable levels of total fumonisin in maize cultures. However, variation in fumonisin production ability was widespread (0.25 - 38 mg kg^{-1}) among isolates. AFLP analysis revealed genotypic variation among the *F. verticillioides* isolates in Ethiopia. Two clusters were identified using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) and Principal Coordinate (PCO) analysis, but there was no clear pattern of clustering of isolates into geographic regions of Ethiopia. *Fusarium verticillioides* isolates that produced the highest and lowest fumonisin levels were distributed throughout the different clusters in the dendrogram and PCO plots. Based on the Analysis of Molecular Variance (AMOVA), the *F. verticillioides* isolates are characterized by high variation between isolates within the same geographic region and very low differentiation between isolates from different regions.

Evaluation of Ethiopian maize cultivars for their resistance to *Fusarium* ear rot and fumonisin accumulation showed the presence of potential sources of resistance. A significant variation in resistance was detected between cultivars. Some maize cultivars consistently showed low level of ear rot severity and fumonisin contamination over the two years experiment, compared to other cultivars. However, none of the maize cultivars tested in this study were completely free from fumonisin contamination. The late maturing type maize cultivars had greater susceptibility to *Fusarium* ear rot and fumonisin contamination compared to early types. *Fusarium* ear rot severity and fumonisin concentration significantly differed between years, and fumonisin concentration in maize grains was positively associated with ear rot severity. Maize cultivars that showed the lowest ear rot severity and fumonisin accumulation may serve as source of resistance for introduction into agronomical elite materials.

Dual culture interaction *in vitro* and in field experiments using seed biopriming revealed that *Trichoderma* species isolates have the potential of suppressing the growth of the *F. verticillioides* as well as fumonisin contamination of maize grains. The *Trichoderma* isolates were growing fast, deterred further growth of the pathogen in the early days of co-inoculation, and later completely controlled *F. verticillioides* by growing over the pathogen after contact. Variation in hyphal growth inhibition and hyphal coiling frequency were observed among the different *Trichoderma* isolates. Among the *Trichoderma* isolates tested, *T. hamatum* isolates (Thm3, Thm6) and *T. harzianum* (Thr2, Thr5) were better in reducing *F. verticillioides* colonization and fumonisin contamination of maize kernels. These isolates may be used as an integral part of *F. verticillioides* and fumonisin management strategies.

Sammendrag

Forskjellige arter av *Fusarium* truer verdens maisproduksjon, inkludert Etiopia. Lite har vært kjent om artssammensetningen og utbredelsen av *Fusarium* spp. samt forurensning av mykotoksinet fumonisin i maiskorn produsert i de forskjellige agroøkologiske soner i Etiopia. Noen begrensede studier viste at *F. verticillioides* er det vanligste patogenet i maiskorn i Etiopia. Det er imidlertid ennå ingen effektive strategier for bekjempelse. Utvikling av mais-genotyper med tilstrekkelig resistens og bruk av biologiske bekjempelsesmidler kan være hensiktsmessige tiltak for å takle problemet. Ulike forsøk ble gjennomført med sikte på å dekke kunnskapshull knyttet til *Fusarium* og fumonisin i mais i Etiopia. For å undersøke komplekset av *Fusarium*-arter og fumonisin-forurensning i maiskorn, ble 200 prøver samlet inn fra 20 forskjellige dyrkingsområder for mais i Etiopia. *Fusarium*-isolater ble identifisert til artsnivå, i hovedsak basert på morfologiske karakterer. Sekvensering av deler av genet som koder for elongeringsfaktor 1-alfa (*EF-1α*) ble utført på representative isolater for å verifisere den morfologiske identifikasjonen. Fumonisin-forurensning ble undersøkt ved hjelp av ELISA (Enzyme Linked Immuno Sorbent Assay). Fumonisin-produksjon av 80 tilfeldig utvalgte *F. verticillioides* isolater ble testet i kulturer av autoklaverte mais, og AFLP (Amplified Fragment Length Polymorphism) analyse ble brukt for å studere den genetiske variasjon i disse *F. verticillioides* isolatene. Et to-årig feltforsøk ble utført for å evaluere etiopiske maissorter for resistens mot *F. verticillioides* og fumonisin-akkumulering i mais. Potensialet for biologisk bekjempelse med lokale *Trichoderma*-isolater ble evaluert ved å måle evnen til å kontrollere *F. verticillioides* infeksjon og fumonisin forurensning både *in vitro* og i feltforsøk.

Resultatene viser at flere sopparter angriper mais i Etiopia. Elleve *Fusarium* spp. ble funnet assosiert med maiskjerner, blant disse dominerte *F. verticillioides*, etterfulgt av artskomplekset *F. graminearum*. Det var forskjeller i artssammensetning og relative forekomst av *Fusarium*-arter mellom ulike dyrkingsområder for mais og mellom agroøkologiske soner. Fumonisin ble påvist i storparten (77 %) av de analyserte prøvene, og konsentrasjonene varierte fra 25 $\mu g\ kg^{-1}$ til 4500 $\mu g\ kg^{-1}$. Frekvensen av soppinfiserte maiskorn og fumonisinforurensning varierte mellom prøver fra de samme områdene, så vel som mellom dyrkingsområder for mais og melllom agroøkologiske soner i Etiopia. Det var mest soppinfeksjon og fumonisin i prøver samlet inn fra de varmeste områdene og laveste høyder over havet. Alle *F. verticillioides* isolerer som ble dyrket maiskulturer i laboratoriet i

denne studien produserte påvisbare nivåer av fumonisin. Men det var stor variasjon i fumonisin-produksjon (0,25 til 38 mg kg^{-1}) blant isolater. AFLP analyse viste betydelig genetisk variasjon blant *F. verticillioides* isolater i Etiopia. To grupper ble identifisert ved hjelp av UPGMA (Unweighted Pair Group Method with Arithmetic Mean) og PCO (Principal Coordinates Analysis) analyse, men det var ingen klare mønster i gruppering av isolater fra forskjellige geografiske regioner i Etiopia. *Fusarium verticillioides* isolater som produserte de høyeste og laveste fumonisinnivåer fordelte seg i ulike klynger i dendrogrammet og PCO analysen. Basert på analyse av molekylær varians (AMOVA), er *F. verticillioides* isolatene karakterisert ved høy variasjon mellom isolater innenfor samme geografiske region og svært lav differensiering mellom isolater fra ulike regioner.

Vurderingen av etiopiske maissorter for resistens mot *Fusarium*-råte i maiskolber og fumonisin-forurensning viste at det finnes potensielle resistenskilder. Det ble funnet et vidt spekter og betydelig variasjon i resistens mellom sorter. Gjennom to års feltforsøk viste noen maissorter konsistent lavt nivå av kolberåte og fumonisin-forurensning sammenlignet med andre sorter. Imidlertid var ingen av de maissortene som ble testet i denne undersøkelsen fullstendig uten fumonisin-forurensning. Sene maissorter var mer mottakelige for *Fusarium*-kolberåte og fumonisin-forurensning enn tidlige sorter. Angrepene av *Fusarium*-kolberåte og fumonisin-forurensning var statistisk signifikant forskjellige fra år til år. Fumonisin-konsentrasjon i maiskorn var positivt assosiert med angrepsgraden av kolbebråte. Maissortene som hadde minst angrep av kolberåte og fumonisin-akkumulering kan være kilde til resistens for innføring i agronomisk foredlingsmateriale.

Dyrkingsforsøk *in vitro* og feltforsøk med biologisk frøbeising viste at isolater av *Trichoderma*-arter har potensiale til å hemme veksten av *F. verticillioides* og redusere fumonisin-forurensning av maiskorn. *Trichoderma*-isolater vokser raskt, hemmer vekst av patogenet i tidlige stadier av dobbelkulturer, og senere stopper veksten av *F. verticillioides* ved å vokse over patogenet etter kontakt. Variasjon i veksthemming av sopphyfer og frekvens av *Trichoderma*-hyfer som vokser rundt patogen-hyfer ble observert mellom ulike *Trichoderma*-isolater. Blant de *Trichoderma*-isolatene som ble testet, var isolatene *T. hamatum* (Thm3, Thm6) og *T. harzianum* (Thr2, Thr5) mest effektive til å redusere *F. verticillioides* kolonisering og fumonisin-forurensning av maiskjerner. Disse isolatene kan bli brukt som en integrert del i bekjempelse av *F. verticillioides* og fumonisin forurensing av maiskjerner.

List of papers included in the thesis:

I. **Tsehaye, H.**, Brurberg, M. B., Sundheim, L., Assefa D., Tronsmo A., and Tronsmo, A. M. 2016. Natural occurrence of *Fusarium* species and fumonisin on maize grains in Ethiopia. (Accepted for publication with modifications: European Journal of Plant Pathology).

II. **Tsehaye, H.**, Elameen, A., Tronsmo, A. M., Sundheim, L., Tronsmo, A., Assefa, D. and Brurberg, M. B. 2016. Genetic variation among *Fusarium verticillioides* isolates associated with Ethiopian maize kernels as revealed by AFLP analysis. (Submitted for publication: European Journal of Plant Pathology).

III. **Tsehaye, H.**, Sundheim, L., Brurberg, M. B., Tronsmo, A., Assefa, D., and Tronsmo A. M. 2016. Fumonisin production by *Fusarium verticillioides* isolates from kernels of maize grown in Ethiopia. (Submitted for publication: African Journal of Microbiology).

IV. **Tsehaye, H.**, Brurberg, M. B., Tronsmo, A., Assefa, D., Sundheim, L., and Tronsmo, A. M. 2016. Evaluation of Ethiopian maize varieties for resistance to *Fusarium verticillioides* and fumonisin accumulation. (Manuscript).

V. **Tsehaye, H.**, Tronsmo, A. M., Sundheim, L., and Tronsmo, A. 2016. Biocontrol potential of native *Trichoderma* species against *F. verticillioides* and fumonisin contamination in field-grown maize. (Manuscript).

1. General introduction

1.1 The maize crop

Maize (*Zea mays* L.) is an annual plant, which belongs to the tribe Andropogoneae of the grass family Poaceae and the genus *Zea* (OGTR 2008). According to archeological and genetic analysis, maize originates in Central America, Mexican highlands. Maize was domesticated from the wild relative plant called teosinte (*Zea mays* subspecies *parviglumys*) 7000 – 10000 years ago (OGTR 2008; Ranum et al. 2014).

Maize is one of the most important cereal crops grown throughout the world over a wide range of environmental conditions. The crop has the potential to produce a great amount of dry matter per hectare (ha), and it is widely grown because of its easiness of cultivation, adaptability to different agroecological zones, versatile food uses and storage characteristics (Fandohan et al. 2003; Shiferaw et al. 2011). Worldwide maize production in 2014 was estimated to 1021million metric tons (FAO 2014). Maize plays an important role in the diet of millions of people; which supplies an energy density of 365 Kcal per 100 g, contains about 72 % starch, 10 % protein, and 4 % fat (Ranum et al. 2014). Maize is also extensively used for animal feed, and it can be processed into a number of industrial products including starch, sweeteners, oil, beverages, glue, industrial alcohol and fuel ethanol (Ranum et al. 2014; Shiferaw et al. 2011).

1.2 Maize in Ethiopia and production constraints

Maize is one of the most important cereal crops in Ethiopia, ranking first in total production (7.2 million tons) and second in area coverage (> 2 million ha) next to teff (*Eragrostis tef* Zucc) (FAO 2014). The crop is believed to be introduced to Ethiopia by Portuguese merchants during the 1600s - 1700s (Haffangel 1961), but currently it is one of the most widely grown crops in different agroecological zones of the country. The mid-altitude humid and sub-humid agroecological zones are the most important maize growing areas (Worku et al. 2012). The weather conditions characterized by warm temperature and adequate amount of rainfall in these zones create favorable conditions for maize cultivation. The crop is mainly produced under rain fed growing condition, and production has increased gradually in the past few years (Fig. 1).

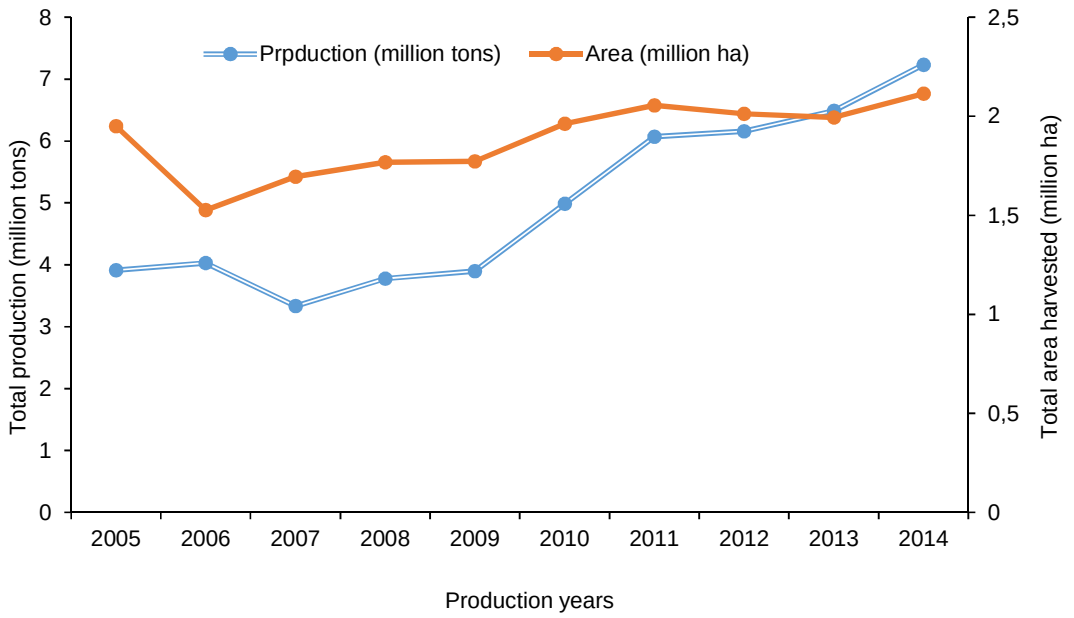


Fig. 1 Historical trends in area and production of maize in Ethiopia between 2005-2014. Source FAO 2014.

Almost all maize grain produced in Ethiopia is consumed as human food (Worku et al. 2012). The smallholder farmers, which comprise for about 80 percent of Ethiopia's population, are both the primary producers and consumers of maize in Ethiopia. Maize is the preferred and lowest cost source of cereal calories, providing 1½ times and 2 times the calories per dollar compared to wheat and teff, respectively (IFPRI 2010). Maize also plays an important role as animal feed and industrial raw material (Worku et al. 2012). Maize will remain a high priority crop to feed the ever-increasing population of the country. This requires a further increase in maize productivity and production in the country through intensification of maize production and reduction of yield losses.

Maize has a higher average yield potential per unit area (3.2 tonnes ha^{-1}), than any other crop in the country. However, the potential of this crop is not fully realized due to heavy pre- and post-harvest losses caused by diseases, insect pests and weeds (Worku et al. 2012). Lepidopterous stem borers that affect maize cause significant losses in Ethiopia, and insect damage also increase fungal infections and mycotoxin contamination. A number of fungal pathogens infect maize at different developmental stages, and all parts of the maize plant are susceptible to certain diseases (Tilahun et al. 2012). Tilahun et al. (2012) have reported more than 47 fungal diseases associated with maize. Much attention should be paid to disease of

the maize ear, because only healthy ears and kernels can ensure high grain yields and qualities. *Fusarium* species are the most common fungal contaminants and causes of ear rot in maize in Ethiopia (Ayalew 2010; Wubet and Abate 2004).

1.3 Significance of *Fusarium* species in maize

The genus *Fusarium* contains economically important plant pathogens, causing various diseases in agriculturally important crops such as maize. *Fusarium* species may cause seedling blight, root rots, stalk rots, ear and kernel rot on maize (Leyva-Madrigal et al. 2015; Logrieco et al. 2002; Munkvold 2003). Two types of ear rot in maize are caused by different *Fusarium* species. These are recognized as *Gibberella* ear rot, also referred to as red ear rot, and *Fusarium* ear rot (pink ear rot) (Das 2014; Logrieco et al. 2002; Mesterházy et al. 2012). *Fusarium* ear rot disease is recognized by white to light-pink cottony mycelium growth on kernels (Fig. 2), and the ear rot occurs on ear tips or as random individual kernels or groups of kernels in scattered areas on the maize ear (Logrieco et al. 2002; Munkvold 2003). Infected kernels also exhibit white streaks, known as "starburst" symptom, radiating from top of kernels (Das 2014). Reddish mold growth, starting from the ear tip and eventually cover large portion of the ear, is a typical symptom of *Gibberella* ear rot. Blue-black perithecia of the telemorph, *G. zeae* can be observed on infected husks and ear shanks (Das 2014). *Fusarium* ear rot is predominantly caused by *F. verticillioides* (Sacc.) Nirenberg. *Fusarium proliferatum* (Matsush.) Nirenberg, and *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun, & Marasas have also been associated with *Fusarium* ear rot (Logrieco et al. 2002; Munkvold 2003). *Gibberella* ear rot is mainly caused by *F. graminearum* (Schwabe), but it may also be caused by other *Fusarium* species including *F. culmorum* (Wm.G. Sm.) Sacc., *F. cerealis* (Cooke) Sacc. and *F. avenaceum* (Fr.) Sacc. (Logrieco et al. 2002; Mesterházy et al. 2012; Munkvold 2003). *Fusarium* ear rot predominates in warmer and drier areas/years, whereas *Gibberella* ear rot has been observed more frequently in cooler areas, and the pathogen requires high humidity from silking to harvest for its development (Dorn et al. 2011; Goertz et al. 2010; Logrieco et al. 2002).

Infection of maize by *Fusarium* species may result in premature death of plants, by interfering with the translocation of water and nutrients to upper plant parts, causing yield losses and reduce grain quality (Presello et al. 2008; Williams et al. 2007). The main concern associated with maize ear rot diseases is that some *Fusarium* species produce secondary metabolites

known as mycotoxins, which render the grain inedible or toxic to humans and domestic animals (Marín et al. 2013; Reddy et al. 2010; Waśkiewicz et al. 2012a). Colonization of maize grains by *Fusarium* ear rot fungi may result in contamination of grains with high level of fumonisins, as well as lower levels of fusarins, fusaric acid, moniliformin and beauvericin, depending on the species involved (Brown et al. 2012; Darnetty and Salleh 2013). *Gibberella* ear rot disease may lead to contamination with deoxynivalenol, nivalenol and zearalenone (Logrieco et al. 2002; Mesterházy et al. 2012). Some data suggested that over 25 % of the world's food crops are affected by mycotoxin contamination each year, with *Fusarium* species playing the significant role for food contamination (FAO 2013).

Fig. 2 Typical symptoms of *Fusarium* ear rot in maize after inoculation with *F. verticillioides*. Photo: Hadush Tsehaye.

1.4 Taxonomy and species concept in *Fusarium*

Fungi in the genus *Fusarium* have been known since 1809 (Summerell et al. 2010). The name *Fusarium* is derived from the Latin word *fusus*, meaning a spindle. The taxonomy of the genus *Fusarium* is complex, and the number of species recognized in the genus has changed (Snyder and Hansen 1945; Nelson et al. 1983; Leslie and Summerell 2006; Summerell and Leslie 2011). The difficulties and unstable taxonomic history of the genus were mainly due to the application of different taxonomic systems that were not standardized (Moretti et al. 2009). Accurate identification requires the use of different suitable markers (Summerell and Leslie 2011). Based on detailed morphological, molecular and biological markers Leslie and Summerell (2006) described 70 different *Fusarium* species, and the number is increasing with the continuous discovery of new species. Three basic concepts are being used to identify

species in *Fusarium.* These are recognized as the morphological, biological and phylogenetic species concepts (Leslie and Summerell 2006).

The morphological species concept uses physical and physiological characters to distinguish *Fusarium* species. A species is a morphologically cohesive group, and members possess morphological characters, that distinguish them from other groups (Leslie and Summerell 2006; Summerell et al. 2010). Differences in shape and size of macroconidia, microconidia and chlamydospores, as well as the presence or absence of these characters, are given the greatest weight when recognizing species morphologically (Leslie and Summerell 2006; Nelson et al. 1983). In addition to these, careful assessment of morphological features such as conidiogenous cells (monophialide or polyphialides), conidial chains and secondary characteristics like color and pigmentation have been employed for morphological classifications of *Fusarium* species (Leslie and Summerell 2006; Nelson et al. 1983). In order to obtain the above morphological structures and identify species correctly, growing isolates on appropriate culture medium and incubation under specific conditions (alternating near UV light and darkness) that promote the development of morphological features is required. The main limitation with morphological species concepts is that the numbers of distinct morphological characters are often limited compared to the great number of species that need to be distinguished (Leslie and Summerell 2006). Differences in morphological structures, such as conidial shape and size, may also be dependent on environmental conditions. Despite these limitations, morphological structures remain as important components of the *Fusarium* species concept, because of their widespread practical use. This could perhaps the primary option especially in resource-limited developing countries, which do not get access to molecular and biological analytical tools.

Biological species refers to groups that have actually or potentially interbreeding individuals, and members of the group share a gene pool, but they are isolated reproductively from other populations (Leslie and Summerell 2006). The biological species concept has been used successfully when tester strains are available. The tester strain can be used in crosses with unidentified isolates to determine whether the unidentified strain is a member of the same biological species or not (Leslie 1995). Defining a biological species for homothallic *Fusarium* species is difficult, due to substantial outcrossing penomena (Leslie and Summerell 2006).

The phylogenetic species concept utilizes molecular markers, usually differences or quantitative measures of genetic relatedness generated based on DNA sequences of selected genes, for defining a species (Summerell et al. 2010). The concept uses genealogies of one or more genes to identify fungal species. Genes commonly used for genealogical concordance phylogenetic species recognition includes ribosomal RNA genes (internal transcribed spacer), intron-poor protein coding genes (polymerases I and II) and intron-rich protein coding genes (translation elongation factor 1-alpha) (Geiser et al. 2004; Taylor et al. 2000). The partial translation elongation factor 1-alpha (*TEF*) gene is widely used for molecular identification of *Fusarium* species. This gene occurs as a single copy in *Fusarium* and shows a high level of sequence polymorphism among closely related species (Geiser et al. 2004; O'Donnell et al. 1998). The phylogenetic species concept may solve the problems with mating (homothallic) and morphological characters associated with biological and morphological species concepts (Taylor et al. 2000).

1.5 Biology and ecology of *Fusarium verticillioides*

1.5.1 Host range and geographic distribution

Fusarium verticillioides is widely distributed throughout the world, mainly under tropical and subtropical environmental conditions. The host range of *F. verticillioides* is broad, but it is mainly associated with maize worldwide (Picot et al. 2010). This fungus has been recovered from a number of important crop plants including sorghum, rice, millet, wheat, beans, sugarcane, cotton, banana, tomato, peanut, pineapple, suger beets, soybean, figs, flax, stone fruits and several grasses (Bacon and Nelson 1994; Das 2014; Scott 2012; Summerell et al. 2010). *Fusarium verticillioides* is recognized as a systemic endophyte and non-obligate plant pathogen (Bacon et al. 2008). Most non-obligate plant pathogens kill their host cells prior to infection and obtain nutrients from nonliving tissue. Depending on the environmental conditions, the endophytic phase varies between a hemibiotroph pathogenic and symptomless biotrophic state (Bacon et al. 2008).

1.5.2 Temperature and water availability

Environmental factors, especially water availability and temperature, significantly affect the pathogen's life cycle (survival, germination, growth and reproduction). *Fusarium verticillioides* is common in warmer and drier areas (Munkvold 2003; Picot et al. 2010). *Fusarium verticillioides* can grow over a wide range of temperatures, but only under higher

water activity (a_w > 0.9) (Marín et al. 2013). Maximum *F. verticillioides* growth has been reported at water activity (0.97 - 0.98) and temperatures between 25 - 30 ℃ (Jurado et al. 2008; Marín et al. 2004). In general, low temperature and water stress often reduce *F. verticillioides* growth, while high temperature and water availability promote higher fungal growth (Jurado et al. 2008; Marín et al. 1999).

1.5.3 Morphological futures

Fusarium verticillioides is characterized by the absence of chlamydospores, and conidia are formed inside the hyphae or conidiophore through an entroblastic process. *Fusarium verticillioides* has small, single-celled, abundant microconidia in long chains (Leslie and Summerell 2006). The conidia are oval to club shaped with a flattened base, and zero-septate. Conidial chains of *F. verticillioides* are produced from monophialides in V-shaped pairs and false heads (Nelson et al. 1983). Macroconidia vary from slightly falcate or sickle-shaped to straight, slender; with the dorsal and ventral surface almost parallel (Nelson et al. 1983). Culture characteristics and pigmenttaion on PDA are variable, ranging from grayish orange to violet grey and dark violet (Leslie and Summerell 2006).

1.5.4 Reproduction and genetics

Fusarium verticilloides has a genome size of about 42 Mb and 11 chromosomes (Ma et al. 2010). The predicted number of genes for this fungus is estimated to be about 14, 179 (Ma et al. 2010). This fungus is a hetrothallic (self-sterile) species, and sexual reproduction in such fungal species requires contribution (cell fusion) from two strains belonging to opposite mating type idiomorphs, which are recognized as *MAT-1* and *MAT-2* (Yun et al. 2000). Asexual reproduction in *Fusarium* is believed to be more frequent than sexual, due to the unequal relative frequency of *MAT-1* and *MAT-2* alleles as well as limited number of female fertile strains (Leslie and Klein 1996). Successful sexual crossing and subsequent perithecial development depends on the compatibility of ‘‘+’’ and ‘‘-’’ (male and female) nucleus carrying the opposite mating type alleles (Leslie and Klein 1996; Leslie and Summerell 2006). In heterothallic fungi, the ascus usually contains eight ascospores with four of them inherited from each opposite mating type. Generally, sexual recombination is believed to be the main source of genetic variation in fungal pathogens (McDonald and Linde 2002).

Gibberlla is the name given for the sexual stage (telemorph) of many *Fusarium* species (anamorphs). The sexual (telemorph) stage of *F. verticillioides* is *Gibberella fujikuroi* mating

population A (Leslie 1995). Individuals within a given mating population can be classified into different vegetative compatibility groups (VCGs) based on their ability to form heterokaryons (cells containg different nucli) with one another (Leslie et al. 1992). Strains belonging to the same VCG may be clones of one another, while strains that are in different VCGs are genetically distinct. Most of the strains in a *F. verticilloides* population are in different VCGs and hence, clones in these populations are rare (Leslie and Kelein 1996; Leslie and Summerell 2006).

1.6 Disease cycle of *Fusarium* ear rot

1.6.1 Survival and sources of inoculum

Infected seeds and crop residue are the sources of inoculum for infecting the succeeding crop plants. *Fusarium* species survive on crop residue as mycelium or other survival structures, including chlamydospores on the soil surface, in periods without host plants (Munkvold 2003; Leplat et al. 2013). Unlike other *Fusarium* species, *F. verticillioides* do not produce chlamydospores, but it can produce thickened hyphae, which prolong its survival capabilities (Leslie and Summerell 2006). Mycelium in infected crop residues can produce several types of infectious propagules such as macroconidia and microconidia (Das 2014; Mesterházy et al. 2012). Microconidia are abundant in *F. verticillioides* and believed to be important for survival and dispersal of the pathogen (Leslie and Kelein 1996). Infected stalks partially buried in the soil are the major overwintering sites and source of *F. verticillioides* inoculum for infection of maize plants (Cotten and Munkvold 1998).

1.6.2 Dispersal and infection process

Fusarium species are known to disperse through various pathways, including dispersal with infected seeds by human transport, movement in soils with water and dispersal as air blown spores (Munkvold 2003; Summerell et al. 2010). Infection may also occur through silks, via wounds created by insects or birds and/or systemically through roots (Mesterházy et al. 2012; Munkvold 2003). *Fusarium verticillioides* and other *Fusarium* species, causing *Fusarium* ear rot, are dispersed primarily by microconidia, because these propagules are abundantly produced and the small size makes wind-blown dispersal easy. Macroconidia seems suitable for water-splash dispersal rather than wind dispersal (Munkvold 2003). The overall disease cycle of *Fusarium* ear rot is illustrated in Fig. 3.

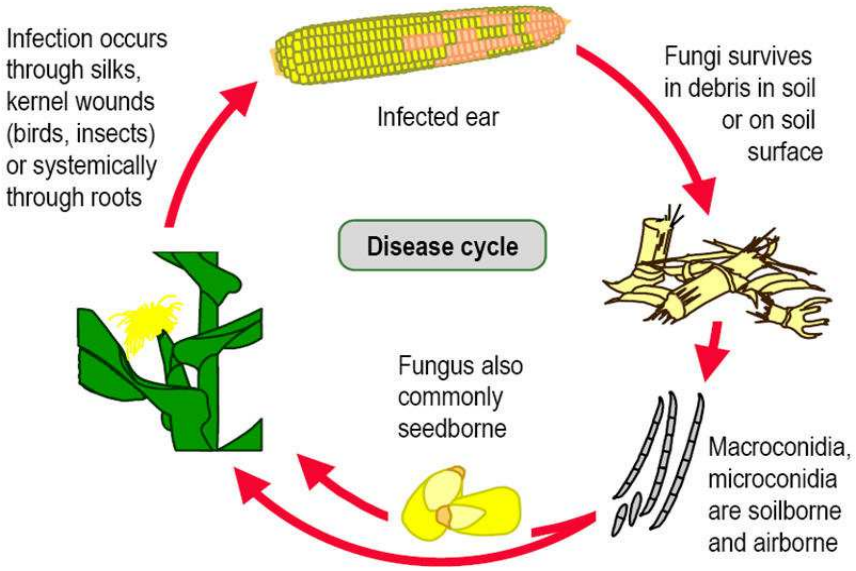


Fig. 3 Disease cycle of *Fusarium* ear rot showing the survival and various infection pathways (www.pioneer.com).

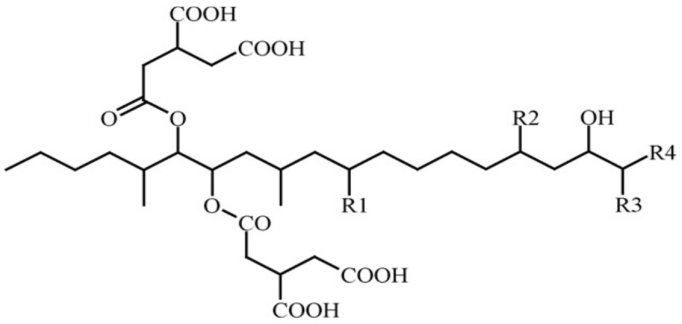
1.7 Fumonsins

Fumonisins are mycotoxins produced by a number of *Fusarium* species, including *F. verticilloides, F. proliferatum, F. temperatum, F. oxysporum, F. globosum, F. napiforme, F. delamine* and *F. nygamai* (Rheeder et al. 2002; Scott 2012; Waśkiewicz et al. 2012a). Fumonisins are also produced by strains of *Alternaria alternate f. sp. lycopersici* (Rheeder et al. 2002) and *Aspergillus niger* (Frisvad et al. 2007). However, *F. verticillioides* and *F. proliferatum* are the primary producers of fumonisins in maize kernels, globally (Picot et al. 2010).

The fumonisin mycotoxins were first isolated in 1988 from *F. verticillioides* cultures in South Africa by Gelderblom et al. (1988) and Bezuidenhout et al. (1988) characterized the molecular structure in the same year. Fumonisins are polar compounds, soluble in water and aqueous solutions of methanol and acetonitrile, but they are not soluble in non-polar solvents (IARC 2002).

At least 28 different fumonisin analogs have been reported from cultures and grain samples (Rheeder et al. 2002). These chemically related secondary metabolites (Fig. 4) has been classified into four main groups, recognized as fumonisins A, B, C, and P series (Rheeder et al. 2002; Waśkiewicz et al. 2012a). Recently new fumonisins have been discovered, such as

the FBX and related metabolites, by LC-MS/MS in *F. verticillioides* cultures (Bartók et al. 2006). The fumonisin B series (FB_1, FB_2 and FB_3) are the most important, naturally occurring compounds; with fumonisin B_1 accounting for 70 - 80 % of the total fumonisin in *F. verticillioides* cultures and in naturally contaminated foods (Rheeder et al. 2002; Waśkiewicz et al. 2012a).



	R_1	R_2	R_3	R_4
FA_1	OH	OH	CH_3CONH	CH_3
FA_2	H	OH	CH_3CONH	CH_3
FB_1	OH	OH	NH_2	CH_3
FB_2	H	OH	NH_2	CH_3
FB_3	OH	H	NH_2	CH_3
FB_4	H	H	NH_2	CH_3
FB_5	OH	H	NH_2	CH_3
FC_1	OH	OH	NH_2	H
FC_3	OH	H	NH_2	H
FC_4	H	H	NH_2	H
FD*	H	H	H	H
FP_1	OH	OH	3-hydroxypyridine	H
FP_2	H	OH	3-hydroxypyridine	H
FP_3	OH	H	3-hydroxypyridine	H

*Hydroxy group between R_1 and R_3 replaced by hydrogen atom.

Fig. 4 Structures of different groups of fumonisins (Zöllner and Mayer-Helm, 2006).

Fumonisins are the most common contaminants of maize and maize based food and feed products worldwide (Marín et al. 2013; Picot et al. 2010; Waśkiewicz et al. 2012a). Fumonisins have also been reported from other commodities such as, sorghum, rice, wheat, soybean, cowpea, beans (navy, mung, adzuki), coffee, grapes, figs and asparagus (Scott 2012; Waśkiewicz et al. 2012a; Zöllner and Mayer-Helm, 2006). However, the contamination level in these crops is usually lower than that of maize. Fumonisins are resistance to thermal degradation, they cannot be destroyed by cooking, and they can, therefore, easily enter the human food chain (Shephard et al. 2012). These points to the importance of testing human and animal foodstuffs for the presence of fumonisins to avoid the associated health hazards.

1.7.1 Animal and human mycotoxicity

Toxicological studies of FB_1 have showed that fumonisin could cause a number of animal diseases including leukoencephalomalacia in horses (Marasas et al. 1988), pulmonary edema and hydrothorax in swine (Haschek et al. 2001), nephrotoxic, hepatotoxic and hepatocarcinogenic in rats (Gelderblom et al. 1996; Scott 2012). Horses and swine are the animal species most susceptible to fumonisin (Voss et al. 2007). Consumption of fumonisin contaminated maize has been associated with a high rate of esophageal cancer (Rheeder et al. 1992) and neural tube defects (Missmer et al. 2006). Fumonisin B_1 is classified as ‘‘possible carcinogenic to humans’’ (group 2B carcinogen) by the International Agency for Research on Cancer (IARC) (IARC 2002). Animal and human health problems related to these mycotoxins are almost exclusively associated with the consumption of contaminated maize or products made from maize (Marín et al. 2013; Reddy et al. 2010; Rheeder et al. 2002).

The toxicity of fumonisin are explained by their ability to disrupt the sphingolipid metabolism through inhibition of the enzyme ceramide synthase, an enzyme that catalyses the acylation of sphinganine and recycling of sphingosine (Marín et al. 2013; Wang et al. 1991). The consequence of this inhibition includes disruption of the synthesis of sphingolipids as well as increases intracellular accumulation of free sphinganine and sphingosine, which initiate a complex of events that may cause toxicity of cells (Soriano et al. 2005; Voss et al. 2007).

1.7.2 Role of fumonisin on plant pathogenesis

The role of fumonisins in plant pathogenesis during *F. verticillioides* colonization of maize is not clear. Some earlier evidences indicate that fumonisins might impose some phytotoxic activity on maize seedlings (Doehlert et al. 1994; Lamprecht et al. 1994). Severe disease symptoms, such as necrotic leaf lesion, seedling blight and stunting, have been reported, when maize seedlings are inoculated with fumonisin producing *F. verticillioides* strain (Williams et al. 2007). Myung et al. (2012) also observed the presence of lesions coincided with accumulation of fumonisin in plant tissue. Fumonisin B_1, produced by *F. verticillioides*, was also observed to modulate maize β-1,3-glucanse activity, which is known to be involved in plant defense responses (Sánchez-Rangel et al. 2012). Contrary to these results, a maize ear inoculation study, using fumonisin-nonproducing mutant strains, showed that fumonisins are not required for pathogenesis, since the mutant strains were as aggressive as the fumonisin-producing parent strains to infection of maize ears and causing ear rot symptom (Desjardins et al. 2002). It has also been observed that fumonisin B_1 producing and nonproducing *F.*

verticillioides strains did not differ in their ability to cause seedling blight (Desjardins et al. 2007). Thus, the importance of fumonisins in pathogenesis on maize is not clear.

1.7.3 Analytical detection and quantification methods

Analytical methods available to detect and quantify fumonisins in food matrix includes: thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), capillary chromatography gas coupled with mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC-MS/MS) (Scot et al. 2012; Turner et al. 2009; Waśkiewicz et al. 2012a). Many of the above methods demand high technical knowledge of experts and laboratory equipment. Thus, alternative methods based on antibodies such as ELISA have been introduced for easy estimation of fumonisins in large number of samples (Goryacheva et al. 2007). These immunochemical methods for fumonisin analysis have become popular, and the method of choice because of simple application, quicker and relatively low cost compared to the other chromatographic methods (Turner et al. 2009). The limitation of competitive ELISA kits is that they are for single use, and possess a limited detection range due to the narrow sensitivity of the antibodies, thus the cost increases for bulk sample testing (Turner et al. 2009).

1.7.4 Legislation and maximum tolerable limits

To address food safety concerns due to fumonisins several countries have developed regulations for maximum tolerable limit of the toxin in food and feed (EC 2007; FDA 2001; FSA 2007). The European Union has set maximum limits of 4 mg kg^{-1} for total fumonisin in unprocessed maize, 1 mg kg^{-1} in maize and maize-based foods intended for direct human consumption, 0.8 mg kg^{-1} in maize-based breakfast and snacks, and 0.2 mg kg^{-1} in Baby foods (EC 2007). The recommended levels of fumonisins concentration in animal feed is 5 mg kg^{-1} for equines, 20 mg kg^{-1} for swine, 60 mg kg^{-1} for cattle being raised for slaughter, and 100 mg kg^{-1} for poultry being raised for slaughter (FDA 2001; Voss et al. 2007). Currently, there are no established guidelines in Ethiopia for maximum tolerable limits of fumonisins allowed in food and animal feed. Researchers in Africa have been suggesting that the provisional maximum tolerable limit should be based on detailed knowledge of use and taking into account maize consumption in various communities (Shephard et al. 2013).

1.8 Factors affecting *Fusarium* infection and fumonisin production

Fusarium infection and fumonisin production in maize are influenced by several biotic and abiotic factors and their interaction (Miller 2001; Parsons and Munkvold 2010; Picot et al. 2010). The genetic makeup of the pathogen is important for fumonisin production (Seo et al. 2001; Sagaram et al. 2006).

1.8.1 Genetic makeup of the pathogen strains

In *F. verticillioides*, fumonisin production is controlled by a collection of genes recognized as the fumonisin biosynthetic gene (*FUM*) cluster, which consist of 17 coregulated genes (Proctor et al. 2003; Visentin et al. 2012). Isolates that have this gene cluster in their genetic constitution are able to produce fumonisin while those who lack this gene cannot synthesize the toxin. Previous molecular studies indicate critical roles of *FUM1*, *FUM6*, *FUM8* and *FUM21* in FB_1 biosynthesis; disruption resulted in significant reduction in FB_1 synthesis (Proctor et al. 2003; Seo et al. 2001; Visentin et al. 2012). Several additional genes that do not exist in the *FUM* gene cluster appear to influence fumonisin biosynthesis both positively and negatively. Some of the genes that are known to regulate fumonisin biosynthesis include *FCC1, FCK1, PAC1, ZFR1, GBP1, GBB1, CPP1, AREA, FST1* and *FvVE1* (Picot et al. 2010; Sagaram et al. 2006).

1.8.2 Temperature and moisture availability

Moisture and temperature conditions during the growing season, as well as during storage are key environmental factors for growth of *Fusarium* spp. and fumonisin contamination (Picot et al. 2010; Pitt et al. 2013; Waśkiewicz et al. 2012b). The optimal temperature for fumonisin production by *F. verticillioides* is in the range of 20 °C to 30 °C (Marín et al. 2004; Samapundo et al. 2005), and no fumonisin production was observed below 10 °C (Marín et al. 1999). Optimal water activity (a_w) for fumonisin biosynthesis were determined to be 0.95 - 0.99 a_w (Lazzaro et al. 2012; Marín et al. 1999). The effect of temperature seems more pronounced when a_w is lower than the optimum for growth of the pathogen (Samapundo et al. 2005). Expression of *FUM* gene associated with fumonisin biosynthesis has been found to be markedly induced at 20 °C, under suboptimal condition for fungal growth and in response to increasing water stress (Marín et al. 2010). Water stress may be an important factor for fumonisin buildup in field when water availability decreases due to changes in rainfall patterns (Jurado et al. 2008). On the other hand, wet conditions in the grain after harvest

creates favorable condition for fungal growth and fumonisin contamination (Cao et al. 2014; Mesterházy et al. 2012). Bush et al. (2004) found maximal fumonisin content at 20 % kernel moisture while the toxin was not detected, when kernel moisture content was higher than 35 %. Maize kernels that was not reach physiological maturity accumulated no fumonisins. Generally, warm and dry conditions during the early grain filling stage, together with decline in precipitation appear to be favorable for fumonisin contamination of maize in the field (Cao et al. 2014; Miller 2001; Pascale et al. 2002; Shelby et al. 1994).

1.8.3 pH and nutrient factors of the substrate

Nutritional conditions, such as sugar availability, appear to be important for fumonisin production, since positive relationship is demonstrated between fumonisins production and sugar concentration (Jiménez et al. 2003). *In vitro* studies indicated that sugar sources, especially amylopectin, are key factors that modulate the fumonisin biosynthesis (Bluhm and Woloshuk 2005; Waśkiewicz et al. 2012b). Similarly, a decrease amino-acid concentration (N-depletion) induces *FUM* gene expression and increases fumonisin production by *F. verticillioides* (Jiménez et al. 2003; Kohut et al. 2009). Fumonisin biosynthesis is also influenced by pH. Low pH is usually required for optimal fumonisin production (Keller et al. 1997), whereas lower level of fumonisin has been observed under high pH (alkaline) condition (Flaherty et al. 2003). In the field, low pH and high amylopectin content may be readily develop in decaying host tissues where starch is being metabolized (Picot et al. 2011).

1.8.4 Insect damage

Kernels wounded by insect feeding are susceptible to *Fusarium* infection and fumonisin contamination (Cao et al. 2014; Mesterházy et al. 2012; Miller 2001). Insects serve as vectors, transferring inoculum between plants or causing wounds and enabling entry of the fungus into the plant. A variety of insect species has been reported as agents in the dispersal of *F. verticillioides* and increase of fumonisin contamination. Some of the most frequently reported insect pests in this regard are the maize stem borers (*Ostrinia nubilalis* and *Sesamia nonagrioides*) (Fandohan et al. 2004; Folcher et al. 2009), Angoumois grain moth (*Sitotroga cerealella*) (Cao et al. 2014) and thrips (*Frankliniella occidentalis*) (Parsons and Munkvold 2010). Blandino et al. (2009) found up to 67 % reduction in fumonisin concentration in plots where the European corn borer was controlled. A significant reduction in mycotoxin (trichothecenes, fumonisins and zearalenone) levels has observed, when maize stalk borers were controlled by insecticide treatments (Folcher et al. 2009).

1.9 *Fusarium* ear rot disease and fumonisin management

1.9.1 Host plant resistance

Breeding maize for genetic resistance is the most effective way to control maize ear rot and fumonisin contamination. Maize hybrids and inbred lines often differ in their response to *Fusarium* infection and fumonisin accumulation (Eller et al. 2008; Löffler et al. 2010; Small et al. 2012). Two resistance mechanisms against *Fusarium* infection are known, namely resistance to initial penetration and resistance to the spread of the pathogen in host tissue (Mesterházy et al. 2012). Resistance to fumonisin contamination is heritable and controlled by several gene regions in maize (Robertson et al. 2006). Resistance to fumonisin contamination is not significantly affected by the pollen source; thus, evaluations can be effectively performed on open-pollinated plants (Starr et al. 2006). Hybrid vigor is an important disease resistance parameter, and a 27 % reduction in ear rot and 30 % lower fumonisin content have been recorded in hybrids compared to their inbred parents (Hung and Holland 2012). Maize varieties with a long vegetation period are considered most susceptible to ear rot and fumonisin contamination (Battilani et al. 2008; Löffler et al. 2010).

In maize genotypes, several morphological and genetic factors have been reported as important for resistance to ear rot and fumonisin contamination. Maize genotypes with good husk cover exhibit less fumonisins (Cao et al. 2014); husk leaves that extend beyond the ear tip and adhere tightly to the developing ear excludes insects that facilitate fungal infection (Butrón et al. 2006). An exposed ear may be more vulnerable to ear rot than one ear enclosed in the husk. Contrary to these results, earlier studies reported that maize cultivars with tight husk cover were more susceptible to *Fusarium* infection, because of slow drying which favors *Fusarium* growth (Fandohan et al. 2004; Warfield and Davis 1996). Maize genotypes with a predisposition for kernel-pop, silk-cut or lateral splits in the kernel pericarp are at greater risk for *Fusarium* infection (Odvody et al. 1997). Similarly, maize inbred lines with softer endosperm (dent) are believed to be more susceptible to fumonisin accumulation than inbred lines with flinty endosperm (Desjardins et al. 2005; Santiago et al. 2013). In contrast to this, Czembor and Ochodzki (2009) and Löffler et al. (2010) found higher fumonisin content in flint than in dent genotypes of maize. However, the tested sets of genotypes in these cases were not the same and may not be contradictory. Production of various defense substances such as accumulation of flavonoids, phenolic compounds and phytoalexins (Sekhon et al. 2006) as well as high levels of phenylpropanoids in their pericarp (Sampietro et al. 2013) have

been related to reduced disease severity and lower fumonisin contamination in maize. Crop plants that are locally adapted and resistant/tolerant to stress factors are generally resistant to toxigenic fungi and fumonisin contamination. While cultivars grown outside their adaptation range appear to be susceptible to toxigenic fungi and accumulate more fumonisins (Doko et al. 1995). Cultivars that display low visual ear rot disease severities often results in lower fumonisin contamination (Bolduan et al. 2009; Robertson et al. 2006).

1.9.1.1 *Inoculation methods and screening for resistance*

Selection of resistant genotypes often relies on artificial inoculation methods, performed either using one well characterized isolate or a mixture of fungal strains. Natural infestations are not as severe as infestations after artificial inoculations, and ear rot disease epidemics are also sporadic in nature (Mesterházy et al. 2012). Maize is most susceptible to *Fusarium* infection during the R_2 (blistering) growth stage, and susceptibility decreases in later developmental stages (Clements et al. 2003). During the R_2 growth stage, kernels are very small and white in color, and the fluid that fills the kernels is clear in color. This stage begines 10 – 14 days after silking. The cob size is nearly complete, and silk begin to dry and darken to a brown color (Ritchie et al. 1993). Thus, to differentiate genotypic differences for resistance, artificial inoculations have been performed at this susceptible growth stage to ensure enough infection and uniform distribution of the pathogen among plants throughout the field. Depending on major modes of fungal entry into the maize ear, two distinct inoculation methods have mainly been used. One of the techniques simulates fungal entry through the silk by injecting a conidial suspension into the silk channel of maize ears (Eller et al. 2008). The other technique encourages fungal entry into kernel wounds by injecting a conidial suspension into artificially wounded kernels (Chungu et al. 1996). Husk penetration (kernel wounding) mimics natural inoculation by insect and silk channel injection mimics spores splashed onto silks by rain, or carried by wind blow (Eller et al. 2008). With the silk channel inoculation, the infection first proceeds down the silk to the kernels, whereas in the other technique infection results in the spread of the fungus from infected kernels to neighboring kernels (Mesterházy et al. 2012). Comparison of different inoculation methods indicated that penetrating husks with pin bars and injecting inoculum down the silk channel were best able to discriminate different levels of resistance to fungal infection and fumonisin accumulation (Chungu et al. 1996; Clements et al. 2003). Silk infection is the predominant pathway for kernel infection; therefore, artificial silk inoculation might be the appropriate

method for evaluating genetic resistance to *F. verticillioides* infection and fumonisin accumulation (Mesterházy et al. 2012).

1.9.2 Biological control methods

Biological control is a sustainable solution for plant disease control, since its effect is long-term with few undesirable side effects compared to other pest control options (Bandyopadhyay et al. 2003; Vinale et al. 2008). Several fungal and bacterial strains have been identified as biological control agents of plant pathogens. Bacterial strains belonging to *Bacillus*, *Agrobacterium*, *Pseudomonas*, and *Streptomyces*, and fungi in the genera *Trichoderma* and *Gliocladium* are widely used biocontrol agents for the control of plant pathogens (Pereira et al. 2010; Vinale et al. 2008). *Trichoderma* species are the most common fungal biocontrol agents that have been studied, and they are deployed in several countries for the control of plant pathogens. Promising control of fumonisin producing *F. verticillioides* have been reported in several studies, using different *Trichoderma* spp. including *T. harzianum*, *T. pseudokoningii* and *T. atroviride* (Bandyopadhyay et al. 2003; Chandra Nayaka et al. 2010; Ferrigo et al. 2014; Sempere and Santamarina 2009). The antagonistic effects of *Trichoderma* spp. against plant pathogenic fungi are through several mechanisms, including production of volatile and non-volitile antibiotics to suppress target pathogens, competition for space and nutrients, hyperparasitism and production of lytic enzymes that result in killing of the pathogen (Howell 2003; Vinale et al. 2008). Some *Trichoderma* stains can also reduce damage from biotic and abiotic stresses by promoting plant growth, or by inducing host resistance (Mastouri et al. 2010; Vinale et al. 2008).

Endophytic bacterial strains such as *Bacillus amyloliquefaciens* and *Enterobacter hormaechei* have been observed to provide good control of *F. verticilloides* and fumonisin (Pereira et al. 2010). Some bacterial strains (*Sphingopyxis* sp.) isolated from soil are capable of removing mycotoxins during digestion by enzymatic detoxification. This process is recognized as biotransformation, and the commercial product *FUMzyme* is capable of degrading FB_1 into non-toxic form (HFB_1) in the gastrointenal tract of animals (Heinl et al. 2010).

1.9.3 Cultural practices

Cultural practices employed for the control of *Fusarium* infections and mycotoxin contamination are directly related to the epidemiology of the pathogen. Severe *Fusarium* infection in maize is usually associated with continuous maize monocropping or growing

maize in short rotations with wheat and vice versa (the other way round) (Munkvold 2003). Repeated planting of maize and other cereal crops in the same field may favor *Fusarium* infection by increasing the fungal inoculum (Fandohan et al. 2004). Since the sources of inoculums are infected seed, soil, and crop residue, management practices based on crop rotation with non-host crops and reduction of inoculum using tillage practices are among the tactics used for mycotoxin management (Munkvold 2003). Management of infected crop debris can reduce the inoclum in subsequent seasons. Tillage hides the inoculum in infected residue and prevents splash dispersal, enhances decomposition, and thus reduces survival of the inoculum (Leplat et al. 2013). Increased fumonisin contamination has been associated with delayed harvest, due to late-season rains. Early harvest may help reduce the level of fumonisin contamination, in years conducive for fumonisin contamination (Cao et al. 2014; Munkvold 2003).

Creating suitable growing condition or avoiding stress factors for the plant are strategies to reduce fungal infection. *Fusarium verticillioides* causes little damage to kernels and produces little fumonisin under good growing conditions for the maize plant (Pitt et al. 2013), whereas stress conditions usually enhance fungal infection and fumonisin contamination (Picot et al. 2010). *Fusarium* ear rot development and fumonsisin contamination are aggravated by drought condition (Miller 2001; Parsons and Munkvold 2010). Thus, agricultural practices minimizing water stress such as farm moisture conservation and supplementary irrigation may reduce the problem. Insect damage is the most important factor that promotes fungus infection and fumonisin contamination of maize kernels (Miller 2001; Parsons and Munkvold 2010). Therefore, emphasis should be given to cultural practices for insect management. Control of ear feeding insects using insecticide application and manipulation of sowing date significantly reduce *Fusarium* infection and fumonisin contamination (Blandino et al. 2009). Appropriate drying of grains before storage is also an important factor in determining post-harvest fungus infection and fumonisin contamination (Cao et al. 2014). Storing grains at low moisture level below 15 % reduces fungal contamination. *Fusarium* species cannot grow well when the water activity (a_w) is below 0.9, and fumonisin synthesis stops once kernels are dried (Pitt et al. 2013).

2.0 The thesis

This thesis focuses on *F. verticillioides* and fumonisin management in maize in Ethiopia and consists of five manuscripts referred to as paper I – V in the text, which are developed based on data generated from field surveys, laboratory analysis and field experiments. Briefly, project justification, methods, main results and discussions, conclusions and future perspectives are described in the sections below. Details of methodology, results and discussion can be found in the individual papers.

2.1 Project justification

Ear rots caused by various *Fusarium* species are among the most common fungal diseases of maize worldwide, including in Ethiopia (Ayalew 2010; Czmebor et al. 2015; Leyva-Madrigal et al. 2015; Logrieco et al. 2002; Stumpf et al. 2013). When disease severity is high, yield losses can be substantial, and grain quality may deteriorate (Presello et al. 2008; Vigier et al. 1997). However, the main problem with maize ear rot diseases is the contamination of grains with mycotoxins, which may seriously affect the health of humans and domestic animals (Marin et al. 2013; Reddy et al. 2010; Waśkiewicz et al. 2012). The mycotoxins known as fumonisins are the most common contaminants of maize, particularly when grown in warmer regions (Marin et al. 2013; Picot et al. 2010; Waśkiewicz et al. 2012). *Fusarium verticillioides* is the dominant species in tropical and subtropical maize growing environments, and the fungus is responsible for fumonisin contamination of maize kernels (Das 2014; Logrieco et al. 2002; Mesterházy et al. 2012; Picot et al. 2010). Most *F. verticillioides* strains are capable of producing fumonisin in maize kernels, but the amount of toxin production differs among isolates (Atukwase et al. 2012; Covarelli et al. 2012). Heavily contaminated grain may cause significant economic losses to farmers, because the value of the produce is reduced in the marketplace, or heavily infected grains have to be discarded.

The distribution, species composition and predominance of *Fusarium* species as well as fumonisin contamination in maize grains greatly vary in different maize growing areas and between years (Dorn et al. 2011; Goertz et al. 2010; Stumpf et al. 2013). These variations in natural occurrence are mainly due to differences in environmental conditions, primarily temperature and precipitations prevailing in the maize growing areas (Cao et al. 2014; Doohan et al. 2003; Picot et al. 2010). Agronomic practices, including cropping system and tillage, can also influence the occurrence of *Fusarium* species, as infected crop debris on the soil

surface support their saprotrophic survival and are source of inoculum for succeeding plants (Cotton and Mulkvold 1998; Munkvold 2003; Leplat et al. 2013). Drought and insect damage are the most important factors in determining the risk of *Fusarium* infection and fumonisin contamination of maize grains (Miller 2001; Parson and Munkvold 2010a).

In Ethiopia, as detailed studies are very limited, few publications are available regarding the incidence of *Fusarium* spp. in the country. These surveys indicated that *F. verticillioides* is the most prevalent pathogen on maize kernels (Ayalew 2010; Wubet and Abate 2004). Very little is known about the species composition and prevalence of *Fusarium* spp. as well as fumonisin contamination levels on maize grains in several maize growing agroecological zones of Ethiopia. The environmental conditions are variable within short distance in a geographic region in Ethiopia. The agroecological conditions may determine the cropping system and the occurrence of *Fusarium* species. Knowledge regarding the composition and prevalence of *Fusarium* spp. infecting maize in a particular geographical area are basis for the design of management strategies. This information is needed in targeting the predominant pathogen species in the target environment in order to reduce the risk of mycotoxin contamination in food and feed. Information is also lacking with respect to genetic variation, aggressiveness and fumonisin-producing ability of *F. verticillioides* isolates in Ethiopia. The levels and distribution of genetic variation within pathogen species may affect the evolution of the species and host plant resistance (McDonald and Linde 2002). Pathogen diversity may determine the dynamics of disease. Therefore, development of appropriate disease management strategies requires thorough understanding of the most prevalent pathogen, details of the pathogen biology and the factors leading to epidemics.

Effective management of the disease in the field is essential, to reduce losses and potential health hazards associated with consumption of fumonisin-contaminated grains. Currently, there is no sole effective measure available for control of *F. verticillioides* and fumonisin contamination, but there is a need to develop appropriate integrated disease management strategies. Chemical control is often ineffective as the fungus can transmit vertically from infected seed or soil into the plant tissue (Bacon et al. 2008; Mesterházy et al. 2012). Dependence on chemical pesticides may lead to development of new variants of pathogen resistant to fungicides. Moreover, the use of fungicides is controversial because of public concern about residues, which may have undesirable side effect on the environment and human health. Cultural methods such as physical removal of infected plant debris, crop

rotation, tillage and manipulation of planting time may reduce the disease to some extent. These methods do not provide sufficient control, because, the pathogen inoculum is abundant in nature and dispersed by several means (Fandohan et al. 2004; Munkvold 2003). Genetic resistance and biological control should form the basis for sustainable and environmentally friendly management of *F. verticillioides* and fumonisin contamination of grains. Studies in other countries reveald that genetic variation for resistance to *Fusarium* ear rot and fumonisin contamination exsists in maize genotypes (Eller et al. 2008; Loffler et al. 2010; Santiago et al. 2013; Small et al. 2012). Efforts must continue to identify maize genotypes with acceptable sources of resistance to the pathogen and fumonisin accumulation. In Ethiopia, little information is available regarding the resistance level of maize genotypes to *Fusarium* ear rot and fumonisin contamination. Resistance levels of existing maize cultivars commonly grown in the country are not known. Because, the maize variety development program mainly focuses on total yield, with little attention to disease resistance. Control of *F. verticillioides* in maize is challenging, especially when the fungus grows systemically inside the plant system as endophyte. The use of biocontrol agents could be a promising strategy for managing such type of infection. The filamentous fungus genus *Trichoderma* is one of the major biocontrol agents getting attention due to its multiple action to suppress soilborne and seedborne pathogens in various crops. Therefore, it would be curucial to check for naturaly occurring *Trichoderma* species isolates and assess their ability to control the *F. verticillioides* pathogen. The present study was carried out with the following aims:

2.2 Study objectives

The overall objective of the present study was to generate knowledge about *Fusarium* and fumonisin in maize in Ethiopia, thereby to reduce yield losses and improve food safety.

The specific objectives of the present study were:

I. To identify *Fusarium* species associated with maize kernels from different major growing areas of Ethiopia, to assess fumonisin contamination levels, and to try to elucidate the effect of different climatic conditions on the fungal infection and fumonisin contamination level.

II. To assess genetic variation among *F. verticillioides* isolates, collected from major maize growing areas present in four different geographical regions of Ethiopia, and to

estimate the genetic differentiation within and between these *F. verticillioides* isolates from different regions.

III. To determine fumonisin production ability by a collection of *F. verticillioides* isolates associated with kernels of maize produced in different agroecological conditions in Ethiopian.

IV. To assess differences in Ethiopian maize cultivars for resistance to *F. verticillioides* infection and fumonisin accumulation after artificial inoculation, and to select maize cultivars with good level of resistance.

V. To evaluate the antagonistic potential of native *Trichoderma* species in suppressing the growth of *F. verticillioides* under *in vitro* assays, as well as to evaluate the effectiveness of selected *Trichoderma* species isolates as biocontrol agents to reduce *F. verticillioides* colonization and fumonisin contamination of maize grains following seed biopriming under field conditions.

2.3 Materials and methods

All survey work and field experimentation were conducted in Ethiopia (trials in Mekelle University), while laboratory activities such as mycological studies, molecular analyses, and fumonisin analysis were performed at the Norwegian Institute of Bioeconomy Research, Norway between 2012 and 2015.

Maize grains sample collection and mycological analysis. Post-harvest maize grain samples were collected during July to August of 2012, from 20 different major maize growing areas, which belong to seven different agroecological zones of Ethiopia (Paper I and III). Totally 200 maize grain samples (10 samples from each area), with nearly 1 kg sample size were collected randomly from each sampling site with in area. For isolation and enumeration of fungal species, maize kernels were surface-sterilized, using 1 % sodium hypochlorite (NaOCl) solution, and five kernels were plated on petri dishes containing a modified Czapek-Dox Iprodione Dichloran Agar (CZPD) as used by Halstensen et al. (2006). *Fusarium* species were identified, mainly based on morphological characters according to the procedures described by Leslie and Summerell (2006). Sequencing of the translation elongation factor 1-alpha (*EF-1α*) gene region was accomplished as described by O'Donnell et al. (1998) and Geiser et al. (2004) for representative isolates (Paper I) to support the morphological identification.

Fumonisin analysis. In all cases, fumonisin was extracted using 70 % methanol with a shaker (1000 rpm) for 3 min. The extracts were filtered through a Whatman no. 1 filter paper and the filtrate collected for assessment. The concentration of total fumonisin in the extracts was quantified using a competitive Enzyme Linked Immunosorbent Assay (ELISA) kits (RIDASCREEN® Fumonisin, R-Biopharm AG, Darmstadt, Germany), according to the manufacturer's procedures (Papers I, III, IV and V). The absorbance was measured using a microplate reader at 450-nm, and fumonisin concentration of samples were evaluated using RIDA®SOFT Win software (Art. Nr. Z9999, R-Biopharm AG, Darmstadt, Germany). The minimum detection limit of the kit was 0.025 mg kg^{-1}, and all samples were run in duplicate wells.

DNA extraction and AFLP analysis. For all molecular analyses (paper I and II) DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) according to the guidelines of the manufacturer. Amplified Fragment Length Polymorphism (AFLP) was used to study the genetic variation among 80 *F. verticillioides* isolates associated with kernels of maize, representing four geographic regions, (Southern, South-western, Central-western and Northern), of Ethiopia. The AFLP procedure was performed according to Vos et al. (1995), as modified by Leslie and Summerell (2006), using six different *Eco*RI/*Mse*I primer combinations (Paper II). FAM-dye labeled *Eco*RI primers was used for the selective amplification. The fragments were separated in an ABI3730 DNA Analyser (Applied Biosystems, USA) and analysed using GeneMapper v4.0 (Applied Biosystems, USA). AFLP fragments were scored manually, with 1 = presence of the band and 0 = absence of the band. Clustering and dendrogram construction were performed in NTSYS-pc version 2.2 (Exeter Biological Software, Setauket, NY, USA) software package (Rohlf 2005) using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) (Sneath and Sokal 1973). Principal coordinate analysis (PCO) was also used to detect clustering among the *F. verticillioides* isolates. Analysis of molecular variance (AMOVA) was carried out in order to estimate the genetic variation within and between isolates from different geographic regions, using the Arlequin software package version 3.0 (Excoffier et al. 2005).

Determination of *in vitro* fumonisin production ability by *F. verticillioides* isolates. Fumonisin production ability the *F. verticillioides* isolates was determined, using autoclaved maize kernels as cultivation medium. *Fusarium verticillioides* isolates used for this study were obtained from kernels of maize grown in different agroecological zones of Ethiopia. The

F. verticillioides isolates were grown on Mung Bean Agar for conidia production (Dill-Macky 2003). Conidial suspension of the different *F. verticillioides* isolates were prepared by adding sterile distilled water and a concentration of 10^6 conidia mL^{-1} was prepared. For preparation of the cultivation medium, 100 g maize kernels and 100 mL of sterile water in 500 mL glass jars were autoclaved at 121℃ for one hour on three consecutive days (Vismer et al. 2004). The conidial suspensions were inoculated into the autoclaved maize cultures in the 500 mL glass jars (Fig. 5), and the culture materials were incubated in the dark for 4 weeks at 25 °C. After four weeks of incubation, the culture materials were dried, finely ground to powder using a laboratory mill, and the fumonisin content was quantified (Paper III).

Fig. 5 Partial view of culture growing condition of the *F. verticillioides* isolates on maize cultures in 500 ml size marmalade glass jars. Close view showing maize kernels totally colonized by mycelial growth of a *F. verticillioides* isolate (A), and jars placed inside an incubator (B).

Field experiments for management of *F. verticillioides* and fumonisin contamination

Evaluation of maize cultivars. Resistance to *Fusarium* ear rot and fumonisin contamination, caused by *F. verticillioides*, was studied on 15 maize cultivars, at Mekelle, Northern Ethiopia. Inoculum was produced from a *F. verticillioides* isolate (SR-6952), which was obtained from a naturally infected maize kernel. The isolate was the highest fumonisin producer under *in vitro* testing (Paper III). Primary ears of ten maize plants per plot were inoculated with conidial suspension (10^6 spores mL^{-1}) through the silk channel at the R_2 growth stage. At crop harvest *Fusarium* ear rot severity was assessed by estimating the percentage (0 - 100 %) of each ear surface covered by visible symptoms of fungal infection such as rots, discolorations and white mycelial growth on kernels (Loffler et al. 2010; Small et al. 2012). One hundred gram of maize kernel samples from the inoculated ears of each plot were finely grinded using

a laboratory mill (IKA®-Werke, GmbH & Co. KG., Germany) and analysed for fumonisin content (Paper IV).

Biocontrol potential of native *Trichoderma* species isolates. The biocontrol potentials of native *Trichoderma* species were evaluated against *F. verticillioides*. The antagonistic potentials of *Trichoderma* spp. isolates were initially assessed under *in vitro* condition, following the dual culture method (Paper V). The best performing isolates, in terms of overall antagonistic potential against the pathogen under the *in vitro* testing, were selected. These selected *Trichoderma* isolates were evaluated under field condition for their ability to reduce *F. verticillioides* colonization and fumonisin contamination of maize kernels. For the field trial, maize seeds were bioprimed with spore suspension (10^7 spore mL^{-1}) of the different *Trichoderma* spp. isolates before planting, and a spore suspension of the *F. verticillioides* pathogen with the same concentration was applied into autoclaved maize seeds separately. Finally, three autoclaved seeds colonized with *F. verticilloides,* and two viable maize seeds treated with the *Trichoderma* isolates were placed closely together per planting hill in the field. Seeds, treated with Apron Star fungicide, were included in the treatments as positive control. Seeds soaked in sterile water without any biological control agent, but planted together with the *F. verticillioides*-colonized autoclaved seeds, were used as fungal control. In the field, agronomic parameters, such as germination percentage and seedling vigor index, were measured to observe the influence of *Trichoderma* species on plant growth. After crop harvest, 200 maize kernels were taken randomly from each plot and used for assessment of fungal contamination levels using the deep-freeze blotter method (ISTA 2003; Paper V). Subsamples of maize kernels (100 g) from each plot were finely milled and used for fumonisin analysis.

Climatic and agroecological data collection. Weather information, such as daily rainfall, relative humidity, minimum and maximum temperature for the experimental place (Papers IV and V) and for the maize grain sample collection sites (Papers I) were obtained from the Ethiopian Meteorological Agency weather stations closest to the experimental sites. Information on agroecological zone classification of the maize grain sample collecting areas (Papers I and III) were obtained from the Ethiopian Ministry of Agriculture and Rural Development.

Statistical analysis. Differences in occurrence of *Fusarium* spp., fumonisin concentrations, proportion of fungal contaminated kernels per area and fumonisin production ability of the *F. verticillioides* isolates were compared using the non-parametric Kruskal-Wallis one-way ANOVA using SPSS version 22 (IBM SPSS statistics 22, Chicago, Illinois) (Paper I and III). One sample T-test was used to compare the mean fumonisin production between the Ethiopian *F. verticillioides* isolates and the South African reference isolate (MRC826) (Paper III). Descriptive statistical analyses were employed to summarize data on occurrence of *Fusarium* spp. and fumonisin levels per study area and agroecological zones. For field experiments, data were subjected to analysis of variance (ANOVA) using the procedure of Statistical Analysis System (SAS) software (SAS institute, Cary, NC) (Paper IV and V). Differences between treatment means were compared using Fisher's protected least significant difference test (LSD) with $P < 0.05$ level of significance. Where appropriate, regression and correlation analysis were conducted to determine the relationship between the independent and dependent variables (Paper I II and IV).

2.4 Main results and discussion

Natural occurrence of *Fusarium* species and fumonisin on maize kernels in Ethiopia. Mycological analysis showed a wide range of variation in percentage of kernels contaminated with fungi among maize samples (16 - 68 %). On average, 38 % kernels of maize were contaminated by different fungal species including of *Fusarium* species, *Aspergillus*, *Penicillium, Stonecarpella*, *Acremonium, Mucor* and *Rhizopous.* The incidence of these fungal agents on maize kernels were ranged 3.9 - 33 %, with the highest relative prevalence of *Fusarium* species and the least for *Rhizopous.*

Eleven different *Fusarium* species were identified in association with kernels of maize produced in various maize growing areas of Ethiopia. *Fusarium verticillioides* was the most frequently isolated species, followed by the *F. graminearum* species complex (mainly *F. boothii*). *Fusarium pseudoanthophilum, F. oxysporum, F. incarnatum, F. brevicatenulatum* and *F. temperatum* were also commonly isolated, in descending order of prevalence (13.4 - 2.8 %). *Fusarium equiseti, F. subglutinans, F. lacertarum* and *Fusarium* sp. were isolated in minor abundance, with each ≤ 1 % of the total number of *Fusarium* isolates recovered from the maize kernels (Paper I). The diversity of *Fusarium* spp. contaminating maize kernels in this study was high, compared to previous reports (Ayalew 2010; Wubet and Abate 2004).

This could be due to yearly variations in weather conditions that may influence the growth and survival of *Fusarium* species. Dorn et al. (2011) and Goertz et al. (2010) have reported high year-to-year variability in the incidence of *Fusarium* spp. and mycotoxin contamination levels in maize in Switzerland and Germany, respectively. The other possible reason for the detection of great number of *Fusarium* species in the present study is that new *Fusarium* species might have been continuously introducing with maize seeds from abroad via domestic and international research institutions. In fact, great number of areas and agroecological zones were surveyed in the present study compered to the previous assessments. This might have contributed to the observed high diversity of *Fusarium* species compared to the previous limited surveys.

The species composition and prevalence of *Fusarium* species varied greatly among different maize growing areas and agroecological zones of Ethiopia (Paper I). We isolated a maximum of eight different *Fusarium* species in samples from several maize growing areas, but only four species in other areas (Paper I). *Fusarium* species diversity was maximum in the tepid humid and sub-humid mid-highland agroecological zones (11 species each); where as *Fusarium* species diversity was least (six species) in the warm sub-moist lowlands. *Fusarium verticillioides* was isolated from samples collected in all areas, but it was most prevalent in areas and agroecological zones, which are characterized by lower elevation, warm and dry conditions. The *F. graminearum* species complex, the second most common fungal contaminant on maize kernels of Ethiopia, was more abundant in areas characterized by higher elevation, low temperature and wetter conditions (Paper I). This is in agreement with earlier observations in other maize-growing areas that *F. graminearum* flourishes in cooler temperature and wetter conditions than *F. verticillioides* (Dorn et al. 2011; Vigier et al. 1997; Scauflaire et al. 2011).

Fumonisins were present in larger proportion (77 %) of the maize samples analysed, with concentrations ranging from 25 $\mu g\ kg^{-1}$to 4500 $\mu g\ kg^{-1}$. These results are in accordance with those reported by Ayalew (2010), who detected fumonisin in concentrations ranging from 300 to 2400 $\mu g\ kg^{-1}$ in samples collected from areas in eastern and central Ethiopia. Total fumonisin concentration in 7 % of the maize samples exceeded 1000 $\mu g\ kg^{-1}$, the maximum tolerable limit set by the European Union in maize intended for direct human consumption (EC 2007). One sample had levels higher than 2000 $\mu g\ kg^{-1}$, the maximum tolerable limit

recommended by the US Food and Drug Administration (FDA) in food intended for direct human consumption (FDA 2001). This presents a risk for food safety. Fumonisins are faily stable during food processing, and only a very limited amount of toxin content reduced by fermentation and cooking (Marín et al. 2013; Shephard et al. 2012).

Our results indicated that fungal and fumonisin contamination level differed considerably among samples collected from different areas and agroecological zones. Highest fungal contamination and fumonisin concentrations were recorded in samples collected from the warm and humid agroecological zones (Paper I). Proportion of kernels contaminated with fungi was significantly correlated with the recorded climatic data (temperature, relative humidity and rainfall). Likewise, fumonisin concentrations in maize samples were significantly correlated with the recorded relative humidity and temperature data. These results suggest the importance of environmental factors in fungal and fumonisin contamination of maize kernels. Environmental conditions related to decreased precipitation and increased temperature during pollination have been reported as important conditions for *Fusarium* infection and fumonisin contamination of maize grains in the field (Cao et al. 2014; Goertz et al. 2010; Pascale et al. 2002; Shelby et al. 1994). This is because fungal infection in maize ears mainly occurs during silking, and stressed plants are more susceptible to fungal infection (Fandohan et al. 2004; Picot et al. 2010). Thus, minimizing such stress factors for the host plant in the field during such critical growth stages may help to reduce fungal and fumonisin contamination of grains. In addition, we have observed great differences in fungal and fumonisin contamination levels among samples collected from the same area. These results suggest that local farm level factors, other than weather parameters, also have a strong influence on the prevalence of fungal species and fumonisin contamination. Differences in agronomic practices including crop rotation, choice of cultivars, insect pest management, harvesting time and post-harvest grain drying practices applied by different maize growing farmers, may have great impact on fungal and fumonisin contamination of maize kernels (Cao et al. 2014; Fandohan et al. 2004; Logrieco et al. 2002; Munkvold, 2003).

As results of this survey revealed, *F. verticillioides* is the most predominant species associated with kernels of maize produced in different agroecological conditions in Ethiopia. Thus, efforts should target to management of this fungal pathogen. To achieve this goal, basic knowledge related to genetic variation and toxigenic potential of the isolates is crucial.

Genetic variation and fumonisin production ability in *Fusarium verticillioides* isolates from kernels of maize produced in Ethiopia. The AFLP analysis indicated genotypic variation among the *F. verticillioides* entities in Ethiopia. Coefficients of dice similarity ranged from 0.46 to 0.96. No clonal isolates were detected, and eighty different AFLP-genotypes were detected among all isolates analysed (Paper II). UPGMA clustering and PCO analysis differentiated the isolates into two main clusters, but no clear relationships were detected between *F. verticillioides* isolates and their geographic origin. *Fusarium verticillioides* isolates that produced the highest and lowest fumonisin concentrations under *in vitro* testing were distributed among different genetic similarity groups in the dendrogram and PCO plots.

The *F. verticillioides* isolates included in this study were characterized by low level of genetic differentiation, weakly subdivided genetically, between geographical regions (Paper II). Larger proportion of the genetic variation was occurred among isolates from the same geographic region (98.5 %), while small variation occurred between isolates from different geographic origin (1.5 %). This may be partly explained by the unrestricted seed exchange, and consequently, high rate of gene flow or sharing of genetic material between the geographic regions. Gene flow can introduce new allels and maintain diversity within populations, but decreases genetic differentiation between popolations, as alleles are being exchanged (McDonald and Linde 2002). Novel allels produced by mutation in another population might be introduced with infected seeds and increase variation within isolates in the same regions. It may also indicate dispersal potential of the strains. For example, long-distance dispersal of inoculum, such as ascospores, via windborne drifts may contribute to mixing of the regional populations. Besides, sexual reproduction could be an epidemiologically significant contributor to the observed high level of genetic variation in *F. verticillioides* isolates within the same region. Sexual recombination increases genotypic diversity, as it creates novel recombinants (Cumagun et al. 2009; Leslie and Summerell 2006). Genetic diversity of a pathogen population to some degree also depends on the extent of geographic scale considered in the analysis. Because, isolates collected from larger geographic regions representing various agroecological zones may display some levels of genetic variation. In Ethiopia, altitude and agroecological conditions within the geographic regions are very variable, and strains with distinct genetic makeup may have evolved through time, due to selection for alleles in the pathogen population to adapt to a local niche. Results of this study were in agreement with the previous report by Reynoso et al. (2009) in Argentina,

who found high genotypic diversity but very low level of genetic differentiation between *F. verticillioides* populations. Genotypic variation in *F. verticillioides* isolates have been reported in other studies in Brazil (Rocha et al. 2011), Italy (Covarelli et al. 2012) and Iran (Dehkordi et al. 2013).

Our study on toxigenic potential of *F. verticillioides* isolates showed that all isolates tested have the ability to synthesize detectable levels of fumonisin on autoclaved maize kernels. A wide range of differences in total fumonisin production ability was detected among isolates, with concentrations from 0.25 to 38 mg kg^{-1}. In addition, high variation was noticed among isolates collected from the same area and agroecological zones (Paper III). This shows the distribution of the highest fumonisin producing *F. verticillioides* strains are not restricted to specific geographic areas, but highest producers are widespread all over the maize growing areas in Ethiopia. The detected differences in fumonisin production ability among *F. verticillioides* isolates could be primarily due to variation in the inherent genetic makeup of the isolates. Fumonisin production in *F. verticillioides* isolates is regulated by fumonisin biosynthetic gene (*FUM*) cluster (Proctor et al. 2003; Sagaram et al. 2006). Neculotied sequence variation inside the *FUM* gene cluster may explain variation in fumonisin production in *Fusarium* species (Stępień et al. 2011). Several other genes (*FCC1, FCK1, PAC1, ZFR, GBP1, GBB1, CPP1, AREA, FST1* and *FvVE1*) that are not located in the *FUM* cluster are also known to regulate fumonisin biosynthesis (Picot et al. 2010; Sagaram et al. 2006). Additionally, several environmental factors have been reported to influence fumonisin production by *F. verticillioides* isolates (Cao et al. 2014; Marin et al. 1999; Picot et al. 2010; Sagaram et al. 2006). If conditions are not ideal, the fungus may not produce as much of the toxin as its genetic potential. In this study, we used 100 g maize kernels per 500 mL jar for cultivation of each isolate (Fig. 5). This culture substrate to container volume ratio is quite high, and it may prevent the circulation of enough oxygen for the fungal growth in the interior of the culture, which may negatively affect fumonisin biosynthesis. Previous studies showed reduced fungal growth and fumonisin production under oxygen limited culture conditions (Keller et al. 1997; La Bars et al. 1994).

According to the criteria of Nelson et al. (1991), all *F. verticillioides* isolates examined in this study are low fumonisin producers (Paper III). However, a large number of the *F. verticillioides* isolates (57.5 %) tested produced fumonisin concentrations > 4 mg kg^{-1}, which is above the maximum tolerable limit set by the European Union in unprocessed maize (EC

2007). The widespread existence of isolates with a potential to produce this much fumonisin, presents the risk for food safety, taking into account that *F. verticillioides* is the most common pathogen on kernels of maize in Ethiopia (Paper I). The amount of fumonisin production could raise when climatic condtions are more suitable for the fungus, with climate changes. Climatic situations such as increased temperature and drought may stress host plants and favour growth of toxigenic *F. verticillioides* strains (Parsons and Munkvold 2010; Picot et al. 2010). Therefore, it is very important to prevent introduction of more toxigenic strains with import of germplasms from abroad, as well as to develop sound management practices including implementation of good agricultural practices in the field to reduce fumonisin contamination of maize grains.

Management of *Fusarium verticillioides* and fumonisins in maize in Ethiopia

Host plant resistance. Evaluation of Ethiopian maize cultivars for their resistance to *Fusarium* ear rot and fumonisin accumulation after artificial inoculation by a fumonisin producer *F. verticillioides* strain showed the presence of a potential source of resistance (Paper IV). The maize cultivars tested exhibited significantly ($p < 0.05$) different reaction to *F. verticilloides* infection and fumonisin accumulation. Among all the cultivars tested, seven cultivars displayed low ear rot disease severity and fumonisin contamination over the two years trial compared to others (Paper IV). Fumonisin level recorded in these cultivars was 59 - 96 % lower compared to the susceptible line (NSCM-411881(32)), which indicates the presence of potential resistance genes against *F. verticilloides* infection and fumonisin accumulation in the maize germplasms of Ethiopia. This was in line with previous studies in other countries that have reported great variations in *Fusarium* ear rot and fumonisin resistance among maize genotypes (Eller et al. 2008; Santiago et al. 2013; Small et al. 2012). None of the 15 maize cultivars evaluated were completely free from fumonisin accumulation. Eight of the maize cultivars exhibited high level of mean ear rot severity (30 – 50 %) and fumonisin content (29 – 58 mg kg^{-1}) over the two years trial. From this, we can understand that many high yielding maize cultivars, widely grown in Ethiopia; do not possess satisfactory levels of resistance to *F. verticillioides* and fumonisin contamination. We have found greater susceptibility in late maturing type of maize cultivars to *Fusarium* ear rot and fumonisin accumulation than early types (Paper IV). Higher susceptibility of late maturing maize genotypes to fumonisin accumulation compared to early maturing is in agreement with results of Battilani et al. (2008) and Loffler et al. (2010). The underlining reason was that grain moisture content reduces slowly in such cultivars, but water availability plays an important

role for fungal growth and development (Fandohan et al. 2004; Marín et al. 2004). The extended period required to reach physiological maturity may also aggravate the problem of fumonisin accumulation on infected ears although some resistance to contamination is present in late maturing verities (Battilani et al. 2008). This is because fumonisin contamination in maize kernels is a cumulative process; amount may increase with the extended infection period.

Fusarium ear rot severity and fumonisin contamination of maize kernels were significantly influenced by year. *Fusarium* ear rot severity and fumonisin contamination on maize kernels were higher in first year (2013) compared to the second year (2014). This was due to variation in weather factors towards the end of the growing season. After the time of silking, through the months of September to December, temperature was steadily higher while precipitation was lower in 2013 than 2014 (Paper IV). This shows the occurrence of more stressful weather conditions for plant growth in 2013 than in 2014. Conditions that result in plant stress favors *F. verticillioides* infection and fumonisin accumulation (Cao et al. 2014; Munkvold 2003; Picot et al. 2010). Our results are in agreement with the findings of Pascal et al. (2002), who have reported highest fumonisin contamination in seasons characterized by high temperature and low precipitation during the period of pollination. Several other studies have reported that *Fusarium* ear rot development and fumonisin contamination in maize are favored by warm, dry or drought condition during early reproductive stages (Miller et al. 1995; Shelby et al. 1994). These results suggest interaction between maize genotypes and environmental factors could be important in determining resistance to *Fusarium* ear rot and fumonisin contamination. Therefore, assessments aiming at obtaining resistance maize cultivars could be successful when evaluations will be performed across years or over multiple environmental conditions.

Our results shows strong association between *Fusarium* ear rot severity and fumonsin content. This suggests that selection for resistance to *Fusarium* ear rot may eventually result in resistance to fumonisin contamination in maize grains. These results are in harmoney with previous studies that detected strong association between visible ear rot severity and fumonisin content (Bolduan et al. 2009; Clements et al. 2003; Robertson et al. 2006). Therefore, it is sensible to make initial selection using visual rating of ear rot disease severity to exclude cultivars accumulating high levels of fumonisin, and further evaluating the selected materials for fumonisin content to minimize the cost. Because, visual ear rot rating is easier

and less expensive, and large number of maize germplasms may be evaluated over different locations or years. Generally, management of *F. verticillioides* and fumonisin using host plant resistant could be more effective if it could integrate with other environmental friendly controlling strategies such as using biological control agents.

Biological control using *Trichoderma* species. In Paper V, *in-vitro* dual culture interaction and field experiment using seed biopriming revealed that native *Trichoderma* species isolates have a promising potential to suppress growth of *F. verticillioides* and reduce fumonisin contamination of maize kernels. Initial screening based on dual culture interaction demonstrated different antagonistic potential of *Trichoderma* species isolates. Radial growth inhibition of the *F. verticillioides* isolate was as high as 54 % and 78 %, 4 days and 7 days after pairing, respectively. Ten days after incubation, either mycelial growth of the *F. verticillioides* isolate was confined to a limited territory, or it was completely overgrown by the mycelial of *Trichoderma* isolates that do parasitize the pathogen. Variations in antagonistic mechanisms were also noticed for different *Trichoderma* isolates, as there was observable differences in the zone of inhibition before contact as well as hyphal coiling frequencies in the interaction region after contact. A robust zone of inhibition was observed in some *T. harzianum* isolates, showing production of antimicrobial substances for suppressing growth of the pathogen. Several others exhibited coiling structures around the hyphae of the *F. verticillioides* isolate, which indicates the employment of hyperparasitisim for controlling growth of the pathogen. Among the 18 *Trichoderma* isolates evaluated under *in vitro,* 11 isolates that have showed promising antagonistic ability against the *F. verticillioides* isolate were selected for field-testing.

Under field condition, a significantly greater fungal and fumonisin contamination was obtained in maize kernels grown from seeds treated with *F. verticillioides* alone (fungal control) compared to *Trichoderma*-treated and fungicide-treated plots. In all treatments in which *Trichoderma* isolates were involved, lower percentages of kernels contaminated with fungi and decreased in fumonisin concentration were recorded compared to the control (Paper V). Similar to the *in vitro* observation, *Trichoderma* isolates demonstrated different degrees of efficacy in controlling the *F. verticillioides* colonization (37.5 - 77.2 %) and fumonisin contamination (46 - 89 %) of maize kernels over the two years experiment. *Trichoderma hamatum* isolates (Thm3, Thm6) and *T. harzianum* (Thr2, Thr5) were the most effective in reducing *F. verticillioides* colonization and fumonisin contamination of maize kernels,

compared to other *Trichoderma* isolates. We have able to recover some of the *Trichoderma* isolates from kernels of maize after crop harvest using the deep freeze blotter method (Fig. 6 C). These isolates were able to grow on maize kernels as indicated in Fig. 6 D, when plates were incubated under alternating near UV/white fluorescent light (12 h) and dark (12 h) for additional days after colony emergence. This shows these *Trichoderma* isolates can grow systemically in the maize plant tissue and suppress growth of the pathogen. Similar results were reported in other studies demonstrating that certain strains of *Trichoderma* species are the most effective antagonists in controlling *F. verticillioides* (Chandra Nayaka et al. 2010; Ferrigo et al. 2014; Sempere and Santamarina 2009). *Trichoderma* strains vary considerably in their abilities to colonize roots of annual crops, and the most effective strains colonize the roots and provide good protection of the crop in the field (Vinale et al. 2008). The antagonistic mechanism of *Trichoderma* species could be due to competition for space and nutrients, mycoparasitisim (driving nutrients from the host), or to antibiosis by production of inhibitory substances that are effective against the pathogen (Annees et al. 2010; Howell 2003; Vinale et al. 2008). A combination of the mechanisms may play an important role in a high level of antagonism.

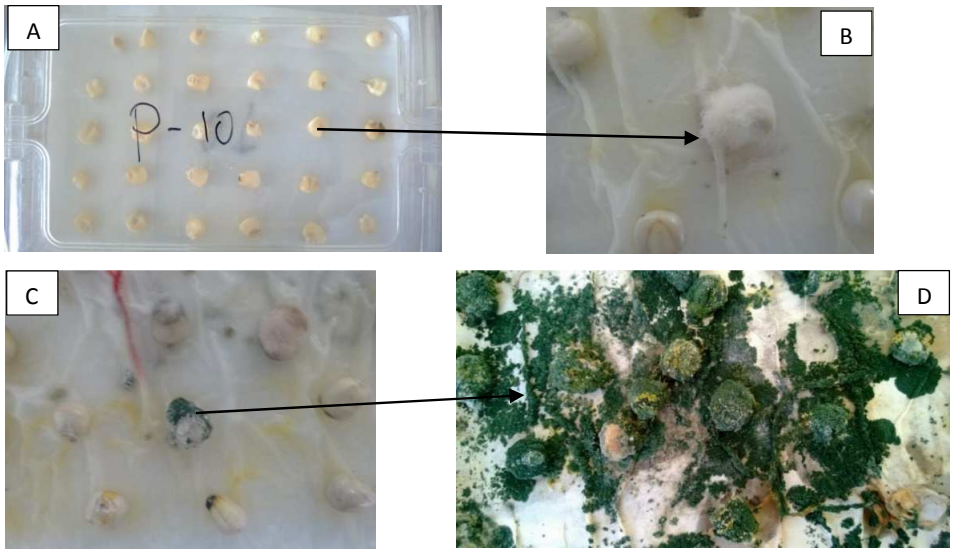


Fig. 6 Maize kernel fungal contamination assessed according to the deep freeze blotter method (Disinfection, incubation at room temperature for 24 h followed by 24 h in -20 °C and 9 days in 12 h alternating cycles of near UV/white light and darkness). A: seeds plated in 18 cm diameter plate, B: *Fusarium* colony growing on maize kernel, C: colony of *Trichoderma* isolate (Thr2) emerging from maize kernel after harvest and D: recovered *Trichoderma* isolate (Thr2) growing on maize kernels and colonizing the whole plate containing maize kernels after additional 15 days of incubation.

Our results also revealed that seed treatment with *Trichoderma* species have various positive effects on growth of the maize plant including enhancing speed of seed germination, boost shoot length and seedling vigor index. This is an additional advantage, compared to other pest management options such as use of pesticides, which frequently causes phytotoxicity. The use of such biocontrol agents is the best alternative for sustainable management of seedborne and soilborne pathogens including *F. verticillioides.* Public concern associated with the use of pesticides and the development of pathogen strains resistant to chemical pesticides can be avoid by the use of biological control.

2.5 Conclusions and future perspectives

Conclusions

Based on evidences from the current study, maize kernels produced in different agroecological conditions in Ethiopia contain a great diversity of *Fusarium* species. A higher diversity of *Fusarium* species occurs associated with maize kernels produced in different maize-growing areas of Ethiopia compared to previous reports. We have identified eleven different *Fusarium* species, and *F. verticillioides* was the most dominant. *Fusarium* species composition and relative prevalence differed depending on maize growing areas and agroecological conditions. A large proportion of maize samples contains fumonisin, but at low concentrations, below the maximum tolerable limit determined by the European Union in most cases. Fungal and fumonisin conamination differed significantly among samples collected from the same area, as well as between samples from different areas and agroecological zones. These variations desplayed strong relationship with the prevailing weather factors such as rainfall, relative humidity and temperature.

Fusarium verticillioides isolates in Ethiopia are generally characterized by the occurrence of little genetic variation between different geographic regions and high levels of variation among isolates within the same region. Genetically, they are genetically interlinked; therefore, host plant resistance measures should concentrate on quantitative resistance, which is effective against the entire *F. verticillioides* population. Fumonisin producing *F. verticillioides* strains are widely distributed in the maize growing areas in Ethiopia, as all *F. verticillioides* isolates tested in this study are able to produce detectable levels of fumonisin.

The widespread occurrence of fumonisin producing *F. verticillioides* strains, in all the maize growing areas of Ethiopia indicates the presence of risk for food safety.

Management of *F. verticillioides* and fumonisin contamination in maize using host plant resistance and biological control agents showed promising results. Ethiopian maize cultivars tested in this study possess different levels of resistance to *Fusarium* ear rot and fumonisin contamination. However, this study identified maize cultivars with high level of resistance to *Fusarium* ear rot and fumonisin contamination. Late maturing maize cultivars are more susceptible to *Fusarium* ear rot and fumonisin contamination than early types. Growing the most resistant cultivars might substantially reduce fumonisin contamination of maize kernels in areas where *Fusarium* ear rot disease is most prevalent. The year of inoculation significantly affects *Fusarium* ear rot severity and fumonisin contamination in maize cultivars. A strong positive relationship exists between *Fusarium* ear rot severity and fumonisin content in maize kernels. *Trichoderma* species isolates evaluated in this study have a great potential to control *F. verticillioides* and subsequently reduce fumonisin contamination of maize kernels. These *Trichoderma* isolates may be useful biocontrol agents as integral part of *F. verticillioides* and fumonisin management in maize in Ethiopia. The antagonistic potential of *Trichoderma* isolates differed and antagonistic activity was not characteristic of a species but that of a strain.

Future perspectives

The observed high levels of differences in samples collected from the same area suggestes, farm level agricultural practices, other than climatic factors, are also important parameters in determining fungal and fumonisin contamination of maize kernels. Therefore, assessments should be continued to analyse fungal and fumonisin contamination in relation to different local agronomic practices. The occurrence of fumonisin on majority of the maize samples suggestes the risk posed for food safety should not be underestimated. Therefoer, efforts should be made including implementation of good agricultural practices to prevent fumonisin contamination of maize in the field to minimize the potential risk to health of consumers. The amount of toxin production may be elevated when condtions are most suitable for the fungus. A variety of harmful mycotoxins other than fumonisin, especially aflatoxins, deoxynivalenol, nivalenol and zearalenone should also be consider in future assessments, as widespread prevalence of fungal species producing these mycotoxins was observed.

Future work is required to investigate the likely cause underlying for the observed high levels of variation within isolates of the same region but little differentiation between isolates from different geographic regions. Investigations on the extent of sexual reproduction in the *F. verticillioides* of Ethiopia could increase our understanding in relation with the observed genetic variation. The study on fumonisin production ability of *F. verticillioides* isolates should resume, considering optimum substrate amount to volume of container for cultivation of the fungus as well as testing using a different maize cultivar, as there is often isolate by cultivar interaction and nutrional composition of the substate is essential for fumonisin production.

The resistance level of high yielding, late maturing maize cultivars, should be improved by introducing alleles from the resistance germplasms. Resistance level of the different maize cultivars currently grown in Ethiopia is not well known. Thus, further evaluation is important by including broad maize collections to increase the chances for selecting maize material with higher resistance levels and develop cultivars with acceptable level of toxin contamination. Evaluations aiming at obtaining resistance maize cultivars could be effective when assessments performed over multiple years or different environmental conditions, since the year of inoculation in this study influenced *Fusarium* ear rot severity and fumonisin contamination levels.

Further studies need to focus to develop techniques for mass multiplication, and to achieve appropriate formulations and delivery systems of the best performing *Trichoderma* isolates for management of *F. verticillioides* and fumonisin. In the meantime, further investigations should be performed to better understand the mechanisms of action the *Trichoderma* isolates used to suppress growth of the pathogen and the toxin it produces. The performance of *Trichoderma* species may also vary with environmental and ecological conditions. Therefore, it is important to evaluate their performance over multiple environmental conditions and their possible synergy with other chemicals used in controlling the pathogen.

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Papers I - V

Paper I

Natural occurrence of *Fusarium* species and fumonisin on maize grains in Ethiopia

Hadush Tsehaye [1,4,*], May Bente Brurberg [2], Leif Sundheim [2], Dereje Assefa [4], Arne Tronsmo[3], Anne Marte Tronsmo [1]

[1] Norwegian University of Life Sciences, Department of Plant Sciences, P.O. Box 5003, NO-1432 Ås, Norway

[2] Norwegian Institute for Bioeconomy Research, Biotechnology and Plant Health Division, P.O. Box 115, NO-1431 Ås, Norway

[3] Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science, P.O. Box 5003, NO-1432 Ås, Norway

[4] Mekelle University, Department of Dryland Crop and Horticultural Sciences, P.O.Box 231, Mekelle, Tigray, Ethiopia

[*] Corresponding author. E-mail address: hadush.beyene@nmbu.no or had031@yahoo.com

Abstract

Fusarium species causing maize kernel rot are major threats to maize production due to reduction in yield as well as contamination of kernels by mycotoxins that poses a health risk to humans and animals. Two-hundred maize kernel samples, collected from 20 major maize growing areas in Ethiopia were analysed for the identity, species composition and prevalence of *Fusarium* species and fumonisin contamination. On average, 38 % (range: 16 to 68 %) of maize kernels were found to be contaminated by different fungal species. Total of eleven *Fusarium* spp. were identified based on morphological characteristics and by sequencing the partial region of translation elongation factor 1-alpha (*EF-1α*) gene. *Fusarium verticillioides* was the dominant species associated with maize kernels (42 %), followed by *F. graminearum* species complex (22.5 %) and *F. pseudoanthophilium* (13.4 %). The species composition and prevalence of *Fusarium* species differed among the areas investigated. *Fusarium* species composition was as many as eight and as few as four in some growing area. The majority of the maize samples (77 %) were found positive for fumonisin with concentrations ranging from 25 μg kg^{-1} to 4500 μg kg^{-1} (mean: 348 μg kg^{-1} and median: 258 μg kg^{-1}). Slight variation in fumonisin concentration was also observed among areas. Overall results indicate widespread occurrence of several *Fusarium* species and contamination by fumonisin mycotoxins. These findings are useful for intervention measures to reduce the impact of the main fungal species and their associated mycotoxins by creating awareness and implementation of good agricultural practices.

Keywords: *Fusarium* spp., Maize, Ear rot, Fumonisin, Ethiopia

Introduction

Maize (*Zea mays* L.) is the most important crop in Ethiopia, cultivated in all parts of the country and in different environmental conditions (CSA 2013; Geleti et al. 2011). In 2013/14, the total maize production in the country was 6.67 million tons harvested from nearly 2 million hectares of land, and this is 26.3 % of the total grain production in the country (CSA 2013). Almost all maize grains produced in Ethiopia is used for direct human food, while the crop residues plays an important role in terms of animal feed (Geleti et al. 2011). *Fusarium* species are the most common fungal pathogens of maize, responsible for various diseases including seedling blight, stalk rot and ear rot (Logrieco et al. 2002). Ear rot disease caused by many *Fusarium* species is a major production constraint of maize throughout the world, including Ethiopia (Ayalew 2010). *Fusarium* spp. cause two distinct types of ear rots in maize, which are recognized as *Gibberella* ear rot and *Fusarium* ear rot (Mesterházy et al. 2012). *Fusarium* ear rot is caused primarily by *F. verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsush.) Nirenberg, and *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun and Marasas, while *F. graminearum* (Schwabe), *F. culmorum* (Wm.G. Sm.) Sacc., *F. cerealis* (Cooke) Sacc. and *F. avenaceum* (Fr.) Sacc. are the main species causing *Gibberella* ear rot in maize (Logrieco et al. 2002; Mesterházy et al. 2012; Munkvold 2003). Infection of maize by *Fusarium* spp. may occasionally cause considerable losses in grain yield and quality deterioration (Logrieco et al. 2002; Vigier et al. 1997), but more importantly grains harvested from infected ears may result in mycotoxin contamination (Mesterházy et al. 2012; Munkvold 2003). Some *Fusarium* spp. can also grow in the plant tissue without causing any visible symptom of infection, but kernels may contain mycotoxins, in trace amounts (Mesterházy et al. 2012; Munkvold 2003). Mycotoxins are poisonous secondary metabolites, produced by certain fungal species including *Fusarium* spp., which are harmful to both human and animal health (Reddy et al. 2010). *Gibberella* ear rot disease frequently leads to contamination with deoxynivalenol, nivalenol and zearalenone mycotoxins whereas *Fusarium* ear rot leads to accumulation of fumonisins (Logrieco et al. 2002; Mesterházy et al. 2012). Mycotoxin contaminated commodities may be rejected in the market and contribute to further economic losses to growers (Waśkiewicz et al. 2012).

The fumonisins are the most common contaminants of maize-based foods and feeds throughout the world (Picot et al. 2010; Reddy et al. 2010; Sundheim and Tsehaye 2015). Maize is contaminated more frequently with high amounts of fumonisin than any other crop

(Sundheim and Tsehaye 2015; Waśkiewicz et al. 2012). Although several *Fusarium* species can produce fumonisins, *F. verticillioides* is considered the primary cause of *Fusarium* ear rot and fumonisin contamination in maize in the tropical and sub-tropical environments (Logrieco et al. 2002; Picot et al. 2010; Sundheim and Tsehaye 2015). The presence of fumonisins in grains is a serious threat because it can cause several health disorders in humans and domestic animals (Waśkiewicz et al. 2012). Consumption of fumonisin contaminated feed causes leucoencephalomalacia in horses (Kellerman et al. 1990), and pulmonary edema and hydrothorax in pigs (Harrison et al. 1990). Fumonisins are also nephrotoxic, hepatotoxic and hepatocarcinogenic in laboratory animals such as rats (Gelderblom et al. 1996). Furthermore, dietary exposure to fumonisin B_1 has been associated with elevated human esophageal cancer incidence (Sydenham et al. 1990) and neural tube defect in humans (Missmer et al. 2006).

High level of maize ear and kernel infection by several *Fusarium* species has been reported in different parts of the globe (Goertz et al. 2010; Ncube et al. 2011; Reyes-Velázquez et al. 2011; Vigier et al. 1997). The species composition and frequency of occurrence of different *Fusarium* spp. and fumonisin contamination varies greatly between different years and maize growing areas (Dorn et al. 2011; Goertz et al. 2010; Ncube et al. 2011). This may largely be caused by unusual or stressful environmental conditions during growth and at the time of harvest, primarily temperature and precipitations (Doohan et al. 2003; Picot et al. 2010). Climatic situations such as reduced precipitation (drought) and the prevalence of ear infecting insect pests often have a major influence on fumonisin contamination of maize kernels (Munkvold 2003). *Fusarium* ear rot is favored by warm and dry conditions, while *Gibberella* ear rot has been associated with cooler and wetter weather situation (Logrieco et al. 2002; Munkvold 2003; Vigier et al. 1997). Agricultural practices such as crop rotation and tillage systems can also influence the occurrence and prevalence of *Fusarium* species as infected crop debris on the soil surface helps the survival of *Fusarium* spp. and serves as a source of inoculum for infection of the next generation of maize plants (Munkvold 2003).

Growing resistant maize cultivars and implementation of good agricultural practices including insect pest management, may help to minimize *Fusarium* infection and subsequent mycotoxin contamination (Mesterházy et al. 2012; Munkvold 2003). Thus, monitoring the composition and abundance of *Fusarium* species causing ear rots of maize and compare that with the climatic conditions is vital to design management strategies including breeding programs for

resistance to the pathogens predominant in the target environment. Despite the importance of maize in Ethiopian agriculture and the well-known threat of mycotoxins to human and animal health, and the legislated regulation of maximum acceptable levels of mycotoxins (EU commission 2006; FDA 2001), very little is known about the species composition and prevalence of *Fusarium* spp. as well as fumonisin contamination levels on maize kernels produced in Ethiopia. The aim of the present study was to identify *Fusarium* species associated with maize kernels from different major growing areas of Ethiopia, to assess their fumonisin contamination levels, and to try to elucidate the effect of different climatic conditions on the fungal infection and fumonisin contamination level.

Materials and methods

Sample collection areas and agroecological zones

Twenty major maize growing areas were selected randomly for collection of maize kernel samples. According to the agroecological classification of Ethiopia, the sample collection areas (Fig. 1) were in seven major agroecological zones; namely: tepid humid mid-highlands (H_3), warm moist lowlands (M_2), tepid moist mid-highlands (M_3), warm sub-moist lowlands (SM_2), cool sub-moist mid-highlands (SM_4), warm sub-humid lowlands (SH_2) and tepid sub-humid mid-highlands (SH_3). The general characteristics of these agroecological zones in terms of elevation, annual rainfall, average temperature and major annual crops grown as described by the Ministry of Agriculture and Rural Development (MoARD 2005) are summarized below (Table 1).

Collection of maize samples and related additional data

During July to August of 2012, a total of 200 maize kernel samples were collected from 20 different major maize growing areas (districts) in Ethiopia, 10 samples from each area (Fig. 1). Maize samples were collected from smallholder farmers. Sampling sites within each area were separated by at least 1 km and at most 5 km from each other. Nearly 1 kg of maize kernel samples were collected randomly from each sampling site within each area and these samples were in storage for 6 - 7 months. Daily maximum and minimum temperature, relative humidity as well as rainfall data for the weather stations closest to the sampling sites were obtained from the Ethiopian Metrological Agency. Thus, climatic data stretching from

seeding to harvesting (May to December 2011), as well as for the storage period (January to June 2012) were considered to include both the field and storage situation, respectively. The duration from harvest to sampling (months of storage time), the seasonal average daily temperature, relative humidity and seasonal total rainfall for each area were as presented in Table 1. Samples were labeled with proper identification codes, placed in cloth bags to prevent condensation that might promote fungal growth. Global positioning system (GPS) co-ordinates and elevation were also recorded at each sampling site.

Table 1 Summary of characteristics of the agroecological zones of Ethiopia, from which maize kernel samples were collected

Agroecological zones	Elevation (masl) [a]	Temperature (°C) [b]	Annual Rainfall (mm) [c]	Major crops [d]
H_3	1600 - 3000	17 - 22.5	1800 - 2200	Co, M, W, RT
M_2	400 - 2000	22.5 - 25	600 - 1400	S, M, T
M_3	1000 - 3600	17 - 20	1000 - 1400	W, T, B, M, Pu
SM_2	400 - 2000	20 - 25	600 - 1000	S, M, T, Fg
SM_4	2800 - 4000	15 - 20	600 - 1000	B, W, T, Pu
SH_2	400 - 2000	22.5 - 27.5	1000 - 1800	S, M, Co
SH_3	1000 - 3200	20 - 22.5	1400 - 1800	S, M, W, Co, RT

[a] masl= meters above sea level; [b] long-term (>30 years data) average temperature and [c] long-term (>30 years) annual rainfall; [d]Co: coffee, M: maize, T: teff, W: wheat, S: sorghum, B: barley, Fg: fingermillets, Pu: pulses, RT: roots and tuber crops (potato, sweet potato, yams, ensete) (Source: MoARD, 2005)

Assessment of kernel infection level, isolation and identification of *Fusarium* spp.

A total of 500 kernels per area (50 kernels per sampling site) were used for determination of fungal infection level and isolation of *Fusarium* species. Maize kernels were surface sterilized by soaking in 1 % sodium hypochlorite (NaOCl) solution for 2 min, rinsed twice in sterile distilled water and dried briefly with sterile paper towels. Then kernels were transferred to CZPD agar plates (a modified Czapek-Dox Iprodione Dichloran Agar) containing propiconazole (0.375 mg L^{-1}) and fenpropimorph (1.125 mg L^{-1}) instead of iprodione (Halstensen et al. 2006). Plates, conaining five kernels each were incubated for 7 to 10 days

at 25 ℃ in the dark. The percentage of kernels contaminated with fungi was recorded by counting the number of kernels from which internal mold contaminants grew (Leslie and Summerell 2006). Colonies that appeared to be *Fusarium* based on shape and color of mycelium were transferred to Spezieller Närstoffarmer Agar (SNA) (Nirenberg 1976), and the identity of *Fusarium* species were confirmed by growing for 7 to 10 days at 20 ℃ under alternating near UV/white fluorescent light (12 h) and dark (12 h) (Leslie and Summerell 2006). Subsequently, single spore isolates were obtained by spreading serial dilutions of spore suspension on water agar plates. After incubation of plates at room temperature (22 ℃) for 16 - 20 h, a single germinating conidium was transferred to new SNA plates (Nirenberg 1976). From this sub-culturing was made into potato dextrose agar (PDA, Difco, Madison, USA) and carnation leaf agar (CLA) (Leslie and Summerell 2006). These cultures containing single spores were incubated for 2 - 6 weeks at 20 ℃ under alternating near UV/white fluorescent light (12 h) and dark (12 h), before identification. The identification of *Fusarium* isolates to species level was primarily achieved using morphological and cultural characters as described by Leslie and Summerell (2006).

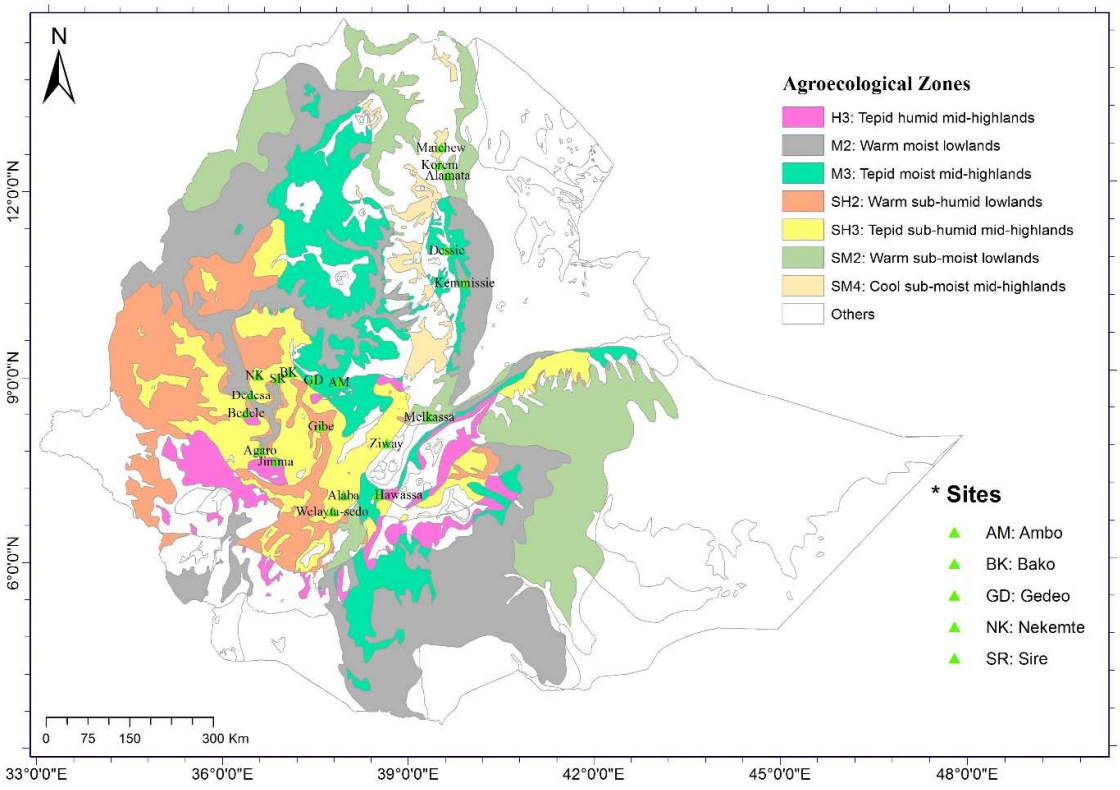

Fig. 1 Maize sample collection sites and agroecological zones of Ethiopia.

Sequencing of the translation elongation factor 1-alpha (*EF-1α*) gene region was performed on representative isolates (Table 3) to support the morphological identification. This gene have been used commonly for appropriate identification of *Fusarium* to species level, because it occurs consistently as a single-copy in the genus *Fusarium*. It shows a high level of sequence polymorphism among closely related species, even when compared to other genes such as calmodulin, β-tubulin and histone H_3 (Geiser et al. 2004). For DNA extraction, pure cultures from single spores were grown on PDA (Difco, Madison, USA) at 22 °C under white light for 7 to 10 days and mycelium was scraped from the surface and ground in liquid nitrogen using a mortar and pestle. DNA was extracted using DNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions. The *EF-1α* gene was amplified in PCR assay using the primer pairs EF1 (5'-ATGGGTAAGGAGGACAAGAC-3) and EF2 (5'-GGAGGTACCAGTCATCATGTT-3') as described by O'Donnell et al. (1998). Amplification reactions were done in volumes of 25 µl containing 2.5 µl 10x PCR buffer (10 mM Tris-HCl; 50 mM ClK; 15 mM $MgCl_2$, pH 8.3), 2 µl dNTPs (2.5 mM), 0.5 µl of each primer (50 µM), 0.125 µl AmpliTaq DNA Polymerase (5U μl^{-1}) (Applied Biosystems, Foster City, CA, USA) and 20 ng of template DNA. The amplification conditions consisted of one cycle of initial denaturation at 95 °C for 5 min, 35 cycles of denaturing at 94 °C for 50 s, annealing at 53 °C for 50 s, extension at 72 °C for 1 min, final extension at 72 °C for 7 min, followed by cooling at 4 °C. Amplified products were submitted for sequencing to GATC Biotech (Cologne, Germany). Sequence data were assembled and analysed using the CLC Main Workbench software 6.9 (Aarhus, Denmark), and consensus sequences were used to search the most related sequences at the GenBank (NCBI-National Centre for Biotechnology Information) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the *Fusarium*-ID database (http://isolate.fusariumdb.org/index.php).

Fumonisin analysis

From each sample, representative kernel sub-samples (100 g kernels) were milled to fine powder using the Micro plant grinding machine (Tianjin Taisite instrument Co., Ltd, China, mesh size 1 mm) and stored at -20 °C. Samples were thawed at room temperature (22 °C) for 13 - 14 hours before fumonisin extraction. Fumonisin was extracted from 10 g samples with 50 ml 70 % methanol on a shaker (1000 rpm) for 3 min. The extract was filtered through a Whatman no. 1 filter paper and the filtrate collected for evaluation. The concentration of total fumonisin in each sample was quantified using a competitive enzyme linked immunosorbent

assay (ELISA) kits (RIDASCREEN®Fumonisin, R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions. ELISA test kits were validated with maize samples of known fumonisin content. Samples with fumonisin concentration exceeding the highest detection limit for the kit were diluted with the extraction solvent, and the obtained results were multiplied by the dilution factor. All samples were run in duplicate wells, and the lowest detection limit of the kit was 0.025 ppm.

Statistical analyses

Differences in occurrence of *Fusarium* spp., fumonisin concentrations and proportion of fungal contaminated kernels per area were compared using the non-parametric Kruskal-Wallis one-way ANOVA. *Fusarium* spp. recorded on maize kernels per area and agroecological zone were also calculated as a percentage of the total number of *Fusarium* isolates per area and agro-ecology. Data on incidence of *Fusarium* spp. on maize samples and relative prevalence in the 20 maize growing areas were pooled to illustrate a countrywide prevalence of the different species. Pearson's correlation coefficients (Araṅkacāmi and Rangaswamy 1995) were calculated to determine the relationship between kernel fungal contamination levels, fumonisin concentrations and the occurrence of *Fusarium* spp. as well as seasonal mean daily temperature, relative humidity and seasonal total rainfall data for the areas. For fumonisin concentrations, all samples were included in the analysis by replacing half value of the minimum detection limit for samples that were below the detection limit. Statistical analysis was performed using SPSS version 22 (IBM SPSS statistics 22, Chicago, Illinois); and all test were performed at a probability level of $P = 0.05$.

Results

Fungal contamination in maize kernel samples

Fungal contamination level, determination of the species composition and prevalence of *Fusarium* species were analysed in a total of 10000 maize kernels, 50 kernels from each of the 200 samples. On average, 38 % (range 16 to 68 %) of the maize kernels were contaminated with several fungal species (Table 2). Among the fungal contaminated kernels, about 33 % were contaminated only by *Fusarium* species, while, the rest was contaminated by one or more of other fungi including *Aspergillus* (29.9 %), *Penicillium* (17.4 %), *Stonecarpella* (12.7

%), *Acremonium* (4 %), *Mucor* and *Rhizopous* species (3.9 %). In some cases, more than one fungus were observed on the kernels. The proportion of kernels contaminated by fungi varied significantly ($p < 0.039$) among maize growing areas. The highest mean fungal contamination of kernels were observed in samples collected from Dedesa (45.2 %), followed by Agaro (43.6 %) and Jimma (42.6 %). Significantly lower fungal contamination were recorded from areas with higher elevation and lower temperature (SM4), such as Maichew (31.2 %) and Korem (31.4 %) (Tables 1 and 2).

The proportion of kernels infected with fungi varied also substantially among samples within areas. For example, kernel infection in samples collected from the Agaro area varied from 30 % to 68 % (Table 2). When data on fungal contamination of kernels were combined and analysed according to agroecological conditions (Fig. 2) the highest kernel contamination was recorded in areas categorized as warm moist lowlands (M2) (43 %) followed by the tepid humid mid-highlands (H3) (42 %). Kernel contamination was lowest (31.3 %) in the cool sub-moist mid-highlands (SM4) compared to the other agroecological zones.

Table 2 Levels of fungal contamination of maize kernels collected in July 2012 from the May-December 2011 growing season in different areas belonging to seven agroecological zones in Ethiopia

[a]Agroecological zones	Area	Altitude [b] (masl)	Rainfall (mm) [c]	[d] Relative humidity (%)	Temperature (°C) [e]		Storage period (Month) [f]	Kernel infection (%)	
					2011	2012		Range	Mean
H_3	Bedele	2015	908	63.0	18.5	19.0	6	28 - 56	41.2 abc
H_3	Jimma	1714	1000	67.4	22.5	22.5	6	28 - 62	42.6 abcd
M_2	Dedesa	1560	915	63.4	23.5	23.8	7	32 - 60	45.2 a
M_2	Kemissie	1450	684	61.3	22.5	23.0	7	28 - 58	40.8 abcd
M_3	Ambo	2150	774	55.6	18.0	18.3	6	24 - 48	38.0 abcde
M_3	Dessie	2490	696	57.0	16.0	16.4	7	20 - 42	33.8 de
M_3	Gedo	2513	746	54.3	17.5	17.8	6	16 - 48	34.2 cde
M_3	Hawassa	1716	884	65.0	22.5	22.7	6	26 - 50	39.8 abcd
SH_2	Gibe	1206	738	63.4	24.5	24.8	7	30 - 60	41.2 abcd
SH_3	Agaro	1685	1037	65.3	21.4	22.0	6	30 - 68	43.6 ab
SH_3	Alaba	1953	806	61.7	19.0	20.0	6	24 - 56	36.8 bcde
SH_3	Bako	1743	709	61.0	19.7	20.0	6	26 - 52	36.8 bcde
SH_3	Nekemte	2100	986	61.2	20.0	20.2	6	26 - 58	38.0 abcde
SH_3	Sire	1869	784	60.0	20.0	20.3	6	24 - 56	35.2 cde
SH_3	W/sedo	2046	872	66.2	22.0	22.4	6	26 - 60	41.0 abcd
SH_3	Ziway	1642	682	62.2	21.5	22.0	7	26 - 54	36.2 bcde
SM_2	Alamata	1524	537	57.0	21.0	22.0	7	26 - 46	38.8 bcde
SM_2	Melkassa	1550	588	54.2	20.0	20.6	7	22 - 48	36.4 bcde
SM_4	Korem	2490	780	56.0	16.0	16.5	6	18 - 44	31.4 e
SM_4	Maichew	2450	759	55.0	17.5	17.8	7	16 - 46	31.2 e

[a]Agroecological zones- H_3: Tepid humid mid-highlands; M_2: Warm moist lowlands; M_3: Tepid moist mid-highlands; SH_2: Warm sub-humid lowlands; SH_3: Tepid sub-humid mid-highlands; SM_2: Warm sub-moist lowlands; and SM_4: Cool sub-moist mid-highlands (Source: MoARD 2005), [b] elevation of a representative location for sample collection area; [c] total rainfall for the period from seeding to harvesting (May to December 2011) [d] seasonal mean relative humidity for the storage period (January - June 2012); [e] seasonal average daily temperature of sample collection areas stretching from May to December 2011) and for the storage period (January - June 2012); [f] duration (months) between crop harvest and sampling; within columns, means followed by the same letter are not statistically different according to LSD (0.05).

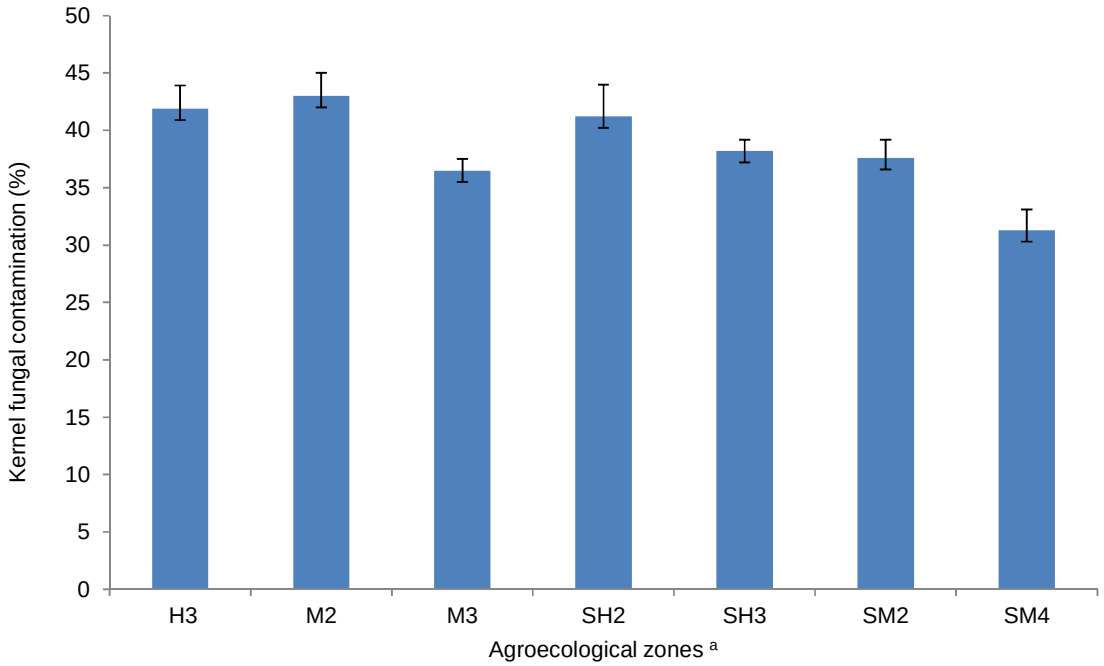

Fig. 2 Fungal infection levels of maize kernels from different agroecological zones of Ethiopia. Error bars represent standard error of the mean; [a]Agroecological zones- H_3: Tepid humid mid-highlands; M_2: Warm moist lowlands; M_3: Tepid moist mid-highlands; SH_2: Warm sub-humid lowlands; SH_3: Tepid sub-humid mid-highlands; SM_2: Warm sub-moist lowlands; and SM_4: Cool sub-moist mid-highlands (Source: MoARD 2005); Bars indicate standard error of the mean.

Identification of *Fusarium* species associated with maize kernels

A total of 1254 *Fusarium* isolates were recovered and identified from the maize kernel samples assessed. Microscopic analysis of morphological fungal structures and *EF-1α* gene sequencing revealed the presence of eleven different *Fusarium* species associated with maize kernels grown in different major maize growing areas of Ethiopia. *Fusarium verticillioides* was the most abundant species, representing 42 % of the total number of *Fusarium* isolates recovered from the maize kernels, followed by *F. graminearum* species complex (22.5 %), *F. pseudoanthophilum* (13.4 %) and *F. oxysporum* (7.5 %) (Fig. 3). *Fusarium incarnatum, F. brevicatenulatum* and *F. temperatum* were less frequent, constituting about 2.8 to 4.8 % of the *Fusarium* species isolated (Fig. 3). *Fusarium equiseti, F. subglutinans* and *F. lacertarum* were among the rarely isolated species. For isolates categorized as unidentified *Fusarium* sp. based on morphology, no good match was found in the NCBI and *Fusarium* ID databases.

The *EF-1α* gene sequences for this *Fusarium* sp. was most similar to isolates of the *Gibberella fujikuroi* species complex with 93 % resemblance (Table 3).

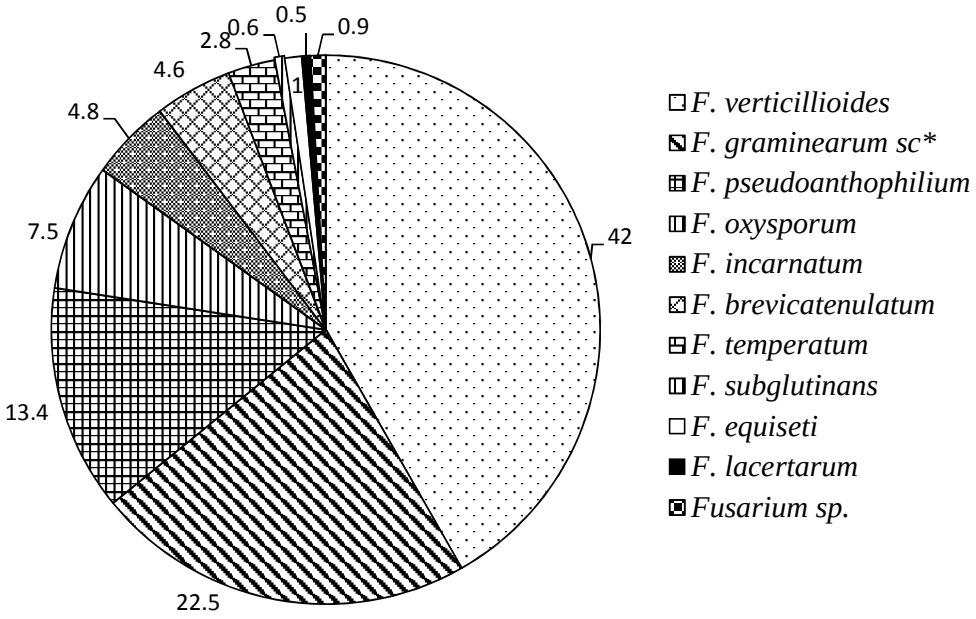

Fig. 3 Relative prevalence (%) of *Fusarium* spp. in maize kernels in Ethiopia in 2012. *sc: *Fusarium graminearum* species complex mainly *F. boothii*.

Species composition and prevalence of *Fusarium* species in the major maize growing areas and different agroecological zones of Ethiopia

The species composition and prevalence of *Fusarium* species isolated in different areas varied, as presented in Fig. 4. Eight different *Fusarium* species were isolated from Korem and Dessie areas, seven *Fusarium* species were isolated from Agaro, Bedele, Bako, Gibe, Dedesa and Kemissie areas; while in Nekemte, Ziway, Melkassa and Alamata four species were isolated (Fig. 4). The total count of each *Fusarium* species varied significantly ($p < 0.05$) among areas investigated except for *F. pseudoanthophilum.*

Table 3 List of *Fusarium* isolates subjected to *EF-1α* gene sequence analysis and sequence with best match of identity in the GenBank and *Fusarium*-ID

Isolate code	Morphological and *EF-1α* based identification	GenBank accession number and sequence with best match	Similarity (%)
AR312	*F. verticillioides*	KC964129.1	100
KM251	*F. verticillioides*	FD_01387_EF-1a	99.4
AW314	*F. verticillioides*	FD_01388_EF-1a	99.9
AG411	*F. verticillioides*	FD_01388_EF-1a	99.9
KR222	*F. verticillioides*	KP732012.1	100
MC142	*F. verticillioides*	FD_01387_EF-1a	100
AG683	*F. graminearum* sc [a]	FD_01128_EF-1a	99.5
BD920	*F. graminearum* sc [a]	FD_01128_EF-1a	99
KR332	*F. graminearum* sc [a]	FD_01131_EF-1a	99.7
GD312	*F. graminearum* sc [a]	FD_01130_EF-1a	99.8
ML322	*F. pseudoanthophilium*	AF160264.1	99
MC351	*F. pseudoanthophilium*	AF160264.1	99
MC411	*F. pseudoanthophilium*	AF160264.1	98
WS212	*F. oxysporum*	KJ418427.1	99
GD431	*F. oxysporum*	KJ418427.1	99
AM521	*F. oxysporum*	KJ418427.1	99
KR511	*F. oxysporum*	KF574857.1	99.9
DS6951	*F. oxysporum*	FD-00117_EF-1a	99.7
DS6941	*F. oxysporum*	FD-00809_EF-1a	100
AW6411	*F. subglutinans*	KC194168.1	100
Z61051	*F. temperatum*	JX987073.1	100
AG821	*F. temperatum*	JX987074.1	100
GI112	*F. temperatum*	KC964121.1	99
AB6642	*F. temperatum*	KC964121.1	100
KR211	*F. temperatum*	KC964121.1	100
KR521	*F. temperatum*	JX987074.1	100
DS6722	*F. brevicatenulatum*	AF160265.1	99
GI412	*F. brevicatenulatum*	AF160265.1	99
BK950	*F. brevicatenulatum*	AF160265.1	98
BD6651	*F. brevicatenulatum*	AF160265.1	98.4
AR111	*F. incarnatum*	JF270215.1	99
AR6732	*F. incarnatum*	JF270267.1	99
KR533	*F. equiseti*	KP732019.1	98
AM61043	*F. lacertarum*	JF740828.1	99
WS622	*Fusarium* sp. [b]	FD_01767_EF-1a	93.4
WS631	*Fusarium* sp. [b]	FD_01767_EF-1a	93.

[a] *F. graminearum* sc: *F. graminearum* species complex mainly *F. boothii*; [b] *Fusarium* sp: *Fusarium* sp. nested within the *Gibberella fujikuroi* species complex with *EF-1α* showing 93% identity to FD_01767_EF-1a

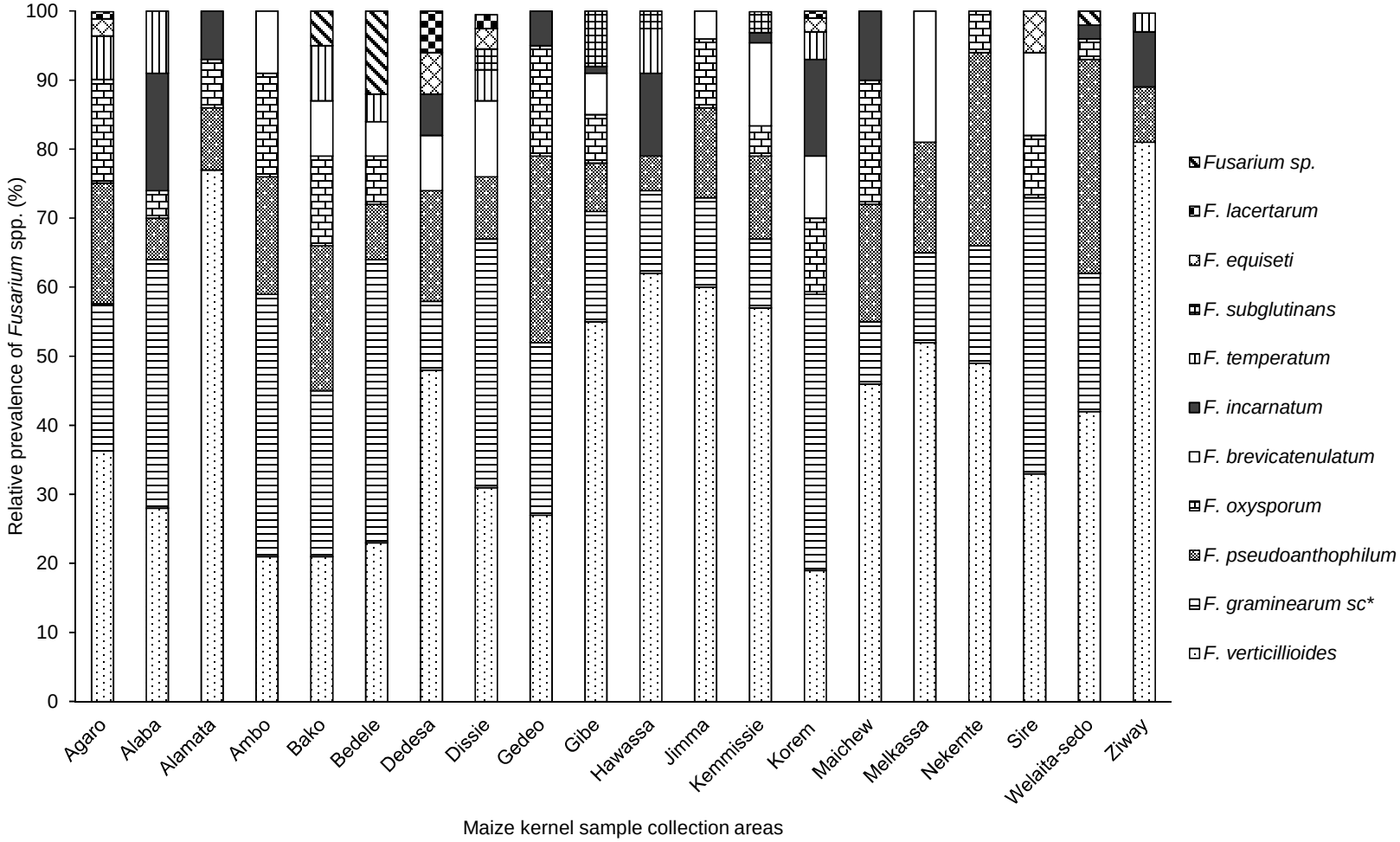

Fig. 4 Species composition and prevalence of *Fusarium* spp. on maize kernels in different growing areas in Ethiopia, during 2012. *sc: *Fusarium graminearum* species complex mainly *F. boothii*.

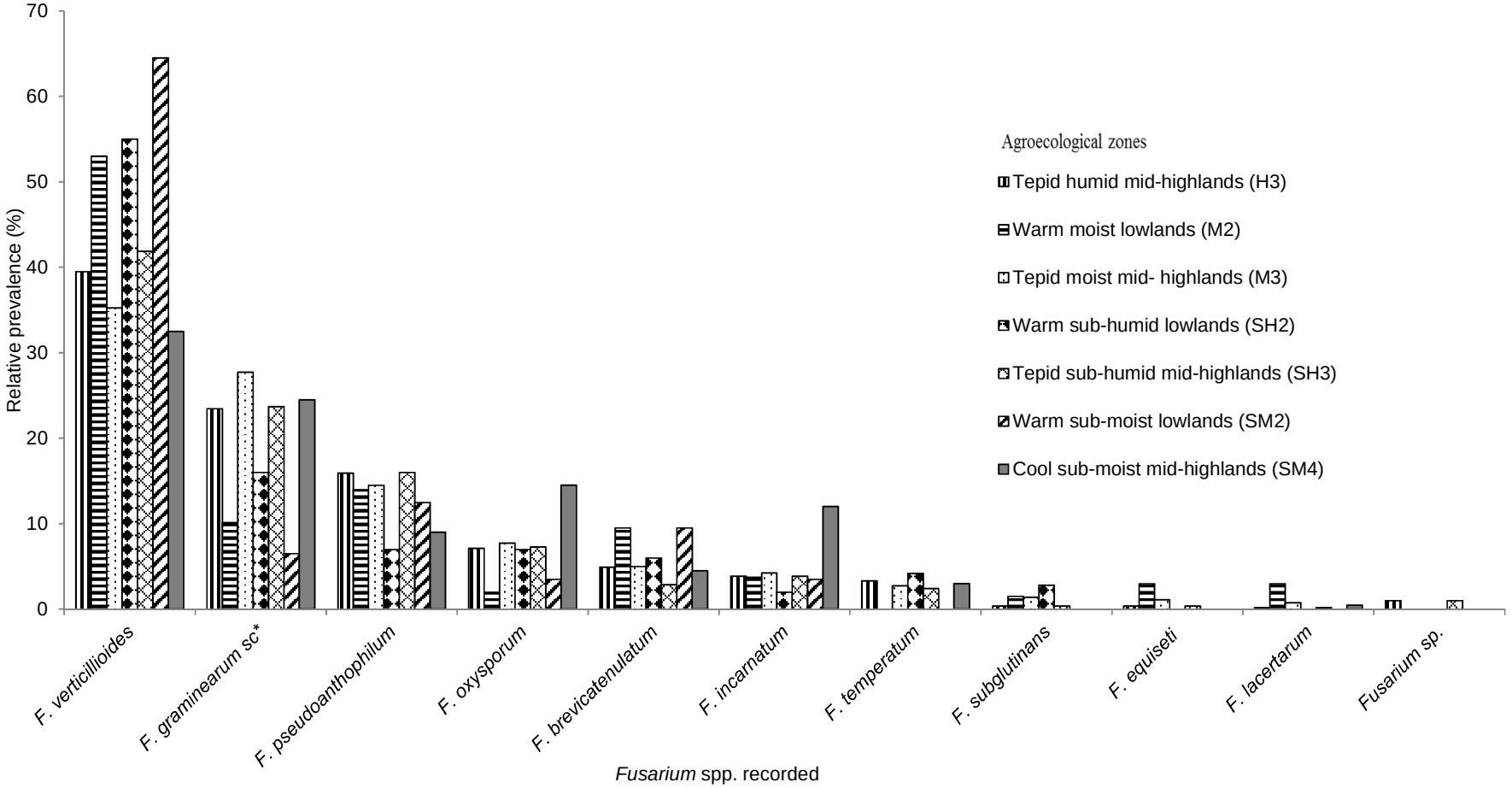

Fig. 5 Prevalence of *Fusarium* spp. on maizes kernels from different agroecological zone of Ethiopia, in 2012. *sc: *Fusarium graminearum* species complex mainly *F. boothii*.

The most prevalent *Fusarium* species on maize kernels in several maize growing areas were *F. verticillioides* followed by *F. graminearum* species complex. *F. verticillioides* occurred in all areas investigated, representing more than two-third of the *Fusarium* species isolated in some areas (Fig. 4). The relative prevalence of *F. verticillioides* was 81 % in Ziway and 77 % in Alamata, while in Korem, Bako and Ambo; it was only from 19 to 21 %. The *Fusarium graminearum* species complex was recorded in 90 % of the areas assessed, but it was predominant in areas with higher elevation and lower temperature (Fig. 4 and Table 2). According to the molecular identification results, *F. boothii* appeared to be the dominant member of the *F. graminearum* species complex associated with maize kernels in Ethiopia (Table 3). *Fusarium oxysporum, F. incarnatum, F. brevicatenulatum* and *F. temperatum* were among the less prevalent *Fusarium* species, but occurred in several areas up to 20 % (Fig. 4).

The data on *Fusarium* species occurrence in the different maize growing areas were grouped into the respective agroecological zones of Ethiopia, and the results were as presented in Fig. 5. *Fusarium verticillioides* was recorded in all agroecological zones assessed, but was most prevalent in the SM_2-zone (64.5 %), the SH_2-zone (55 %) and the M_2-zones (53 %) which are characterized by lower elevation, warm and dry conditions. The *F. graminearum* species complex was less prevalent in these zones and more abundant in zones generally characterized by low temperature and wetter conditions, such as the M_3-zone, SM_4-zone and the SH_3-zone (Fig. 5). The prevalence of *F. pseudoanthophilum* was similar in several agroecological zones investigated except the SH_2-zone. The highest relative prevalence of both *F. incarnatum* and *F. oxysporum* was in the SM_4-zone. *Fusarium temperatum, F. equiseti, F. subglutinans, F. lacertarum* and *Fusarium* sp. were detected in only some agroecological zones and with less than 5 % prevalence (Fig. 5).

Fumonisin contamination in maize samples

Fumonisin was detected in maize samples collected from all but one of the maize growing areas sampled (Table 4). The majority of the 200 samples (77 %) contained fumonisin at concentrations ranging from 25 μg $kg^{-1}$to 4500 μg kg^{-1}. The overall mean and median fumonisin concentration in samples were 348 μg $kg^{-1}$and 258 μg kg^{-1}, respectively. Fumonisin levels were comparatively higher than the overall mean in samples from Jimma (mean: 918.6 μg kg^{-1}, median: 501.5 μg kg^{-1}), Ziway (mean: 577 μg kg^{-1}, median: 392 μg kg^{-1}), Alamata

(mean: 533 µg kg^{-1}, median: 392.5 µg kg^{-1}) and Hawassa (mean: 523 µg kg^{-1}, median: 455 µg kg^{-1}). The fumonisin level in one sample from Jimma exceeded the maximum tolerable limit of 2000 µg kg^{-1} set by the US Food and Drug Administration in food intended for direct human consumption (FDA 2001). In total, about 7 % of the maize samples exceeded the maximum tolerable limit set by the European Union in maize flour (> 1000 µg kg^{-1}) (EU commission 2006). These were three samples from Ziway, two from Kemmissie, two from Alamata, two from Jimma, and one each from Alaba, Hawasa, Nekemte, Dissie and Korem. Bako was the only area where none of the samples were contaminated with detectable level of fumonisin (Table 4). The Sire area had also fewer incidents of fumonisin-contaminated samples, with a mean concentration far below the mean for all the samples. When fumonisin data was grouped into the agroecological zones of Ethiopia, the highest mean fumonisin concentration was recorded in the H_3-zone (568.8 µg kg^{-1}) followed by the SH_2-zone (422 µg kg^{-1}) and the M2-zone (420.5 µg kg^{-1}). The lowest mean fumonisin concentrations were observed in samples from the M_3 and SH_3 zones (Fig. 6).

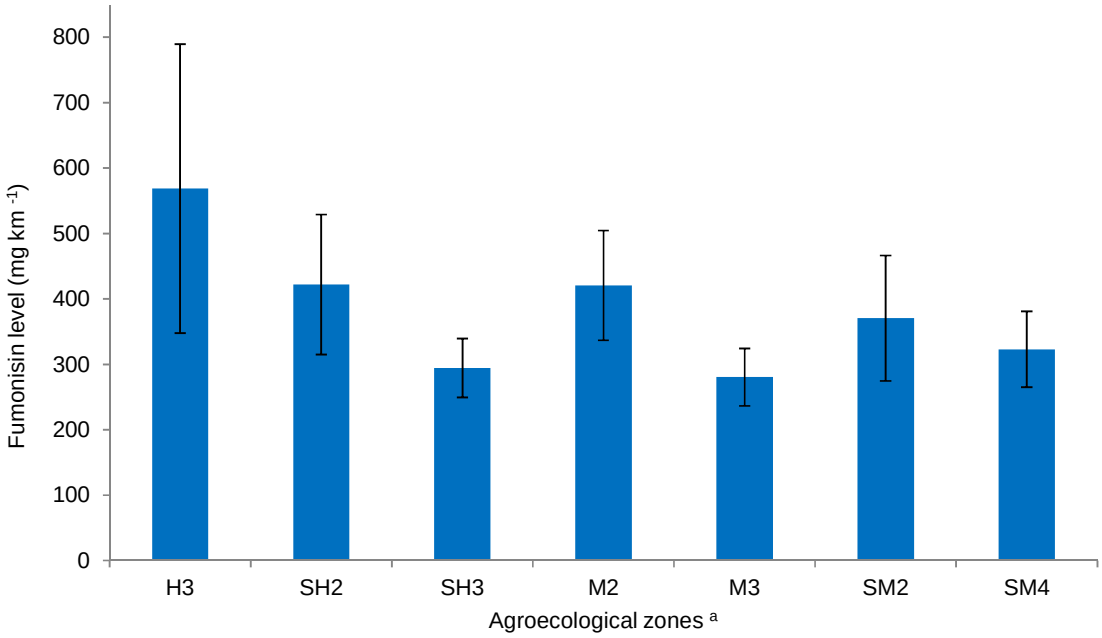

Fig. 6 Mean fumonisin level in maize kernels from different agroecological zones of Ethiopia. Error bars represent standard error of the mean; [a]Agroecological zones- H_3: Tepid humid mid-highlands; M_2: Warm moist lowlands; M_3: Tepid moist mid-highlands; SH_2: Warm sub-humid lowlands; SH_3: Tepid sub-humid mid-highlands; SM_2: Warm sub-moist lowlands; SM_4: Cool sub-moist mid-highlands (Source: MoARD 2005); bars indicate standard error of the mean.

Table 4 Fumonisin contamination levels of maize samples from different maize-producing areas of Ethiopia, in 2012

Maize growing areas	Samples containing fumonisin (%)	Fumonisin concentrations (μg kg^{-1})		
		Range	Median	Mean
Agaro	90	72 - 772	395	395 bcd
Alaba	60	86 - 1380	121	316 bcd
Alamata	100	30 - 1370	393	533 bc
Ambo	90	94 - 324	146	159 cde
Bako	0	< 25	< 25	< 25 e
Bedele	100	25 - 846	142	219 bcde
Dedesa	50	639 - 743	326	345 bcde
Dissie	90	39 - 1070	75	231bcde
Gedeo	40	325 - 664	169	210 bcde
Gibe	70	29 - 840	432	422 bc
Hawassa	100	248 - 1138	455	523 bc
Jimma	100	62 - 4500	502	919 a
Kemissie	90	99 - 1090	368	496 bc
Korem	90	25 - 1060	233	307 bcde
Maichew	100	89 - 697	297	339 bcde
Melkassa	70	27 - 807	82	208 bcde
Nekemte	80	108 - 1010	193	297 bcde
Sire	30	44 - 125	13	33 de
Welayta-sedo	100	49 - 986	423	432 bc
Ziway	90	110 - 1530	392	577 ab
Average	77		258	348

Samples have been stored for 6-7 months after harvest by small-scale farmers and ten samples were analysed from each area; within columns, means followed by the same letter are not statistically different according to LSD (0.05).

Correlation between occurrence of *Fusarium* species, kernel contamination, fumonisin and climatic data

The total contamination of kernels with fungi was significantly correlated with the recorded temperature ($r = 0.794$, $p < 0.001$) and rainfall ($r = 0.500$, $p < 0.029$) for the growing season (2011). Similar positive correlation of kernel contamination with the recorded temperature ($r = 0.791$, $p < 0.001$) and relative humidity ($r = 0.761$, $p < 0.001$) for the storage period was observed. Positive correlation was also observed between the occurrence of some of the *Fusarium* spp. and kernel contamination levels (Table 5). Significantly strong positive correlation ($r = 0.862$, $p < 0.001$) was observed between the incidence of *F. verticillioides*, the primary producer of fumonisin, and fumonisin concentrations in maize samples. There was a significant positive correlation between fumonisin concentration with temperature recorded in the growing season ($r = 0.533$, $p < 0.016$), relative humidity ($r = 0.521$, $p < 0.018$) and temperature recorded for the storage period ($r = 0.518$, $p < 0.019$). However, there was no or poor correlation between rainfall data recorded for the growing season and fumonisin contamination ($r = 0.276$, $p > 0.238$) (Table 5).

Discussion

The results reported in the present study indicate that maize grown in different ecological conditions in Ethiopia contains a wide range of *Fusarium* species. Several *Fusarium* spp., both common and less frequent pathogens of maize were isolated from maize kernels. *Fusarium verticillioides* was the predominant one, found in all areas investigated and representing 42 % of the total number of isolates recovered. It was, however, most prevalent in the low altitude and high temperature areas. The consistent recovery of *F. verticillioides* throughout all maize growing areas and Ethiopian agroecological zones indicates its intimate association with the plant and its adaptation to the tropical climate. This is in line with previous observations of Ayalew (2010) that this *Fusarium* species is dominant in maize grain produced in Ethiopia although predominance levels differ. In some parts of eastern and central Ethiopia, Ayalew (2010) has reported that 99 % of the *Fusarium* spp. on the internal and external surface of maize kernels was *F. verticillioides*. The high prevalence of *F. verticillioides* on maize kernels is also in agreement with observations made in other countries such as South Africa (Ncube et al. 2011), Mexico (Reyes-Velázquez et al. 2011) and Kenya (Bii et al. 2012).

Table 5 Simple linear correlations coefficients (r) between climatic data, *Fusarium* spp., kernel infection level and fumonisin contamination

Variables	Fumonisin (µg kg^{-1})		Total fungal infection of kernels (%)	
	Corr. (r)	p-values	Corr. (r)	p-values
Rainfall 2011 (mm) [a]	0.276	0.238	0.500	0.029
Temperature 2011 (°C) [b]	0.533	0.016	0.794	0.001
Relative humidity (%) [c]	0.521	0.018	0.761	0.001
Temperature 2012 (°C) [d]	0.518	0.019	0.791	0.001
F. verticillioides [e]	0.862	0.001	0.683	0.001
F. graminearum species complex [e]	0.241	0.001	0.475	0.010
F. pseudoanthiphilium [e]	0.604	0.001	0.626	0.001
F. oxysporum [e]	0.210	0.003	0.350	0.001
F. brevicatenulatum [e]	0.156	0.027	0.230	0.001
F. semitectum [e]	0.212	0.003	0.050	0.483
F. temperatum [e]	0.325	0.001	0.319	0.001
F. subglutinans [e]	0.275	0.001	0.129	0.001
F. equiseti [e]	0.152	0.031	0.178	0.038
F. lacertarum [e]	0.045	0.526	0.167	0.017
Fusarium sp. [e]	0.121	0.089	0.148	0.036
Total fungal infection of kernels (%)	0.671	0.001	-	-

[a] total amount of rainfall from seeding to harvest (May to December 2011); [b] seasonal mean daily temperature from seeding to harvest (May to December 2011); [c] seasonal mean relative humidity for the storage period (January to June 2012); [d] seasonal mean daily temperature for the storage period (January to June 2012); [e] total number of isolates recorded or count value and r: correlation coefficient.

Among the *Fusarium* spp. reported in this study, the *F. graminearum* species complex is the second major contaminant of maize kernels in Ethiopia and it is most important in areas characterized by high elevation, low temperature and wet conditions. Observations of *F. graminearum* thriving in cooler temperature and wetter conditions than *F. verticillioides*, have also been reported from other maize-growing areas in the world (Vigier et al. 1997; Dorn et al. 2011; Scauflire et al. 2011). *Fusarium pseudoanthophilum* and *F. oxysporum* were also

among the common *Fusarium* spp. on maize kernels isolated in the current study. Leslie and Summerell (2006) indicated that *F. pseudoanthophilum* could be found in hot dry areas and wet tropical regions, while species from the *F. oxysporum* complex are cosmopolitans that can be recovered from different climatic conditions. In addition to the above-mentioned *Fusarium* spp., a large number of other species such as *F. incarnatum, F. brevicatenulatum, F. temperatum, F. equiseti, F. subglutinans, F. lacertarum* and *Fusarium* sp. were detected on maize kernels. Many of these species occurred in low frequency in several areas and agroecological zones in Ethiopia. Some of these species could be saprophytes saprobes or opportunistic colonizers of maize plants, however, they may still cause yield losses and mycotoxin contamination of the kernels. To our knowledge, this is the first report of the occurrence of *F. temperatum, F. brevicatenulatu, F. pseudoanthophilum, F. incarnatum, F. equiseti* and *F. lacertarum* in maize in Ethiopia.

The current study shows the presence of considerable variation in species composition and prevalence of *Fusarium* species in the different maize growing areas and agroecological zones in Ethiopia. Variation in fungal and fumonisin contamination levels were also high. The reason for this could partly be due to the variation in climatic factors in the maize growing areas, mainly temperature, humidity and precipitation. The influence of climatic conditions on the species composition and prevalence of *Fusarium* spp. may be due to a direct effect on growth, production and dispersal of inoculum, but also an indirect effect on soil and vegetation type, which may influence saprophytic survival (Doohan et al. 2003; Munkvold 2003). The positive correlations of fungal kernel contamination with temperature and rainfall data as well as fumonisin concentration with relative humidity and temperature data found in this study also show the strong effect of environmental factors. Weather conditions characterized by high temperature and low rainfall in the growing season, particularly after silking, favor colonization of maize ears by pathogenic *Fusarium* species and subsequent fumonisin contamination (Goertz et al. 2010; Vigier et al. 1997). Contamination of kernels with *Fusarium* species and fumonisin may also increase considerably with wet or humid weather condition in the later growing season, especially at the time of crop harvest and during drying (Munkvold 2003).

In addition to climatic factors, agricultural practices such as crop rotation (previous crop), insect pest management, choice of cultivars, tillage systems and crop residue management, post-harvest drying and handling practices applied by different maize growing small-scale

farmers, may have great impact on kernel contamination, the prevalence of *Fusarium* species on maize kernels and subsequent fumonisin contamination. The pronounced differences recorded for percentage of kernels contaminated by fungal agents and fumonisin concentration among samples collected from the same areas points to the importance of different on-farm agricultural practices and postharvest handling activities employed by different maize growers. Some farmers store the unshelled cobs on tree branches and the grains in underground pits before transfer into the house; such practice may expose to late rain showers and hence high fungal infection. In some parts of Ethiopia, maize is often grown in short rotations preceding small grain cereals such as wheat and sorghum or maize monocropping. Such repeated planting of maize and other cereals in the same field may lead to the high incidence of *Fusarium* spp. and other fungal contaminants by increasing the amount of inoculums. Logistics regression modeling of cropping systems employed to predict fumonisin contamination level in maize showed that preceding crop, maturity class of hybrids, grain moisture and harvesting week significantly affects the level of fumonisin contamination (Battilani et al. 2008).

The results obtained in the present study indicate that about two-third (77 %) of the maize samples were contaminated with fumonisin, which indicate a widespread occurrence of the toxin in maize grown in Ethiopia. These results are in accordance with those reported by Ayalew (2010), with detected fumonisin concentrations ranging from 300 to 2400 µg kg^{-1} in 17 samples collected from areas in eastern and central Ethiopia. However, the fumonisin concentrations obtained in the maize samples in the present study (mean: 348 and median: 258 µg kg^{-1}) were lower than reported from other neighboring countries in eastern and southern Africa (Bii et al. 2012; Nucbe et al. 2011; Sundheim and Tsehaye 2015). Under good growing condition, the fungus is a commensal, causing small damage to kernels and little fumonisin formation (Pitt et al. 2013). In the Bako and Sire areas, no or relatively few samples contaminated with fumonisins were detected (Table 4). A reason for this could be that these areas are located nearby the national center for maize research where farmers can get access to improved maize hybrids that are resistance to insect pests, fungal infection and fumonisin contamination.

In general, higher fumonisin concentration was found in samples collected in the humid and warmest areas, and fumonisin concentration was positively correlated with relative humidity and temperature, while there was poor correlation between total seasonal rainfall and

fumonisin concentration. This is in agreement with previous reports from different countries (Goertz et al. 2010; Ncube et al. 2011).

The highest mean total fumonisin was recorded in the H_3 zone (Fig. 6) although the prevalence of *F. verticillioides*, the primary producer of fumonisin, was highest in the SM_2 zone (Fig. 5). This indicates an important role of humidity in fumonisin contamination, as the H_3 zone is more humid than the SM_2 zone. Meteorological data recorded for the year 2011 indicate the presence of late season rainfall during the time of harvesting in the areas included in the H_3 zone (Jimma and Bedele) but not in areas included in the SM_2 zone (Alamata and Melkassa). The exceptionally highest fumonisin concentration (4500 gm kg^{-1}) recorded in the current study was also from the H_3 zone and this may exaggerate the mean fumonisin concentration in this zone.

From the present study, it becomes clear that a great diversity of *Fusarium* species infects maize kernels in Ethiopia but species composition and relative prevalence differs depending on area and agroecological conditions. Pooled results over all areas investigated indicated that *F. verticillioides* is the predominant species on maize kernels in Ethiopia followed by the *F. graminearum* species complex. Overall results indicate widespread occurrence of fumonisin mycotoxins on maize kernels in Ethiopia. These findings will serve as an important foundation for any study on *Fusarium* and fumonisins in maize in Ethiopia, and points to the most important *Fusarium* spp. for designing control strategies. Further assessment need to be continued in the future to analyse the prevalence of *Fusarium* spp. and fumonisin contamination in relation to agronomic practices over different years, to observe trends in fumonisin contamination and food safety. The observed widespread prevalence of different toxigenic fungal species such as *Aspergillus* spp., *F. graminearum* species complex, *F. subglutinans* and others may indicate the possibility of contamination of maize kernels by several mycotoxins other than fumonisin. Thus, the occurrence of other frequent contaminants of maize and harmful mycotoxins such as aflatoxins, deoxynivalenol, nivalenol and zearalenone should also be consider in future assessments.

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Paper II

Genetic variation among *Fusarium verticillioides* isolates associated with Ethiopian maize kernels as revealed by AFLP analysis

Hadush Tsehaye [1,4,*], Abdelhameed Elameen [2], Anne Marte Tronsmo [1], Leif Sundheim [2], Arne Tronsmo [3], Dereje Assefa [4], May Bente Brurberg [2]

[1] Norwegian University of Life Sciences, Department of Plant Sciences, P.O. Box 5003, NO-1432 Ås, Norway

[2] Norwegian Institute for Bioeconomy Research, Biotechnology and Plant Health Division, P.O. Box 115, NO-1431 Ås, Norway

[3] Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science, P.O. Box 5003, NO-1432 Ås, Norway

[4] Mekelle University, Department of Dryland Crop and Horticultural Sciences, P.O.Box 231, Mekelle, Tigray, Ethiopia

[*] Corresponding author. E-mail address: hadush.beyene@nmbu.no or had031@yahoo.com

Abstract

Amplified fragment length polymorphism (AFLP) was used to study the genetic variation among 80 *F. verticillioides* isolates from kernels of Ethiopian maize, collected from 20 different maize growing areas in four geographic regions. A total of 213 polymorphic fragments were obtained using six *Eco*RI/*Mse*I primer combinations. Analysis of the data based on all 213 polymorphic AFLP fragments revealed high level of genetic variation in the *F. verticillioides* entities in Ethiopia. About 58 % of the fragments generated were polymorphic. The genetic similarity among *F. verticillioides* isolates varied from 46 % to 94 % with a mean Dice similarity of 73 %. Unweighted Pair Group Method with Arithmetic Average (UPGMA) analysis revealed two main groups and four subgroups. The principal coordinate analysis (PCO) also displayed two main groups that agreed with the results of UPGMA analysis, and there was no clear pattern of clustering of isolates according to geographic origin. Analysis of molecular variance: (AMOVA) showed that only 1.5 % of the total genetic variation was between geographic regions, while 98.5 % was among isolates from the same geographic regions of Ethiopia. Eighty distinct haplotypes were recognized among the 80 isolates analysed. Hence, breeding efforts should concentrate on quantitative resistance that is effective against all genotypes of the pathogen.

Keywords: AFLP, AMOVA, *Fusarium verticillioides,* genetic variation

Introduction

Maize (*Zea mays* L.) is currently one of the most important food crops in Ethiopia, grown under rain fed as well as irrigated agriculture. Maize has high yield potential, and it is a source of important nutrients. The crop plays a key role in food security for millions of people in Ethiopia (Worku et al. 2012). Fungal diseases are among the primary constrains for maize production in Ethiopia, of which *F. verticillioides* (Sacc.) Nirenberg (teleomorph *Gibberella fujikuroi* (Sawada) Wollenw. is the most frequently isolated fungal pathogen from maize kernels (Ayalew 2010). This Ascomycete fungus is a common fungal pathogen of maize worldwide, causing various diseases such as root rot, stalk rot and ear rot (Brown et al. 2012; Darnetty and Salleh 2013). *Fusarium verticillioides* infection may cause reduced grain yield and quality in maize (Presello et al. 2008). During growth on maize, the fungus may contaminate the grains by mycotoxins mainly, fumonisins (FB_1, FB_2, FB_3) (Ono et al. 2010; Rocha et al. 2011), and trace amounts of several other toxins including fusarins, fusaric acid, and moniliformin (Brown et al. 2012; Darnetty and Salleh 2013). Globally, maize infected with *F. verticillioides* is considered to be the major sources of high level of fumonisin contamination (Waśkiewicz et al. 2012). Although there is variation among *F. verticillioides* isolates in fumonisin production, a majority of the isolates are able to synthesize the toxin (Covarelli et al. 2012; Rocha et al. 2011). Fumonisins are associated with several mycotoxicoses in various animal species, including leukoencephalomalacia in horses (Kellerman et al. 1990), pulmonary edema in swine (Harrison et al. 1990), and nephrotoxic and hepatotoxic effects in experimental animals (rodents) (Waśkiewicz et al. 2012). In humans, fumonisin contaminated food is considered a risk factor for esophageal and liver cancer, birth (neural tube) defects and cardiovascular problems (Waśkiewicz et al. 2012). Some *F. verticillioides* strains are associated with high morbidity and mortality in immunosuppressed patients (Chang et al. 2013).

Despite the importance of *F. verticillioides* in Ethiopia, nothing is known with respect to its genetic variation, aggressiveness, and potential for sexual recombination. Previous analyses of field samples of maize grains from some parts of eastern and central Ethiopia showed fumonisin contamination levels ranging from 0.3 to 2.4 mg kg^{-1} (Ayalew 2010). There are no effective measures available for control of *F. verticillioides,* and there is a need to develop appropriate disease management strategies. Genetic resistance should form the basis for sustainable management of *F. verticillioides* epidemics, and knowledge about the genetic

structure of the pathogen population is essential for developing resistant maize genotypes. Characterization of genetic diversity is an essential prerequisite, so that breeding materials can be tested against representative isolates in order to be effective to a number of genetically diverse pathogen isolates.

Several molecular techniques have been used to assess the genetic variation among *F. verticillioides* isolates in different parts of the world, for example restriction fragment length polymorphism of the internal transcribed spacer rDNA (RFLP-ITS) (Patiño et al. 2006), random amplified polymorphic DNA (RAPD) (Daie Ghazvini et al. 2011; Ono et al. 2010), inter simple sequence repeat (ISSR) (Chang et al. 2013), amplified fragment length polymorphism (AFLP) (Covarelli et al. 2012; Rocha et al. 2011; Reynoso et al. 2009) and genomic sequence analysis (Brown et al. 2008; Dehkordi et al. 2013). Such analytical techniques have made it possible to identify a particular species, to determine genetic relatedness among individuals and pathogen populations, differentiate subpopulations or formae speciales adapted to a specific host, distinguish lineage differing in fumonisin production, group individual races/strains of a pathogen according to their host cultivars preference and geographic origin. In this study, we used AFLP to characterize *F. verticillioides* isolates obtained from maize grains produced in Ethiopia. This method has been increasingly used in analysis of *Fusarium* populations, and to estimate genetic relatedness in *F. verticillioides* isolates (Covarelli et al. 2012; Reynoso et al. 2009; Rocha et al. 2011). Previous studies from other countries showed pronounced intraspecific genetic variation among *F. verticillioides* isolates (Covarelli et al. 2012; Cumagun et al. 2009; Dehkordi et al. 2013; Rocha et al. 2011). In Argentina, Reynoso et al. (2009) have also found high genotypic diversity within populations, but limited or no detectable genetic subdivision between populations. Based on the fact that fungal reproduction in nature is consistent with random mating, we expect individuals within *F. verticillioides* isolates to be genetically diverse. The objectives of the present study were to assess genetic variation among *F. verticillioides* isolates collected from major maize growing areas present in four different geographical regions of Ethiopia, and to estimate the genetic differentiation within and between these *F. verticillioides* isolates from different regions.

Materials and Methods

Fungal isolates

Fusarium verticillioides were isolated from kernels of maize, collected from 20 different maize growing areas (Fig. 1), representing four geographic regions (South, South-west, Central-west and North) of Ethiopia. Altogether 200 maize grain samples were collected (10 samples from each of 20 different areas), and sampling sites within areas were separated at least by 1 km and at most approximately by 5 km distance from the other. Maize kernels were surface disinfected (1 % NaOCl for 2 min) and washed twice with sterile distilled water. Then fifty kernels were plated (five kernels per Petri dish) on a modified Czapek-Dox Iprodione Dichloran Agar (CZID), where the fungicides propiconazole 0.375 mg L^{-1} and fenpropimorph 1.125 mg L^{-1} were used instead of iprodione (Halstensen et al. 2006). The plates containing maize kernels were incubated for 5 - 7 days at 25 °C in the dark (Leslie and Summerell 2006). After confirming the identity of *Fusarium* colonies, by first growing on Spezieller Närstoffarmer Agar (SNA) for 7 - 10 days at 20 °C under alternatively near ultraviolet/white fluorescent (NUV) light (12 h) and dark (12 h), a spore suspension was prepared in sterile water and spread onto Water Agar (WA) plates for single spore isolation (Leslie and Summerell 2006). These plates were incubated at room temperature (22 °C) for 16 - 20 h and single germinating conidia were removed and transferred to a new SNA plates. Subsequently, sub-culturing was made on potato dexterous agar (PDA, Difco, Madison, USA) and carnation leaf agar (CLA) plates, incubated at 20 °C with alternative photoperiod (12 h NUV-light, followed by a 12 h dark period) from two up to six weeks for morphological identification to species according to the procedure described by Leslie and Summerell (2006). For confirmation of morphological identity, the translation elongation factor-1 alpha gene (*EF-1α*) region was sequenced for representative isolates (six isolates), as described by O'Donnell et al. (1998). A total of 80 *F. verticillioides* isolates were analysed in this study, with 3 to 5 isolates selected randomly from each maize growing area. One *F. verticillioides* isolate (MRC826), obtained from the South African Medical Research Council was also included as a reference isolate.

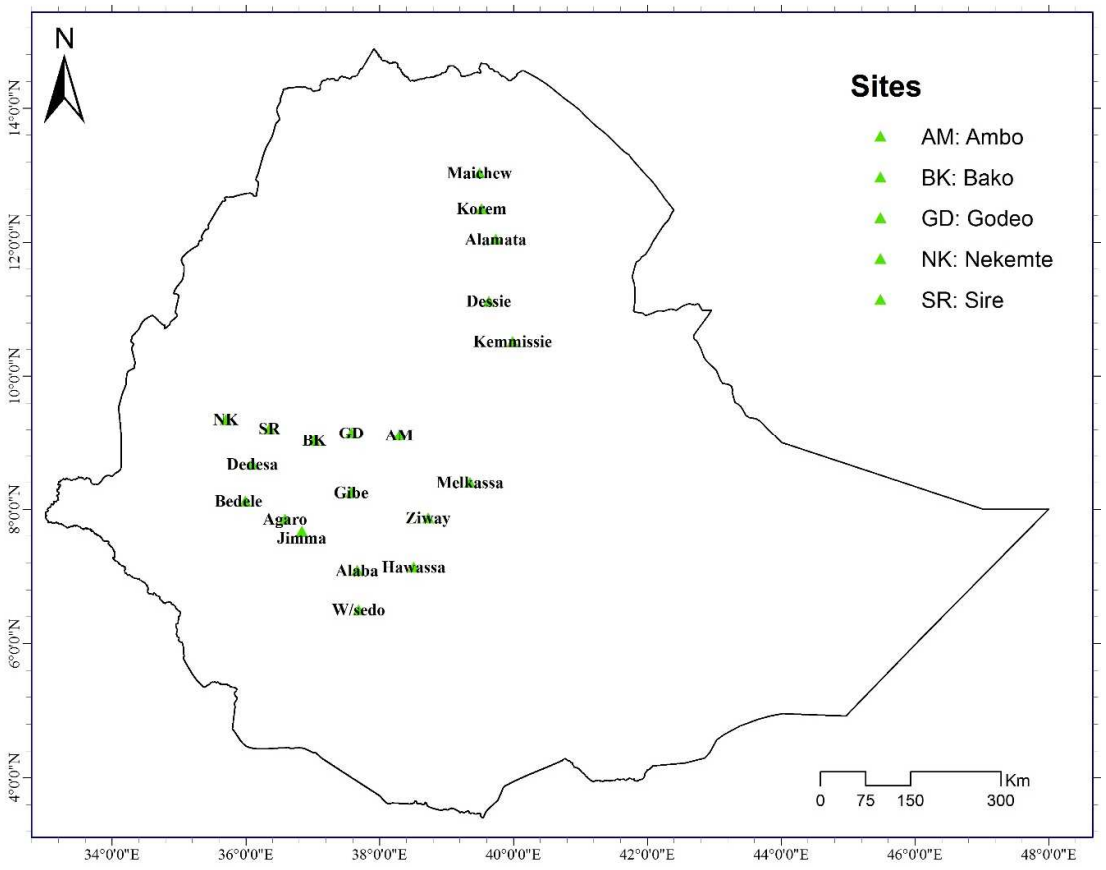

Fig. 1 Map of Ethiopia showing approximate geographic location of maize seed collection areas in North (five areas), South (five areas), South-west (four areas) and Central-west (six areas) from which *F. verticilloides* isolates were obtained. Maize seed sample collection sites in each areas were separated by a distance of at least 1 km and at most 5 km from the nearest other; Δ: sites; AM: Ambo, BK: Bako, GD: Gedo, NK: Nekemte, SR: Sire.

DNA extraction

For extraction of genomic DNA, mycelium from pure cultures obtained from single spores was grown on PDA (Difco, Madison, USA) at 22 °C under white light for 7 to 10 days. The mycelium was scraped from the surface of the agar plates and ground into fine powder with liquid nitrogen, using a mortar and pestle. DNA was extracted from approximately 100 mg mycelium of each isolate, using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. The concentration of DNA was estimated by using nano-drop spectrophotometry measurements and quality was checked by agarose gel electrophoresis.

AFLP analysis

AFLP analysis was performed as described by Vos et al. (1995), with modifications that included the use of fluorescently labelled primers instead of radioactive labelling as described by Leslie and Summerell (2006). A total of twelve *Eco*RI and *Mse*I primer combinations were initially tested, using a subset of isolates from the same *F. verticilloides*. The generated finger prints were evaluated for overall clearness of banding pattern and number of polymorphic markers produced. The selective amplification reaction was performed using six different primer combinations, and *Eco*RI primers were end labeled with fluorescent dye (6-FAM). PCR reaction was performed as previously described by Bonants et al. (2000). The fluorescently labelled PCR products were analysed using an ABI3730 DNA Analyser (Applied Biosystems, USA). One µl of PCR product was added to a loading buffer containing 8.75 µl Hi-Di formamide (Applied Biosystems, USA) and 0.25 µl of GeneScan 500 LIZ size standard (Applied Biosystems, USA). GeneMapper v4.0 (Applied Biosystems, USA) was used to derive and visualize the fragment lengths of the labeled DNA-fragments, using the known fragment lengths of the LIZ-labeled marker peaks. To check the repeatability of the results, fifteen samples of extracted DNA were repeated with all the six primer combinations.

Data Analysis

Band profiles for each isolate and primer combination were scored manually; using '1' for presence of a band at a particular position and '0' for absence of the band. The final binary data set were used to calculate genetic similarity using Dice coefficients. The clustering and dendrogram construction was performed using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) (Sneath and Sokal 1973). A principal coordinate analysis (PCO) was also performed to detect clustering and structure in the *F. verticillioides* isolates. All analyses were performed using the NTSYS-pc version 2.2 (Exeter Biological Software, Setauket, NY, USA) software package (Rohlf 2005).

Arlequin version 3.0 (Excoffier et al. 2005) was used for analysis of molecular variance (AMOVA) in order to estimate the genetic variation within and among regional populations (isolates from different geographic regions). All loci, polymorphic and monomorphic fragments, were considered for this analysis to avoid overestimation of genetic diversity. Genetic differentiation of the isolates from different regions was estimated from the calculated

population mean value of fixation index (F_{ST}), pair-wise for the isolates from each geographic region against the isolates from the other regions (Weir and Cockerham 1984). Thus, this can be expressed as $F_{ST} = (\pi_B - \pi_S)/\pi_B$, where F_{ST} is a measure of allelic diversity of randomly chosen alleles (individuals) within a population from a certain geographic region relative to that of the entire population (all 80 isolates in this study), π_B represents the average number of pairwise differences between two individuals (alleles) sampled from different regional populations and π_S refers to the average pairwise differences when sampled from the same regional-population. The significance of F_{ST} values was tested by 1023 permutations, and Nei's mean gene diversity were calculated for each regional population. All computations were carried out using the Arlequin software package version 3.0 (Excoffier et al. 2005).

The relationship between genetic similarity groups and fumonisin producing ability of the different *F. verticillioides* isolates (Tsehaye et al. unpublished) was calculated by Pearson correlation test procedure in SPSS version 22 (IBM SPSS statistics 22, Chicago, Illinois). The mean Dice similarity value (73 %) was used to separate isolates in to groups, resulting in a total of 13 genetic groups and correlated with the fumonisin concentrations produced under in vitro conditions.

Results

AFLP analysis of the 80 *F. verticillioides* isolates, using the six primer combinations, generated a total of 368 fragments. Of these fragments 213 (58 %) were polymorphic, while 155 (42 %) were monomorphic. Primer pairs differed in terms of the total number of fragments amplified and the number of polymorphic fragments generated (Table 1). The number of amplification products scored ranged from 48 to 77 for each primer combination. The highest number of polymorphic bands (46) was recorded using the primer combination *Eco*RI+AC/*Mse*I+CA (Table 1). The fragment sizes of the amplification products generated and considered for scoring ranged from 50 to 420 bp.

Analysis of the data based on all 213 polymorphic AFLP fragments revealed a pronounced genetic variation within the Ethiopian *F. verticillioides*. Eighty distinct genotypes were recognized among the 80 isolates analysed. Dice similarity coefficient showed that the genetic similarity among *F. verticillioides* isolates varied from 46 % (between isolates Kemmissie-1 and Sire-3) to 94 % (between isolates Sire-1 and Bako-4). The mean Dice similarity among

F. verticillioides isolates was 73 %, while the similarity between *F. verticillioides* isolates from Ethiopia and the reference strain from South Africa (MRC826) ranged from 53 % to 87 %.

Table 1 Number of AFLP fragments generated, polymorphic fragments and fragment sizes considered during scoring of *Fusarium verticillioides* isolates from Ethiopia

Primer combination	Number of AFLP fragments	Number of polymorphic fragments	Polymorphisms (%)	Fragment size range (bp)[a]
*Eco*RI+AC/*Mse*I+CA	77	46	60	51 - 420
*Eco*RI+GA/*Mse*I+CA	68	40	59	57 - 415
*Eco*RI+GG/*Mse*I+CA	48	23	48	53 - 310
*Eco*RI+GA/*Mse*I+CC	64	39	61	52 - 385
*Eco*RI+AC/*Mse*I+CG	56	38	68	50 - 320
*Eco*RI+GA/*Mse*I+CG	55	27	49	53 - 350
Total number of AFLP fragments	368	213	-	-
Mean	61	35.5	58	-

[a] fragment sizes considered for scoring as present or absent

A graphical representation of genetic similarity between the isolates was obtained by generating a dendrogram (Fig. 2) from the similarity matrix based on Dice coefficient and the UPGMA clustering method. Both the dendrogram and PCO plot showed two major clusters, with group A containing 71 isolates and group B containing 9 isolates (Fig. 2). Further subgroups can be identified with each main group splitting further into two subgroups. The reference isolate from South Africa (MRC826) clustered with the larger group A (Fig. 3). Results from the cluster analysis demonstrated the absence of relationship between *F. verticillioides* isolates and their geographic origin, although in some cases isolates from the same areas grouped together (Fig. 2). Some *F. verticillioides* isolates obtained from the same area appeared to be very distantly related, while many other isolates from the different geographical regions of Ethiopia were genetically closely related to each other as indicated in Fig. 2 and 3.

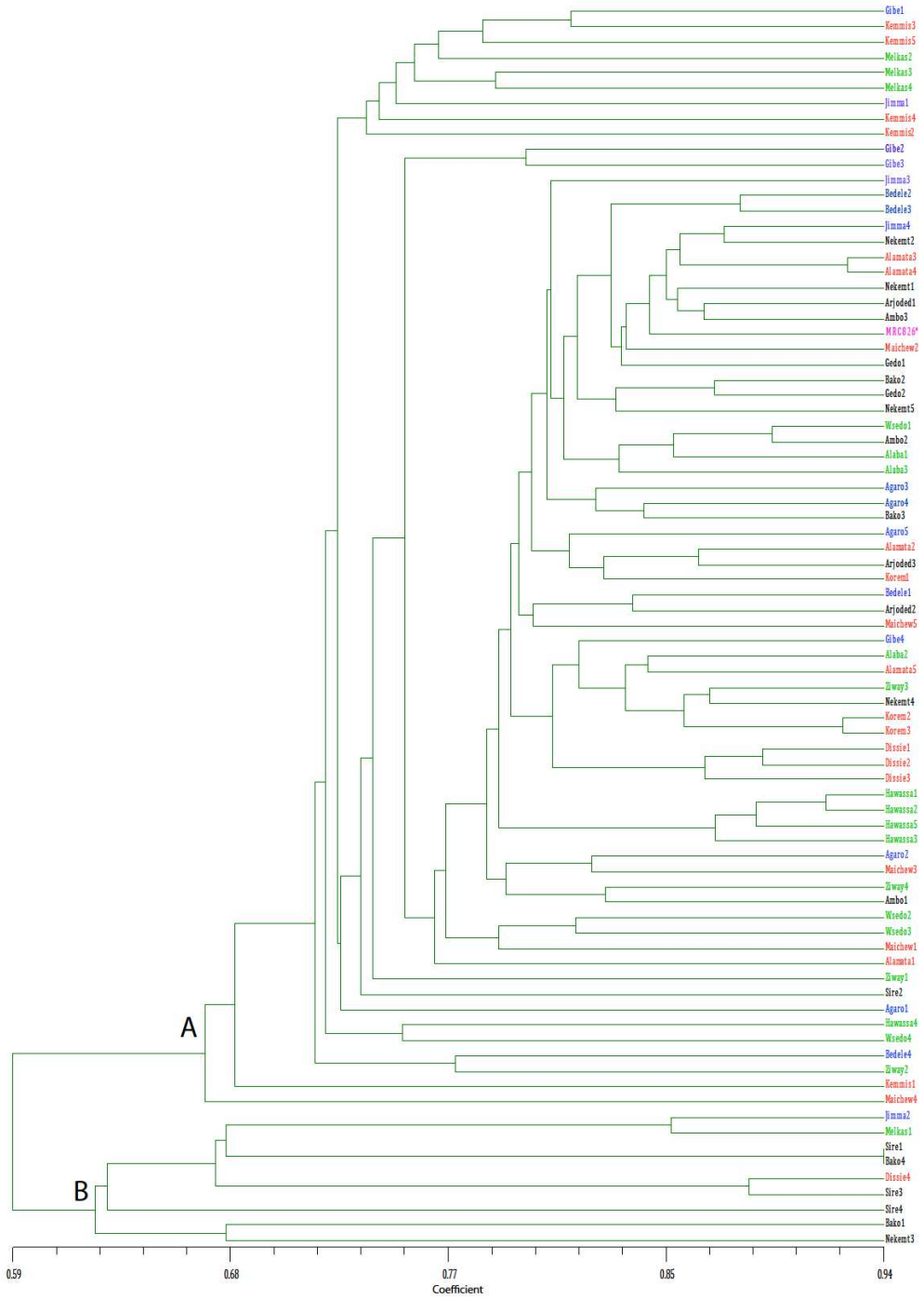

Fig. 2 Dendrogram of 80 *Fusarium verticillioides* isolates obtained from kernels of maize produced in different geographic regions of Ethiopia and a reference isolate (MRC826) from South Africa, generated by UPGMA cluster analysis using 213 AFLP markers. The scale from 0.59 to 0.94 indicates genetic similarity calculated using Dice similarity coefficient in NTSYS (Rohlf 2005). The two main clusters are remarked as A & B.

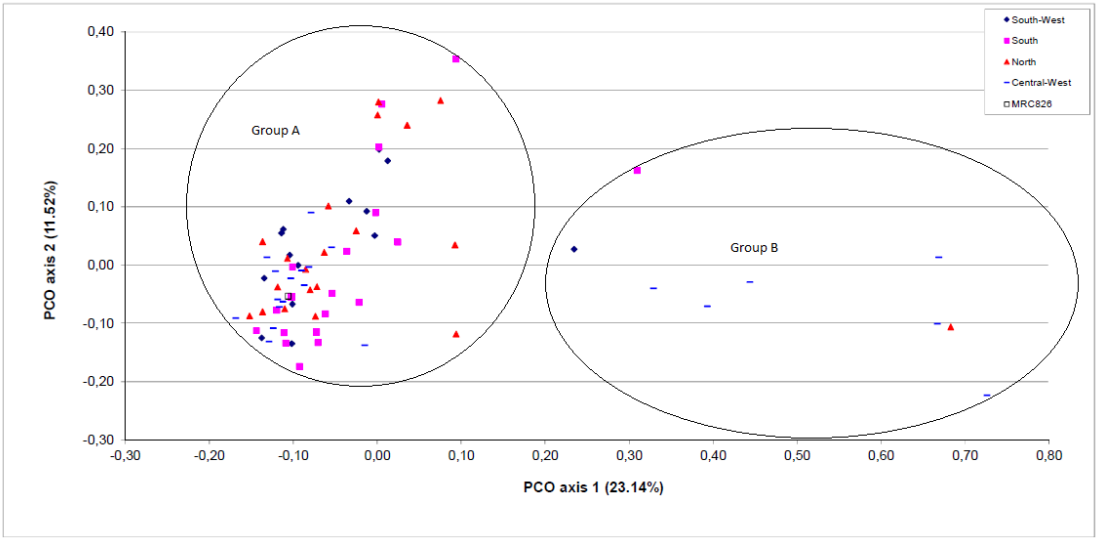

Fig. 3 Principal coordinate analysis plot of the 80 *Fusarium verticillioides* isolates obtained from kernels of maize produced in different geographic regions of Ethiopia and a reference isolate (MRC826), summarizing genetic similarity, outcome from AFLP analysis scores using 213 polymorphic markers.

With regard to morphological or culture characteristics, *F. verticillioides* isolates included in this study were categorized in two types, namely isolates with ample aerial mycelial (white cream color) and isolates with scarce aerial mycelial growth (pink to orange color) on PDA. There was no clustering of isolates according to the above morphological groups, as both morphological types were distributed in group A and group B in the dendrogram (Fig. 2).

AMOVA results for estimation of variance components revealed that most of the total genetic variation (98.5 %) was among the *F. verticillioides* isolates within the same geographic region, while the genetic variation between isolates of different geographical origin was very small (1.5%) (Table 2). The low level of genetic differentiation obtained in this study (F_{ST} = 0.015), indicates high level of exchange or sharing of genetic material among the geographic regions.

The pair-wise genetic analysis indicated that the genetic distance between *F. verticillioides* isolates from different geographic regions were very low (Table 3), which indicates little genetic differentiation between isolates of different geographic region. The highest level of genetic differentiation was observed between isolates of South-western and Central-western regions of Ethiopia (0.030), while the smallest genetic differentiation (0.001) was between

the Southern and South-western geographic regions of the country (Table 3). The highest Nei's mean gene diversity was recorded among isolates of the Central-Western region (0.218), whereas the least mean gene diversity was within the South-western region (0.155) (Table 3).

Table 2 Analysis of molecular variance (AMOVA) of the 80 Ethiopian *Fusarium verticillioides* isolates using 213 AFLP markers

Source of variation	d.f [a]	Sum of squares	Variance components	Percentage of variation
Between isolates from the different geographic regions	3	122.4	0.48	1.5
Among individuals within the same geographic region	76	2379.9	31.3	98.5
Total	79	2502.3	31.8	

Fixation index (F_{ST}): 0.015

[a]d.f: degree of freedom

Table 3 Gene diversity and pairwise genetic differentiation between *Fusarium verticillioides* populations obtained from four geographic regions of Ethiopia, as calculated by the pair-wise genetic distance method (Weir and Cockerham 1984)

Geographic regions	Population fixation index (F_{ST}): pairwise differences				Nei's mean gene diversity
	South-west	South	North	Central-west	
South-west	0.000				0.155
South	0.001^{ns}	0.000			0.165
North	0.006^{ns}	0.008^{ns}	0.000		0.169
Central-west	0.030*	0.021*	0.019^{ns}	0.000	0.218

* Significant at $p < 0.05$, ns: statistically non-significant ($p > 0.05$)

No significance correlation ($r = 0.200$, $p = 0.113$) was found between genetic similarity groups and fumonisin producing ability of the *F. verticillioides* isolates. *F. verticillioides* isolates that produced the highest and lowest fumonisin concentrations under *in vitro* condition were distributed among the different genetic similarity groups in the dendrogram and PCO plots.

Discussions

In the present study *F. verticillioides* isolates, obtained from kernels of maize produced in different geographic regions of Ethiopia, were analysed for their genetic variation using AFLP markers. The genetic similarity between *F. verticillioides* isolates from different regions varied from 46 % to 94 % with a mean Dice similarity of 73 %. This indicates that all the isolates analysed belong to the same species, which is consistent with the morphological identification. According to Leslie et al. (2007), strains of the same species often share at least 60 % of AFLP fragments, while those that share less than 40 % of the fragments belong to different species.

Eighty distinct AFLP-genotypes were identified among the 80 isolates analysed, indicating a noticeable genotypic variation in the Ethiopian *F. verticillioides* isolates. Similar wide level of genotypic diversity based on AFLP analysis have been reported in *F. verticillioides* in other parts of the world, such as Brazil (Rocha et al. 2011), Argentina (Reynoso et al. 2009), Italy (Covarelli et al. 2012) and Iran (Dehkordi et al. 2013). Ren et al. (2012) has also reported great genetic variation in *F. verticillioides* isolates from various geographic regions of China using SSR markers. Chang et al. (2013) used several molecular markers to differentiate *F. verticillioides* strains isolated from plants and humans, and they reported high levels of genetic variation among the phytopathogenic isolates compared to the clinical isolates from humans. Their hypothesized reason is that clinical strains may be acquired in the field after incidental exposure to plants affected by the pathogen.

Results of the present study indicates that most of the variation recorded occurred within isolates from the same geographic region, and very little variation occurred between isolates from different geographic origin. Frequent sexual reproduction may be a key contributor to the observed great genotypic variation among *F. verticillioides* isolates within the same region. It is well known that sexual recombination increases genetic diversity, as it creates novel recombinants (Cumagun et al. 2009; Leslie and Summerell 2006). *Fusarium verticillioides* can reproduce both asexually and sexually. The fungus is heterothallic with two different mating type alleles *MAT-1* and *MAT-2*. Thus, strains with different mating types from diverse hosts and geographic areas can interact and form perithecia (Leslie 1995; Leslie and Summerell 2006). Furthermore, genetic diversity of a pathogen population to some extent also depends on the extent of geographic scale analysed. Isolates collected from larger

geographic areas, representing various agroecological zones, such as is the case with the present study, may show quite great levels of genetic variation. In Ethiopia, altitude and agroecological conditions within the geographic regions are very variable, and genetically different strains may have evolved independently from local populations. This tendency is observed in the dendrogram (Fig. 2) as some isolates collected from the same area have clustered together in small subgroups. This is probably due to selection for alleles in the pathogen population to adapt to a local niche. A very small genetic differentiation between isolates of different geographic origin may be explained by a high rate of seed exchange and consequently gene flow between the geographic regions. Gene flow increases genetic variation within the same region because it brings in new alleles, and it decreases genetic differentiation between regions, because alleles are being exchanged. Infected maize seeds could be the main vehicle for dissemination of pathogen genotypes throughout Ethiopia. There is free movement of seeds within Ethiopia as well as continuous introduction of planting material via seed companies and research institutions importing from international research centers. Thus, new strains with distinct genetic makeup may continuously be introduced with seeds, and such strains may contribute to the observed great genetic variation among isolates within the same region. Long-distance dispersal of ascospores could also to some extent contribute in mixing of the regional populations. Aerial propagules of *Fusarium* species are common with potential origin in broad geographic regions (Lin 2013; Schmale et al. 2006). Schmale et al. (2006) observed that long-distance movement of spores effectively masks biogeography of *Fusarium graminearum.* The long-distance dispersal of *Fusarium* is reported to occur both by airborne drifts or dust storms and movement in soil (with water) (Leslie and Summerell 2006). Several isolates analysed in this study showed greater genetic similarity with the reference isolate from South Africa, than their similarity with other isolates from maize in Ethiopia. These results suggest spread and long-range dispersal of *F. verticillioides,* and that the pathogen is not geographically isolated. However, *F. verticillioides* may rather be considered to consist of a large interbreeding population.

Cluster analysis based on UPGMA and PCO, using Dice similarity coefficients, categorized *F. verticillioides* isolates into two major groups and subgroups. The main groups and subgroups on the AFLP dendrogram and PCO contained isolates from all geographic regions of Ethiopia, and there was no clear association between *F. verticillioides* isolates and their geographic areas of origin (Fig. 3). This could probably be due to free dispersal of the fungi in nature via several means. These results are consistent with a study from Brazil (Rocha et

al. 2011) and Italy (Covarelli et al. 2012) who demonstrated a similar lack of a relationship between isolates and geographic origins. In contrast to the present study, Daie Ghazvini et al. (2011) has reported a genetic relationship among DNA polymorphic patterns generated using RAPD markers and geographic origin of *F. verticillioides* isolates. Our results were also in contrast with other reports that observed grouping in *F. verticillioides* isolates according to different sites or localities from which the *F. verticillioides* isolates were collected (Ono et al. 2010; Pamphile and Azevedo 2002). All *F. verticillioides* isolates included in this study were able to produce detectable level of fumonisin under *in vitro* testing, but displayed a wide range of variation in fumonisin production ability (0.25 - 38 mg of the toxin per kg fungal and maize kernel biomass; Tsehaye et al. unpublished). However, we did not find any correlation between AFLP profiles and the amounts of total fumonisin produced by the different *F. verticillioides* isolates. The observed weak relationship or not finding any differentiation related to fumonisin producing ability of isolates may be due to several reasons. The ability of a *F. verticillioides* strain to produce fumonisin has been associated with the presence of a 42.5 kb fumonisin biosynthesis gene cluster (*FUM*) (Proctor et al. 2003), and previous studies revealed that sequence variation inside the *FUM* gene cluster may explain variation in fumonisin production (Stępień et al. 2011). However, other factors, e.g. environmental, might regulate the metabolic route and expression levels that results in a wide variation of fumonisin levels. Some level of clustering of *F. verticillioides* isolates according to fumonisin producing ability (Covarelli et al. 2012; Daie Ghazvini et al. 2011), host preference and co-evolution processes have been observed in some other studies (Dehkordi et al. 2013; Pamphile and Azevedo 2002).

In conclusion, the phylogenetic analysis revealed that the *F. verticillioides* isolates in Ethiopia are genetically diverse. Much of the genetic variation was within the same geographic regions, but little genetic differentiation was observed among isolates from the different geographic regions. This probably indicates the presence of a frequent sexual reproduction in *F. verticillioides* in Ethiopia or it might indicate continuous introduction of new strains mainly with planting material. High rate of seed exchange and consequently, gene flow between the geographic regions and long-distance dispersal of ascospores may also contribute in mixing of the regional populations. A high level of genetic variation in a pathogen population may allow rapid selection of strains that are resistant to fungicides, or strains that are more pathogenic. Therefore, breeding measures should concentrate on quantitative resistance, which is effective against all genotypes of the pathogen. Efforts should emphasize on

prevention of the introduction of new aggressive strains with planting material. Findings obtained in this study provide important baseline information on *F. verticillioides* in Ethiopia, with respect to genetic variation and could be used for designing management strategies. The source for the observed high level of genetic diversity calls for investigation into the mechanisms for how the *F. verticillioides* maintain such diversity, particularly whether this is due to high sexual reproduction or not. The separation of *F. verticillioides* isolates into two major clusters and further subgroups may also require additional work to determine if there is differences in pathogenicity characteristics.

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Supplementary material 1 Geographic origin, altitude and GPS coordinate of maize grain sample collection sites in Ethiopia from which *F. verticilloides* isolates were obtained

Isolate name [a]	Geographic region	GPS coordinate of sample collecting sites		Altitude
		North	East	
Agaro-1	South-west	7º 51.006’	36º 35.009’	1685
Agaro-2	South-west	7º 51.682’	36º 35.192’	1718
Agaro-3	South-west	7º 52.226’	36º 34.093’	1697
Agaro-4	South-west	7º 50.078’	36º 34.004’	1567
Agaro-5	South-west	7º 50.043’	36º 35.789’	1620
Alaba-1	South	7º 07.044’	37º 57.005’	1753
Alaba-2	South	7º 06.763’	37º 56.129’	1953
Alaba-3	South	7º 08.542’	37º 57.841’	1876
Alamata-1	North	12º 24.004’	39º 36.048’	1524
Alamata-2	North	12º 25.603’	39º 36.872’	1587
Alamata-3	North	12º 22.503’	39º 37.094’	1498
Alamata-4	North	12º 24.245’	39º 54.221’	1479
Alamata-5	North	12º 23.069’	39º 32.048’	1573
Ambo-1	Central-west	8º 58.053’	37º 52.019’	2150
Ambo-2	Central-west	8º 58.894’	37º 50.102’	2167
Ambo-3	Central-west	8º 59.408’	37º 54.096’	2207
Bako-1	Central-west	9º 06.769’	37º 10.042’	1743
Bako-2	Central-west	9º 08.806’	37º 09.732’	1736
Bako-3	Central-west	9º 07.807’	37º 08.432’	1689
Bako-4	Central-west	9º 06.169’	37º 09.086’	1652
Bedele-1	South-west	8º 27.021’	36º 21.011’	2015
Bedele-2	South-west	8º 29.078’	36º 22.086’	1908
Bedele-3	South-west	8º 26.021’	36º 21.424’	1896
Bedele-4	South-west	8º 25.214’	36º 23.402’	1906
Dedesa-1	Central-west	8º 44.001’	36º 25.053’	1560
Dedesa-2	Central-west	8º 43.041’	36º 26.900’	1490
Dedesa-3	Central-west	8º 45.041’	36º 24.010’	1486
Dessie-1	North	11º 07.015’	39º 38.000’	2490
Dessie-2	North	11º 08.501’	39º 40.021’	2250
Dessie-3	North	11º 07.211’	39º 37.032’	2310
Dessie-4	North	11º 06.876’	39º 38.605’	2213
Gedo-1	Central-west	9º 00.047’	37º 26.058’	2513
Gedo-2	Central-west	9º 00.579’	37º 27.977’	2435
Gibe-1	South-west	8º 14.056’	37º 34.028’	1206
Gibe-2	South-west	8º 15.062’	37º 35.206’	1234
Gibe-3	South-west	8º 14.698’	37º 33.028’	1201
Gibe-4	South-west	8º 13.698’	37º 34.928’	1204
Hawassa-1	South	7º 02.021’	38º 27.033’	1716
Hawassa-2	South	7º 03.241’	38º 28.017’	1714
Hawassa-3	South	7º 03.941’	38º 28.763’	1702
Hawassa-4	South	7º 03.822’	38º 27.013’	1694

[a] *Fusarium verticillioides* isolate name derived from source area from where the maize kernel samples collected. In each of the twenty areas assessed, sampling sites were separated by at least 1 km and a maximum of 5 km from each other.

Supplementary material 1 (continued)

Isolate name [a]	Geographic region	GPS coordinate of sample collecting sites		Altitude
		North	East	
Hawassa-5	South	7º 04.021’	38º 28.733’	1724
Jimma-1	South-west	7º 40.009’	36º 50.220’	1714
Jimma-2	South-west	7º 38.972’	36º 51.520’	1695
Jimma-3	South-west	7º 40.495’	36º 51.812’	1719
Jimma-4	South-west	7º 39.396’	36º 49.126’	1732
Kemise-1	North	10º 43.055’	39º 52.019’	1450
Kemise-2	North	10º 42.130’	39º 52.890’	1467
Kemise-3	North	10º 44.026’	39º 52.321’	1550
Kemise-4	North	10º 43.047’	39º 51.110’	1440
Kemise-5	North	10º 41.025’	39º 51.991’	1370
Korem-1	North	12º 30.607’	39º 31.022’	2495
Korem-2	North	12º 32.229’	39º 31.625’	2486
Korem-3	North	12º 33.507’	39º 31.822’	2464
Maichew-1	North	12º 46.059’	39º 32.039’	2450
Maichew-2	North	12º 46.993’	39º 32.659’	2520
Maichew-3	North	12º 46.046’	39º 33.436’	2321
Maichew-4	North	12º 44.795’	39º 31.003’	2432
Maichew-5	North	12º 45.788’	39º 30.786’	2456
Melkassa-1	South	8º 24.020’	39º 21.001’	1550
Melkassa-2	South	8º 24.201’	39º 21.803’	1547
Melkassa-3	South	8º 25.321’	39º 23.108’	1542
Melkassa-4	South	8º 25.829’	39º 22.322’	1542
Nekemte-1	Central-west	9º 05.008’	36º 33.053’	2100
Nekemte-2	Central-west	9º 04.531’	36º 31.122’	2017
Nekemte-3	Central-west	9º 03.434’	36º 31.120’	2087
Nekemte-4	Central-west	9º 05.912’	36º 33.890’	1896
Nekemte-5	Central-west	9º 06.946’	36º 34.151’	1872
Sire-1	Central-west	9º 02.009’	36º 52.020’	1714
Sire-2	Central-west	9º 02.301’	36º 51.128’	1729
Sire-3	Central-west	9º 03.423’	36º 51.326’	1869
Sire-4	Central-west	9º 02.544’	36º 52.897’	1846
Welayta-sedo-1	South	6º 51.042’	37º 45.048’	2046
Welayta-sedo-2	South	6º 52.236’	37º 45.157’	1987
Welayta-sedo-3	South	6º 50.112’	37º 45.032’	1964
Welayta-sedo-4	South	6º 51.042’	37º 43.218’	1852
Ziway-1	South	7º 59.020’	38º 38.013’	1642
Ziway-2	South	7º 57.654’	38º 36.107’	1664
Ziway-3	South	7º 59.927’	38º 38.632’	1631
Ziway-4	South	7º 59.018’	38º 36.312’	1648
MRC826	South Africa			

[a] *Fusarium verticillioides* isolate name derived from source area from where the maize kernel samples collected. In each of the twenty areas assessed, sampling sites were separated by at least 1 km and a maximum of 5 km from each other.

Paper III

Fumonisin production by *Fusarium verticillioides* isolates from kernels of maize grown in Ethiopia

Hadush Tsehaye [1,4,*], Leif Sundheim [1,2], May Bente Brurberg [1,2], Arne Tronsmo [3], Dereje Assefa [4], Anne Marte Tronsmo [1]

[1] Norwegian University of Life Sciences, Department of Plant Sciences, P.O. Box 5003, NO-1432 Ås, Norway

[2] Norwegian Institute for Bioeconomy Research, Biotechnology and Plant Health Division, P.O. Box 115, NO-1431 Ås, Norway

[3] Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science, P.O. Box 5003, NO-1432 Ås, Norway

[4] Mekelle University, Department of Dryland Crop and Horticultural Sciences, P.O.Box 231, Mekelle, Tigray, Ethiopia

[*] Corresponding author. E-mail address: hadush.beyene@nmbu.no or had031@yahoo.com

Abstract

A study was conducted to determine fumonisin-producing ability of 80 *F. verticillioides* isolates from kernels of maize produced in different maize growing areas and agroecological conditions in Ethiopia. The *F. verticillioides* isolates were grown on autoclaved maize cultures for one month, and the fumonisin content was quantified using competitive enzyme linked immunosorbent assay (ELISA). All isolates tested produced detectable levels of total fumonisin in maize culture, with values ranging from 0.25 to 38.01 mg of the toxin per kg of culture material (fungal biomass and maize kernels). Noticeable variation in total fumonisin production was observed among isolates obtained from the same area as well as agroecological zone. The results indicate that the majority (57.5 %) of *F. verticillioides* isolates associated with maize grains in Ethiopia produced fumonisin > 4 mg kg^{-1}; while the rest of the isolates appears to be low fumonisin producers, with 35 % of the isolates producing < 2 mg kg^{-1} total fumonisin. The widespread presence of fumonisin producing strains across all maize growing areas of Ethiopia presents a food safety risk. Thus, efforts should give emphasis to prevention of the introduction of new aggressive strains with planting material and avoiding contamination of maize with fumonisins.

Keywords: fumonisin, *Fusarium verticillioides*, maize, ELISA, food safety

Introduction

Fumonisins are a family of mycotoxins, produced mainly by *Fusarium verticillioides* (Sacc.) Nirenberg, and *Fusarium proliferatum* (Matsush.) Nirenberg. In fact, *F. verticillioides* is the most important fungal pathogen of maize worldwide (Picot *et al*., 2010) and fumonisins are most frequently found in maize food and feed products (Picot *et al*., 2010; Sundheim and Tsehaye, 2015; Waśkiewicz *et al*., 2012). This mycotoxin has also been reported at low levels in a number of other agriculturally important commodities such as sorghum, rice, wheat, barley, beans, asparagus and medicinal plants (Waśkiewicz *et al*., 2012). The pure chemical substance of fumonisin is white hygroscopic material, which is well soluble in water and aqueous solutions of methanol or acetonitrile (IARC, 2002). They are resistance to high temperatures and no substantial reduction achieved by cooking (Shephard *et al.*, 2012).

At least 28 different fumonisin molecules are known, which are extracted from natural samples or synthesized in artificial culture media in laboratory. These are classified into A, B, C and P series based on their chemical structures (Falavigna *et al*., 2012; Picot *et al*., 2010). Members of the fumonisin B series (mainly FB_1, FB_2 and FB_3) are the analogues, which often occur in great quantities in naturally contaminated maize samples (Waśkiewicz *et al*., 2012). Fumonisin B_1 is the most important in food contamination, as it accounts for more than 70 % of all fumonisins found in naturally contaminated food and feed (Nelson *et al*., 1991). Among the minor fumonisin analogues, the C series are known to occur on mouldy as well as normal non-mouldy maize grains, while members of the fumonisin A and P series are secondary metabolites produced in trace amount on artificial culture media in the laboratory (Falavigna *et al*., 2012). Structurally fumonisins belonging to the B-series are characterized by a 20 carbon aminopolyhydroxy-alkyl chain that is diesterified with propane-1,2,3-tricarboxylic acid (Bryla *et al*., 2013). Due to their structural similarity with sphingolipid intermediates sphinganine and sphingosine, they may affect sphingolipid metabolism, which are major components of cell membranes and important components of many signalling pathways, by interfering the enzyme ceramide synthase (Wang *et al*., 1991).

Contamination of agricultural produce by fumonisin is a growing concern globally, as it causes divers and complex adverse effects on the health of humans as well as animals (Waśkiewicz *et al*., 2012). Upon consumption of contaminated feed and following: fumonisin may cause a fatal brain lesion, known as leucoencephalomalacia, in horses (Kellerman *et al*.,

1990) and pulmonary edema and hydrothorax in pigs (Harrison *et al*., 1990). Fumonisins are nephrotoxic, hepatotoxic, carcinogenic and embryo-toxic in laboratory animals (Gelderblom *et al.*, 1996; Waśkiewicz *et al*., 2012). In humans, long-term consumptions of fumonisin contaminated food has been linked with high incidence of oesophageal cancer, and the mycotoxin is consider as a risk factor for liver cancer (Sun *et al*., 2007). The International Agency for Research on Cancer (IARC) has assessed the potential risk of FB_1 to humans, and IARC classified it in Group 2B, as ‘‘probably carcinogenic to humans’’ (IARC, 2002). Fumonisin has detrimental effects on the developing foetus and young infants, implicated in a cluster of cases of neural tube defects (Missmer *et al*., 2006). In maize seedlings, accumulation of fumonisin in roots has been linked with the development of phytotoxic symptoms such as necrotic leaf lesion, seedling blight, and reduced root development (Williams *et al*., 2007). However, there is no clear information on the relationship between fumonisin production and pathogenicity.

The amount of fumonisin produced varies among *F. verticillioides* isolates, and not all isolates produce the toxin (Alakonya *et al*., 2008; Atukwase *et al*., 2012; Covarelli *et al*., 2012). The potential of a strain to synthesize fumonisin has been associated with the presence of the fumonisin biosynthesis gene cluster (*FUM*) (Proctor *et al*., 2003). Furthermore, fumonisin biosynthesis by *F. verticillioides* strains can be affected by environmental factors. Temperature, relative humidity, pH and nutrient status of the substrate on which the fungus grows, have been reported to influence the expression of *FUM* genes and fumonisin production by *Fusarium* strains (Picot *et al*., 2010; Sagaram *et al*., 2006).

Maize is a major component of staple food in Ethiopia. It is the second crop in area coverage (2.12 million ha), and maize has higher average yield (3.2 tonnes/ha), than any other crop in the country (FAOSTAT, 2014). Maize is grown in several agroecological zones of the country. In the northern and eastern parts of the country, which are commonly characterized by water stress (erratic and little rainfall), early maturing, open pollinated genotypes are commonly grown as rain-fed maize. In the central, southern and southwestern parts of Ethiopia, which usually receive sufficient rainfall for extended period of time, medium to late maturity and high yielding hybrids are planted (Worku *et al*., 2012). Due to the high variation in agroecological condition of the maize growing areas of Ethiopia, varieties introduced by research centres and seed agencies to these areas varies in earliness and other properties (Worku *et al*., 2012). Thus, there is a high diversity in the maize genotypes in the farming

system of the country. *Fusarium* ear rot is one of the major challenges for maize production in Ethiopia, and *F. verticillioides* is the most frequently isolated *Fusarium* species from maize kernels (Ayalew, 2010). Previous and recent assessment of maize samples collected from different maize growing areas in the country indicated a widespread occurrence of fumonisin at different concentrations (Ayalew, 2010; Sundheim and Tsehaye, 2015). With the variation in maize genotypes, and agroecological growing conditions in the country, *F. verticillioides* populations are expected to show high variation in fumonisin production ability. At present little is known in Ethiopia regarding the potential for fumonisin production of *F. verticillioides* isolates from domestic maize. This is the first attempt to examine total fumonisin (FB_1, FB_2 & FB_3) production by a collection of *F. verticillioides* strains isolated from kernels of maize produced in different agroecological conditions in Ethiopia.

Materials and Methods

Fungal isolates

A total of 80 *F. verticillioides* isolates were included in this study. All the strains were isolated from maize seed samples, collected from the 20 major maize growing areas (Fig. 1) of Ethiopia in 2012. The sample collection areas represent seven major agroecological zones of Ethiopia, and their characteristics with respect to elevation, temperature, rainfall and major annual crops are presented in Table 1. Maize seeds were surface disinfected by soaking in 1 % sodium hypochlorite (NaOCl) for 2 minutes and washing twice in sterile, distilled water. Fifty seeds of each sample were plated on Petri dishes (five seeds per Petri dish), containing a modified Czapek-Dox Iprodione Dichloran Agar (CZID), where the fungicides propiconazole 0.375 mg L^{-1} and fenpropimorph 1.125 mg L^{-1} were used instead of iprodione (Halstensen *et al*., 2006). The Petri dishes with maize seeds were incubated for 5 - 7 days at 25 °C in the dark. The identity of *Fusarium* colonies were confirmed by first growing on Spezieller Närstoffarmer Agar (SNA) (Nirenberg, 1976) for 7 - 10 days at 20 °C under alternatively near ultraviolet (NUV)/white fluorescent light (12 h) and dark (12 h). Then a spore suspension was prepared in sterile water and spread onto Water Agar (WA) plates for single spore isolation. The plates were incubated at room temperature (22 °C) for 16 - 20 h and single germinating conidia were removed and transferred to a new SNA (Nirenberg, 1976) plates, containing a filter paper, to obtain monosporic cultures (Leslie and Summerell, 2006). Sub-culturing was made on PDA (Difco, Madison, USA) and carnation leaf agar

(CLA) plates, incubated at 20 °C with alternative photoperiod (12 h near UV/white fluorescent light, followed by a 12 h dark period) from two up to six weeks. The morphological identification was according to the procedure described by Leslie and Summerell (2006). For confirmation of morphological identification, sequencing part of the translation elongation factor 1-alpha (*EF-1α*) gene was done for randomly selected (six) isolates, as described by O'Donnell *et al*. (1998). Sequence data were assembled and analyzed using the CLC Main Workbench software 6.9 (Aarhus, Denmark). The consensus sequences were checked for similarity against the GenBank (NCBI-National Centre for Biotechnology Information) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the *Fusarium*-ID database (http://isolate.fusariumdb.org/index.php). A *F. verticillioides* isolate (MRC826), which is a known high fumonisin producer (Vismer *et al*., 2004), obtained from the South African Medical Research Council, was included and used as outgroup reference isolate.

Table 1. Summary of characteristic of the agroecological zones of Ethiopia, from which maize seed samples were collected to isolate *F. verticillioides* strains.

Agroecological zones	Elevation (masl) [a]	Temp (°C) [b]	Annual Rainfall (mm) [c]	Major crops [d]
Tepid humid mid-highlands (H_3)	1600-3000	17 - 22.5	1800-2200	Co, M, W, RT
Warm moist lowlands (M_2)	400-2000	22.5 - 25	600-1400	S, M, T
Tepid moist mid-highlands (M_3)	1000-3600	17 - 20	1000-1400	W, T, B, M, Pu
Warm sub-moist lowlands (SM_2)	400-2000	20 - 25	600-1000	S, M, T, Fg
Cool sub-moist mid-highlands (SM_4)	2800-4000	15 - 20	600-1000	B, W, T, Pu
Warm sub-humid lowlands (SH_2)	400-2000	22.5 - 27.5	1000-1800	S, M, Co
Tepid sub-humid mid-highlands (SH_3)	1000-3200	20 - 22.5	1400-1800	S, M, W, Co, RT

[a] masl: meters above sea level; [b] Temp: Temperature, long-term (> 30 years data) average temperature and [c] long-term (> 30 years) annual rainfall; [d]Co: coffee, M: maize, T: teff, W: wheat, S: sorghum, B: barley, Fg: fingermillets, Pu: pulses, RT: roots and tuber crops (potato, sweet potato, yams, ensete) (Source: MoARD, 2005).

Preparation of maize substrate

Fumonisin production by each isolate was determined, using autoclaved whole maize kernels as cultivation medium. An improved open pollinated maize variety, Melkassa-4, obtained from the Tigray Seed Agency was used as growing substrate for this study. Initially, maize kernels (100 g of kernels and 100 mL of sterile water in 500 mL glass jars) were autoclaved at 121 ℃ for one hour on three consecutive days (Vismer *et al*., 2004). After allowing to settle, moisture levels of the maize kernels were quantified with a moisture analyzer (Perten instruments, model: AM5100, Sweden), and it was approximately 45 % at inoculation.

Inoculum preparation and culture growing condition

For spore production, each *F. verticillioides* isolate was sub-cultured by transferring a small disc of mycelia onto a petri-dish, containing Mung Bean Agar (MBA) (Dill-Macky, 2003). The plates were incubated at 22 °C for 7 - 10 days, with alternative photoperiod (12 h NUV-light followed by 12 h dark periods). Conidia of the different *F. verticillioides* isolates were suspended in sterile water, and final concentration of 10^6 conidia mL^{-1} was prepared using a Kova glasstic slide (spore counting chamber) and inoculated into the maize cultures in 500 mL glass jars. The cultures were incubated in the dark for 4 weeks at 25 ℃, with manual shaking during the first two weeks.

Extraction and fumonisin analysis

After four weeks of incubation, the culture materials (entire fungal mass and maize substrate) were dried in a forced air incubator at 60 °C for three days. The dried samples were finely ground to powder using an ultra-centrifugal mill ZM-200 (Retsch GmbH & Co. KG, Germany).The samples were stored at -20 ℃ until analysis. For fumonisin analysis 5 g thoroughly mixed ground samples were used, and extraction was performed with 25 mL of 70 % methanol using a shaker (1000 rpm) for 3 min. The extract was filtered through a Whatman no. 1 filter paper, and the collected filtrate was analyzed using competitive enzyme linked immunosorbent assay (ELISA) kit (RIDASCREEN®fumonisin, R-Biopharm AG, Darmstadt, Germany), according to the manufacturer's instructions. Evaluation of the results of RIDASCREEN® enzyme immunoassay was performed, using RIDA®SOFT Win software (Art. Nr. Z9999, R-Biopharm AG, Darmstadt, Germany), by plotting the mean relative

absorbance in percentages on the standard curve of known fumonisin concentrations. Fumonisin concentrations above the highest standard were further diluted, and the results obtained were multiplied by the dilution factor. The minimum limit of detection of the kit was 0.025 mg kg^{-1} and all samples were analyzed in duplicates.

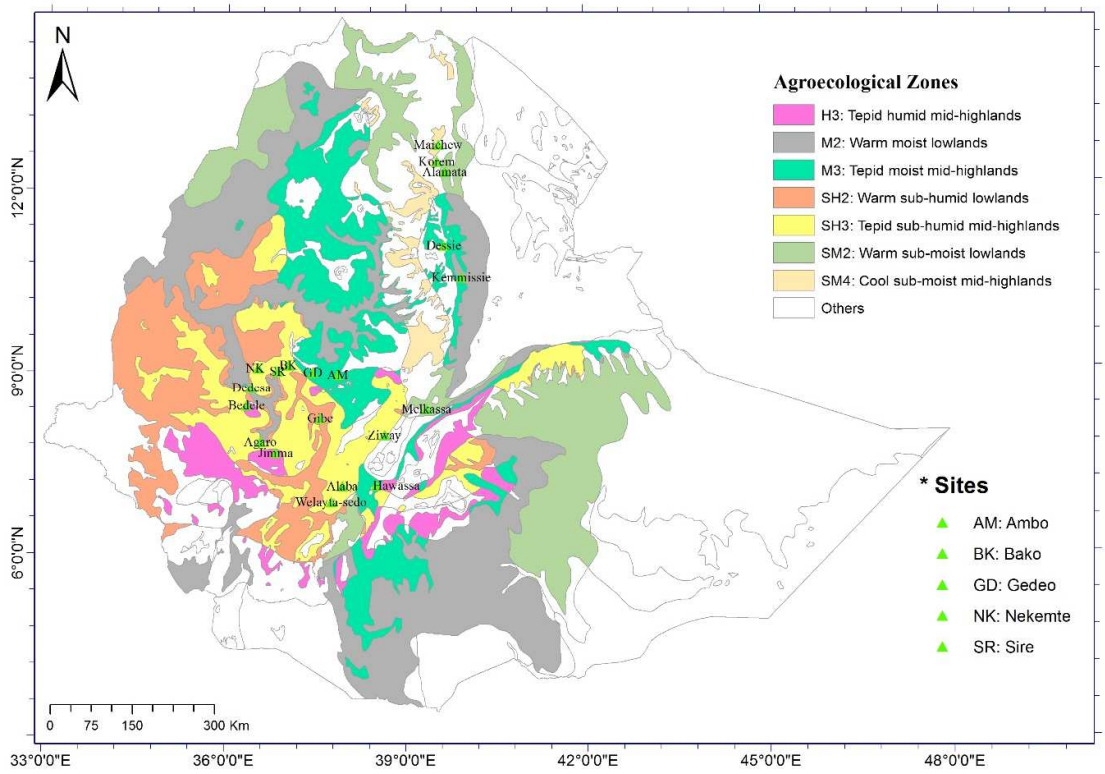

Fig. 1 Maize seed sample collection areas and agroecological zones of Ethiopia from which *F. verticillioides* isolates obtained.

Statistical analysis

Statistical analysis, for comparison of fumonisin production by the *F. verticillioides* isolates, was performed using SPSS version 22 (IBM SPSS statistics 22, Chicago, Illinois). One sample T-test ($P < 0.05$) was used to compare the mean fumonisin production between the test isolates and the South African reference isolate (MRC826). Descriptive statistical analyses were employed to summarize mean fumonisin levels per study area and agroecological zones.

Results

All the 80 *F. verticillioides* isolates analyzed in this study were able to synthesize fumonisin, when grown on autoclaved maize kernels, but there were variation in their ability to produce the toxin (Table 2). One sample T-test analysis revealed significant variation ($p < 0.025$) in fumonisin production potential among the *F. verticillioides* isolates in Ethiopia. Total fumonisin production of the isolates ranged from 0.25 to 38 mg of the toxin per kg of culture material (fungal biomass and maize kernels). There was also substantial variation in total fumonisin production among isolates collected from the same geographic area. For example, among isolates obtained from Sire area, isolate SR-6952 produced the highest amount of fumonisin (38 mg kg^{-1}) of all isolates tested, while isolates SR-3133 and SR-6351 from the same area produced very low level of fumonisin, only 0.66 mg kg^{-1} and 1.82 mg kg^{-1}, respectively (Table 2). Pronounced variation in fumonisin production was also observed among isolates collected from the other areas.

The mean fumonisin level produced by isolates from Gibe, Dedessa, and Hawassa were higher than the level produced by isolates obtained from the others areas. For isolates from Gibe, the fumonisin levels ranged from 7.84 to 33.54 mg kg^{-1} (mean 21.4 mg kg^{-1}), isolates from Dedessa ranged from 9.6 to 24.96 mg kg^{-1} (19 mg kg^{-1}) and isolates from Hawassa produced 1.33 to 34.1 mg kg^{-1} fumonisin, with mean 16.6 mg kg^{-1}. On the other hand, isolates from Bedele and Melkassa produced only low amount of fumonisin. Fumonisin production by isolates from Bedele ranged from 0.95 to 1.57 mg kg^{-1} (mean 1.2 mg kg^{-1}), likewise isolates from Melkassa produced fumonisin in the range of 0.25 to 1.97 mg kg^{-1} and mean of 1.5 mg kg^{-1} (Table 3).

Under the culture conditions employed in this study, all Ethiopian *F. verticillioides* isolates showed significantly ($p < 0.001$) lower fumonisin production than the South-African reference isolate (MRC826). Observed differences were in the range of 60 % - 99.7 % compared to the reference isolate. Generally, the total fumonisin produced by some of *F. verticillioides* strains isolated from kernels of maize of Ethiopia was quite low, since 35 % of the isolates produced fumonisin levels < 2 mg kg^{-1}. Only 25 % of the total isolates produced above 20 mg kg^{-1} fumonisin (Table 2).

Table 2. Total fumonisin levels produced under *in vitro* by different *F. verticillioides* isolated from kernels of maize grown in Ethiopia.

Isolate name	Source area	Fumonisin (mg kg^{-1}) [a]	Isolate name	Source area	Fumonisin (mg kg^{-1}) [a]
AG-122	Agaro	1.82	AW-412	Hawassa	5.42
AG-131	Agaro	10.29	J-332	Jimma	8.40
AG-411	Agaro	1.26	J-512	Jimma	18.88
AG-431	Agaro	34.86	J-541	Jimma	23.22
AG-6632	Agaro	5.87	J-6811	Jimma	0.71
AB-6921	Alaba	4.61	KM-131	Kemmisse	4.94
AB-6821	Alaba	1.95	KM-142	Kemmisse	2.79
AB-522	Alaba	10.14	KM-313	Kemmisse	27.9
AL-6111	Alamata	35.91	KM-322	Kemmisse	6.83
AL-4432	Alamata	1.87	KM-351	Kemmisse	4.32
AL-3443	Alamata	1.87	KR-231	Korem	24.80
AL-6811	Alamata	1.71	KR-252	Korem	11.87
AL-5843	Alamata	11.23	KR-6743	Korem	3.129
AM-212	Ambo	1.36	MC-212	Maichew	1.38
AM-6552	Ambo	2.73	MC-234	Maichew	12.68
AM-6444	Ambo	24.8	MC-422	Maichew	12.22
AR-212	Dedessa	24.96	MC-112	Maichew	1.95
AR-6441	Dedessa	22.88	MC-431	Maichew	33.81
AR-312	Dedessa	9.60	ML-313	Melkassa	1.71
BK-1122	Bako	0.60	ML-6341	Melkassa	1.82
BK-2734	Bako	8.64	ML-6533	Melkassa	1.97
BK-6521	Bako	31.29	ML-6842	Melkassa	0.25
BK-3741	Bako	11.34	NK-112	Nekemte	9.42
BD-112	Bedele	1.57	NK-242	Nekemte	23.68
BD-331	Bedele	0.95	NK-432	Nekemte	10.24
BD-413	Bedele	1.14	NK-6232	Nekemte	1.62
BD-422	Bedele	1.03	NK-6633	Nekemte	25.60

[a] fumonisin concentration in mg of the toxin per kg of culture material (fungal biomass and maize kernel), ($p < 0.001$).

Table 2. (Continued)

Isolate name	Source area	Fumonisin (mg kg^{-1}) [a]	Isolate name	Source area	Fumonisin (mg kg^{-1}) [a]
DS-121	Dessie	0.99	S-3133	Sire	0.66
DS-132	Dessie	2.16	S-322	Sire	22.88
DS-322	Dessie	0.92	S-6351	Sire	1.82
DS-693	Dessie	37.38	S-6952	Sire	38.01
GD-6351	Gedeo	10.27	WS-112	Welayta-Sedo	3.32
GD-6531	Gedeo	2.67	WS-6232	Welayta-Sedo	1.70
G-112	Gibe	7.84	WS-121	Welayta-Sedo	4.56
G-421	Gibe	33.54	WS-6332	Welayta-Sedo	23.20
G-61011	Gibe	30.08	Z-111	Ziway	34.02
G-6331	Gibe	14.09	Z-311	Ziway	1.77
AW-121	Hawassa	18.48	Z-6812	Ziway	4.31
AW-222	Hawassa	34.10	Z-512	Ziway	5.96
AW-231	Hawassa	18.88	MRC-826	South Africa	95.5
AW-314	Hawassa	1.33	Control	Melkassa-4	< 0.025

[a] fumonisin concentration in mg of the toxin per kg of culture material (fungal biomass and maize kernel), ($p < 0.001$).

When the data were combined and analyzed according to the agroecological zones of Ethiopia, highest mean total fumonisin production was observed in isolates originated from the warm, sub-humid lowlands (21.4 mg kg^{-1}), followed by isolates from the warm, moist-lowlands with (13 mg kg^{-1}) (Fig. 2). Isolates from the warm sub-moist lowlands produced the lowest amount of fumonisin (mean 6.4 mg kg^{-1}) (Fig. 2).

Discussion

The present study revealed a widespread occurrence of fumonisin producing *F. verticillioides* strains in maize in Ethiopia. All the 80 isolates tested were able to produce fumonisin, when cultured on maize kernels. These findings are in agreement with results from a previous study in Mexico (Reyes-Velázquez *et al*., 2011), who reported that all *F. verticillioides* isolates obtained from maize were able to produce fumonisin. Likewise, all *F. verticillioides* isolates,

grown on sterile rice grain, analyzed in Brazil by Rocha *et al*. (2011) produced fumonisin B_1. In contrast, Covarelli *et al*. (2012) analyzed fumonisin production by 25 *F. verticillioides* isolates in Italy, and 20 % of them did not produce detectable level of the toxin. In Kenya Alakonya *et al*. (2008) observed that 26 % of the *F. verticillioides* isolates did not produce detectable level of fumonisin.

Table 3. Summary of fumonisin production by *F. verticillioides* strains isolated from kernels of maize produced in different areas of Ethiopia.

Isolate source-area	Agroecological zone [a]	Number of isolates tested	Fumonisin levels (mg kg^{-1})	
			Range	Mean
Agaro	SH_3	5	1.26 – 34.86	10.8
Alaba	SH_3	3	1.95 – 11.98	4.0
Alamata	SM_2	5	1.22 – 35.91	10.4
Ambo	M_3	3	1.36 – 24.8	9.6
Bako	SH_3	4	0.60 – 31.29	13.0
Bedele	H_3	4	0.95 – 1.57	1.2
Dedessa	M_2	3	9.06 – 24.96	19.0
Dessie	M_3	4	0.92 – 37.38	10.4
Gedeo	M_3	2	2.67 – 10.27	6.5
Gibe	SH_2	4	7.84 – 33.54	21.4
Hawassa	M_3	5	1.33 – 34.10	16.6
Jimma	H_3	4	0.71 – 23.22	12.8
Kemmisse	M_2	5	2.79 – 27.90	9.36
Korem	SM_4	3	3.13 – 24.80	13.3
Maichew	SM_4	5	1.38 – 33.81	12.4
Melkassa	SM_2	4	0.25 – 1.97	1.5
Nekemte	SH_3	5	1.62 – 25.60	14.0
Sire	SH_3	4	0.66 – 38.01	15.8
Welayta-Sedo	SH_3	4	1.70 – 23.20	8.2
Ziway	SH_3	4	1.77 – 34.02	11.5
MRC826	-	1	95.5	95.5
Untreated control	-	-	< 0.025	< 0.025

[a]Agroecological zones- H_3: Tepid humid mid-highlands; M_2: Warm moist lowlands; M_3: Tepid moist mid-highlands; SH_2: Warm sub-humid lowlands; SH_3: Tepid sub-humid mid-highlands; SM_2: Warm sub-moist lowlands; SM_4: Cool sub-moist mid-highlands (Source: MoARD, 2005).

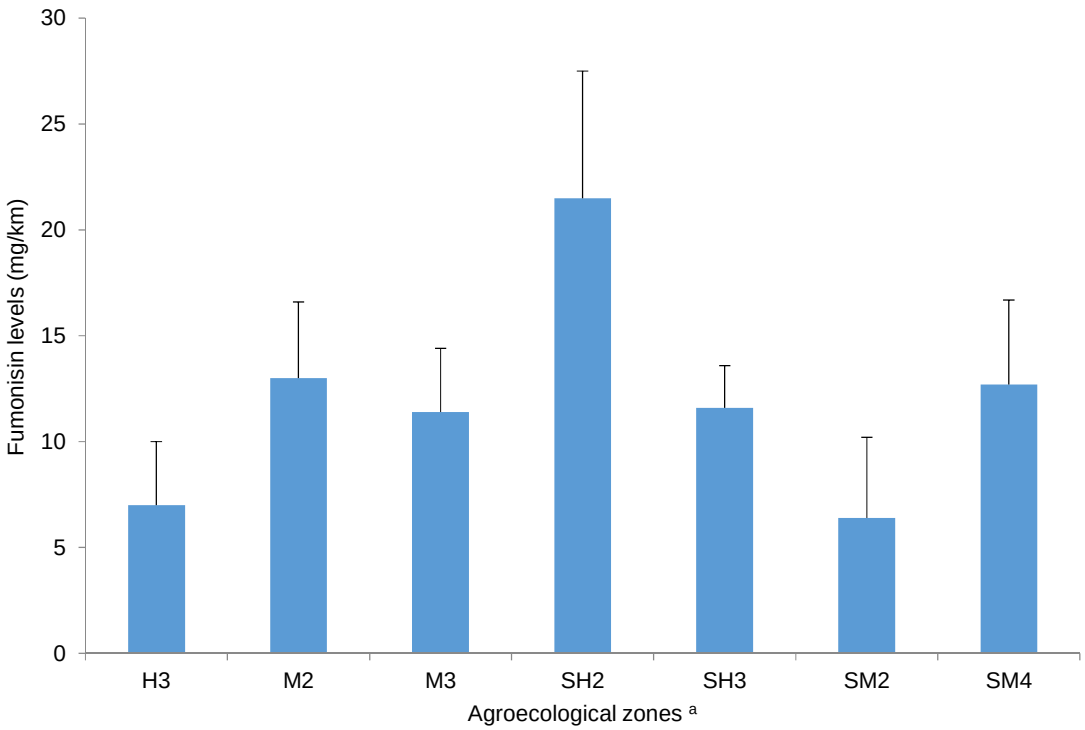

Fig. 2 Fumonisin production by *F. verticillioides* isolates from kernels of maize grown in different agroecological zones of Ethiopia. Data from 20 areas are summarized into their respective agroecological zones. [a]Agroecological zones- H_3: Tepid humid mid-highlands; M_2: Warm moist lowlands; M_3: Tepid moist mid-highlands; SH_2: Warm sub-humid lowlands; SH_3: Tepid sub-humid mid-highlands; SM_2: Warm sub-moist lowlands; SM_4: Cool sub-moist mid-highlands (Source: MoARD, 2005).

A wide-ranging variation in the fumonisin production ability of the *F. verticillioides* isolates was observed in this *in vitro* study, because some of them synthesized the toxin at very low levels (0.25 mg kg^{-1}), while other isolates produced as high as 38 mg kg^{-1} of the toxin. The detected variation in fumonisin production among isolates could be due to variation in the inherent genetic characteristics of the isolates. Previous molecular studies of *F. verticillioides* isolates revealed a positive relationship between the *FUM* gene cluster and fumonisin production (Picot *et al.*, 2010; Sagaram *et al.*, 2006). The amount of transcription products from the key *FUM* genes, *FUM1*, *FUM21* and *FUM8* positively correlates with fumonisin accumulation, but disruption of these genes results in significant reduction in fumonisin production (Fanelli *et al.*, 2013; Sagaram *et al.*, 2006). Additional genes outside the *FUM* gene cluster are also known to regulate fumonisin biosynthesis, including *FCC1, FCK1, PAC1, ZFR1* and *GBP1* (Sagaram *et al.*, 2006). According to (Stępień *et al.*, 2011) inter and

intraspecific variation in fumonisin production by *Fusarium* species can be explained by a high level of sequence variation inside the *FUM* gene cluster.

Fumonisin production by *F. verticillioides* isolates can also be influenced by several factors, including temperature, water activity, pH, oxygen and nutrient composition of the substrate. If conditions are not optimal, the fungus will not achieve the maximum of its genetic potential in synthesizing the toxin. Pervious, *in vitro* studies indicated that the optimal conditions for growth of *F. verticillioides* and fumonisin production are water activity of 0.97 to 0.99 a_w and temperatures between 25 °C to 30 °C (Fanelli *et al*., 2013; Marin *et al*., 1999). Fumonisins are not produced at temperature less than 10 °C and water activity levels below 0.93 (Marin *et al*., 1999). Variable levels of fumonisin production have been observed in liquid (Myro medium) and solid medium (maize and rice patties) when using the same *F. verticillioides* isolates, while isolates that produced high level of the toxin in solid media could not produce similar levels in liquid medium and *vice versa* (Vismer *et al*., 2004). For this reason, the authors suggested that conditions for fumonisin biosynthesis should be optimized for each individual strain. Nutritional factors of the substrate, on which the fungus is cultivated, has also been reported to be an important factor in fumonisin biosynthesis. A positive association has been found between sugar concentration (especially amylopectin) as well as nitrogen starvation with regard to fumonisin production (Picot *et al*., 2010). Well-aerated conditions are reported as important factors for fumonisin biosynthesis. The culture substrate to container volume ratio used in the present study (100 g seed per 500 mL jar) is quite high. This probably makes the culture stuffy, and it does not provides enough air circulation for the fungal growth in the interior of the culture, which may negatively affect fumonisin biosynthesis. La Bars *et al*. (1994) reported lower FB_1 concentration after 3 weeks incubation than after 2 weeks, and this difference in toxin level has been attributed to the decrease of oxygen during incubation. Under oxygen limited conditions, reduced fungal growth and fumonisin production have been reported (Keller *et al*., 1997).

The presence of highest fumonisin producing isolates compared to others across all areas investigated in Ethiopia, demonstrated that the distribution of fumonisin producing *F. verticillioides* strains is not linked to specific geographic regions. Potent strains appear wide spread and uniformly distributed all over the Ethiopian maize growing areas. The observed variation in fumonisin production by *F. verticillioides* isolates obtained from the same geographical areas is in accordance with the results obtained by others (Alakonya *et al*., 2008;

Covarelli *et al*., 2012; Rocha *et al*., 2011). However, the levels of fumonisin observed in this study are lower compared to those reported in Uganda (Atukwase *et al*., 2012), in South Africa (Vismer *et al*., 2004), in Italy (Covarelli *et al*., 2012) and in Mexico (Reyes-Velázquez *et al*., 2011). Depending on the criteria of Nelson *et al*. (1991), *F. verticillioides* strains can be categorized into three groups, high fumonisin producers (above 500 mg kg^{-1}), intermediate (50 mg kg^{-1} to 500 mg kg^{-1}) and low producers (trace to 49 mg kg^{-1}). Based on the above criteria, all of the strains isolated from maize kernels in Ethiopia and tested in this study are low fumonisin producers.

Fumonisin levels produced by a substantial number of the *F. verticillioides* isolates in the present study were very low, with 35 % of the isolates producing < 2 mg kg^{-1}. This may indicates that the fumonisin contamination of maize produced in Ethiopia may be generally low. This seems in line with previous observations made on the natural occurrence of fumonisin in maize samples of Ethiopia. In 100 maize samples from Southern Ethiopia Alemu *et al*. (2009) found mean fumonisin level of 1.68 mg kg^{-1}. Ayalew (2010) analyzed fumonisin levels in 17 maize samples mainly obtained from Eastern Ethiopia and only four of them contained fumonisin levels ranging from 0.3 - 2.4 mg kg^{-1}. However, fumonisin contamination of natural samples could vary year-to-year depending on the climatic and ecological situation. Under field condition, warm temperature and low precipitation after silking are favorable environmental conditions for *F. verticillioides* infection and subsequent fumonisin contamination of maze kernels (Miller, 2001). Stressful environmental conditions during growth such as drought stress increases insect activity and compromise host plant defenses against pathogens, which leads to elevated levels of fumonisin contamination in kernels (Miller, 2001; Picot *et al*., 2010). Late season rainfall at the time of harvest has also been positively associated with *F. verticillioides* infection and fumonisin contamination (Sagaram *et al*., 2006).

In conclusion, the results revealed that fumonisin producing *F. verticillioides* strains are widely distributed in the maize growing areas in Ethiopia. Although the amount produced by several isolates is generally low, the widespread existence of fumonisin producing strains indicates that the risk for food safety should not be underestimated, taking into consideration the fact that *F. verticillioides* is the most frequently isolated *Fusarium* species from kernels of maize in Ethiopia. The amount of fumonisin produced may become higher when stressful environmental condition prevails, which is more suitable for toxin production. Climate

change effects such as increased temperature and limited water availability may stress host plants and favour growth of mycotoxigenic *F. verticillioides* strains. *In vitro* data on fungal growth and mycotoxin production may not relate directly to the situation in the field, thus, the ability of these isolates to synthesize fumonisin under field condition need to be investigated further, taking into consideration the diversity of maize cultivars and the agroecological conditions in the maize growing areas of Ethiopia. It is very important to prevent introduction of more toxigenic strains with germplasms from abroad.

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Paper IV

Evaluation of Ethiopian maize cultivars for resistance to *Fusarium verticillioides* and fumonisin accumulation

Hadush Tsehaye [1,4,*], May Bente Brurberg [1,2], Arne Tronsmo [3], Dereje Assefa [4], Leif Sundheim [1,2], Anne Marte Tronsmo [1]

[1] Norwegian University of Life Sciences, Department of Plant Sciences, P.O. Box 5003, NO-1432 Ås, Norway

[2] Norwegian Institute for Bioeconomy Research, Biotechnology and Plant Health Division, P.O. Box 5003, NO-1431 Ås, Norway

[3] Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science, P.O. Box 5003, NO-1432 Ås, Norway

[4] Mekelle University, Department of Dryland Crop and Horticultural Sciences, P.O.Box 231, Mekelle, Tigray, Ethiopia

*Corresponding author: Hadush Tsehaye; email address: hadush.beyene@nmbu.no or had031@yahoo.com

Abstract

Fusarium ear rot of maize caused mainly by *Fusarium verticillioides* is a major concern to maize production worldwide due to contamination of grains with fumonisin mycotoxin, which have been associated with various toxicoses of animals and humans. Maize cultivars with adequate level of resistance are the best alternative to minimize the problem. The objective of this study was to find sources of resistance to *Fusarium* ear rot and fumonisin accumulation in maize germplasm. In total 15 maize cultivars mainly recommended for the medium to limited rainfall areas were evaluated by means of silk channel inoculation using a fumonisin producing *F. verticillioides* isolate in field trials during 2013 and 2014. The percentage of visible symptoms and mycelium coverage on the inoculated ears was rated at harvest (0 - 100 %), and fumonisin content was quantified using competitive Enzyme Linked Immunosorbent Assay (ELISA). *Fusarium* ear rot severity and fumonisin content were significantly higher in 2013 than 2014. The percentage of visible infected kernels per ear after inoculation, ranged from 5 % to 60 % in 2013 and from 3 % to 40 % in 2014. Fumonisin accumulation in maize cultivars ranged from 2.7 to 76.3 mg kg^{-1} (mean 33.3 mg kg^{-1}) in 2013 and from 1.8 to 52.7 mg kg^{-1} (mean 23 mg kg^{-1}) in 2014. Fumonisin content was positively correlated with ear rot severity ($r = 0.924$, $p < 0.001$) and ($r = 0.908$, $p < 0.001$) in 2013 and 2014, respectively. The fifteen tested maize cultivars differed significantly ($p < 0.001$) in *Fusarium* ear rot severity and fumonisin accumulation. The six cultivars, viz. Berihu, Melkassa-7, Melkassa-2, Melkassa-4, MHQ-138 and BHQP-542 had the lowest fumonisin content over the two years trial. Overall results of this study indicated the presence of potential sources of resistance to *Fusarium* ear rot and fumonisin accumulation in the Ethiopian maize germplasm. Cultivars that showed low disease severity are useful in breeding programs aiming at developing cultivars with higher resistance to *Fusarium* ear rot and fumonisin accumulation.

Keywords: Maize, *Fusarium verticillioides*, *Fusarium* ear rot, fumonisin

Introduction

Maize (*Zea mays* L.) is the most important cereal crop produced by smallholder farmers in Ethiopia, and is cultivated throughout the country under different agroecological conditions. Total estimated maize production by small-scale farmers in Ethiopia in 2014 was 7.2 million tonnes, and the area planted with maize in the same year was estimated at 2.1 million ha (FAO, 2014). *Fusarium verticillioides* (Sacc.) Nirenberg is the most common fungal pathogen associated with maize grains in Ethiopia (Ayalew, 2010; Wubet and Abate, 2004). This fungus can infect the maize plant at all growth phases causing ear rot, stalk rot and root rot (Logrieco *et al.,* 2002; Mesterházy *et al.,* 2012). *Fusarium verticillioides* infection in maize kernels often occurred between the blister stage and the dough stage depending on location and year (Picot *et al.,* 2011). Severe prevalence of maize ear rot is associated with reduced grain yield and quality (Presello *et al.,* 2008). However, the main implication associated with *F. verticillioides* infection of maize is the contamination of grains with harmful mycotoxins, known as fumonisins (Logrieco *et al.,* 2002; Mesterházy *et al.,* 2012). The contamination of grains by fumonisin is of great concern worldwide because it can lead to serious intoxications in humans and domestic animals (Waśkiewicz *et al.,* 2012). It is now well recognized that fumonisins may cause leukoencephalomalacia in horses (Marasas *et al.,* 1988), pulmonary edema and hydrothorax in pigs (Harrison *et al.,* 1990), and are nephrotoxic and hepatotoxic in laboratory model animals (Gelderblom *et al.,* 1996). In humans, consumption of fumonisin-contaminated maize has been implicated for elevated esophageal and liver cancer (Sun *et al.,* 2007), and neural tube defects (Missmer *et al.,* 2006). Taking into consideration the risk it bears to human and animal health, several countries and geographical regions have established guidelines for maximum tolerable limit of fumonsin in maize products intended for human food and animal feed (FDA, 2001; EC, 2007).

In order to reduce the potential health hazard associated with consumption of fumonisin-contaminated grains, effective management of the disease at field level is required. Currently there are no effective control strategies available to prevent *Fusarium* infection and fumonisin accumulation in maize grains. Chemical control is often ineffective as the fungus transmits vertically from seed or soil to the plant (Munkvold *et al.,* 1997) and it may also lead to selection of new strains of the pathogen with resistance to fungicides. Cultural methods such as physical removal of infected plant derbies, crop rotation, and manipulation of planting time may reduce the disease to some extent, but do not provide sufficient control (Fandohan *et al.,*

2004; Munkvold 2003). Breeding for *Fusarium* resistant maize cultivars and cultivation of these seems to be the most promising option for controlling *Fusarium* ear rot disease and reduce the risk of fumonisin-contaminated grains. Resistance to *Fusarium* infection and fumonisin accumulation exists among maize lines, but high or adequate level of resistance may not be present in commercial hybrid cultivars (Eller *et al.,* 2008). Many of the improved maize cultivars are derived from a relatively narrow maize genetic pool, and do not appear to provide adequate level of resistance (Nelson *et al.,* 2008). However, older locally adapted public germplasm may be an important source of resistance for this widespread pathogen. Significantly high variation in resistance to *Fusarium* ear rot and fumonisin accumulation among maize germplasms have been reported in previous studies (Afolabi *et al.,* 2007; Clements *et al.,* 2004; Schjøth *et al.,* 2008; Small *et al.,* 2012).

Screening based on phenotypic traits related to resistance could be advantageous since it is cheaper and faster than mycotoxin analysis. Phenotypic traits associated with ear rot resistance includes maturity (Battilani *et al.,* 20008), husk covering and tightness (Butron *et al.,* 2006; Warfield and Davis 1996), as well as length of the silk channel (Bolduan *et al.,* 2009). Fumonisin concentrations are often much higher in symptomatic kernels than in asymptomatic kernels (Desjardins *et al.,* 1998). Thus, reduction in the proportion of ear surface area infected, may relate to resistance to fumonisin accumulation.

Fusarium ear rot epidemics are sporadic in nature and disease severity strongly influenced by the climatic conditions. For this reason, natural infection do not always give sufficient and homogeneous symptom development for adequately differentiating phenotypic variation in resistance to *F. verticillioides* (Mesterházy *et al.,* 2012). Artificial inoculation often enhances disease severity but may reduce the variation within the treatments by ensuring high inoculum pressure compared with natural infection (Afolabi *et al.,* 2007; Celements *et al.,* 2003; Schjøth *et al.,* 2008). Resistance tests frequently uses artificial inoculation at the susceptible, the R_2 (blister), plant growth stage (Celements *et al.,* 2003) to ensure adequate infection and uniform distribution of the pathogen among plants throughout the field. Inoculation by penetrating the husk with pin bars or injecting inoculum down the silk channel have been used as suitable methods to discriminate between different levels of resistance to *F. verticillioides* infection and fumonisin accumulation in maize cultivars (Eller *et al.,* 2008; Mesterházy *et al.,* 2012). The husk penetration mimics the natural mode of fungal entry into ears via wounds formed by insects or birds. The silk channel infection mimics spores deposited and germinating

through the silk channel (Eller *et al.,* 2008). Previous studies have shown that data from natural infection and artificial inoculation correlates well when silk channel inoculation is used (Mesterházy *et al.,* 2012). Besides, the predominant pathway for maize ear infection by *Fusarium* species is the silk infection (Mesterházy *et al.,* 2012), therefore, artificial silk inoculation might be the appropriate method for evaluating genetic resistance to *F. verticillioides* infection and fumonisin accumulation.

In order to incorporate resistance to ear rot and fumonisin contamination into agronomical elite maize cultivars, identification of cultivars with adequate resistance level is crucial. In Ethiopia, very little information is available regarding the resistance level of maize cultivars to *Fusarium* ear rot or fumonisin contamination, as maize development program mainly focuses on total yield advantage. Therefore, the objective of this study was to assess differences in Ethiopian maize cultivars for resistance to *F. verticillioides* infection and fumonisin accumulation after artificial inoculation, and to select maize cultivars with good level of resistance.

Materials and Methods

Field study site

This study was conducted during the rainy season in 2013 and 2014 at the experimental field site of Mekelle University main campus, northern Ethiopia (Fig. 1). The site is located at 13º 29’ N latitude and 39º 30’ E longitude, at an altitude of 2130 m above sea level. Semi-arid climatic condition with short growing season, erratic and low amount of rainfall is general characterize of the area. The average annual rainfall is about 600 mm, and most of the rain (70 – 80 %) falls in the main crop-growing season June through September (Araya *et al.,* 2011). The mean annual minimum temperature is about 12 ºC, while the mean annual maximum temperature is 28 ºC. The soil type at the experimental site is Cambisols with silt-clay texture (Araya *et al.,* 2011).

Planting materials and experimental design

Fifteen maize cultivars, eight open pollinating composites, five hybrids, one local landrace and one ear rot susceptible inbreed line were evaluated for resistance to *Fusarium* ear rot and

fumonisin accumulation caused by *F. verticillioides*. The susceptible inbred line, with low resistance (NSCM411881(32)), was chosen on the basis of its previous history, as one of the maize hybrid (BH541) developed from this parent material was withdraw from the production system due to its susceptibility to ear rot (Personal communication with Mr. Brhanu Taddesse, coordinator of National maize development program in Ethiopia). The maize cultivars were very heterogeneous in terms of plant height, days required to maturity as well as kernel characteristics such as texture and color (Table 1).

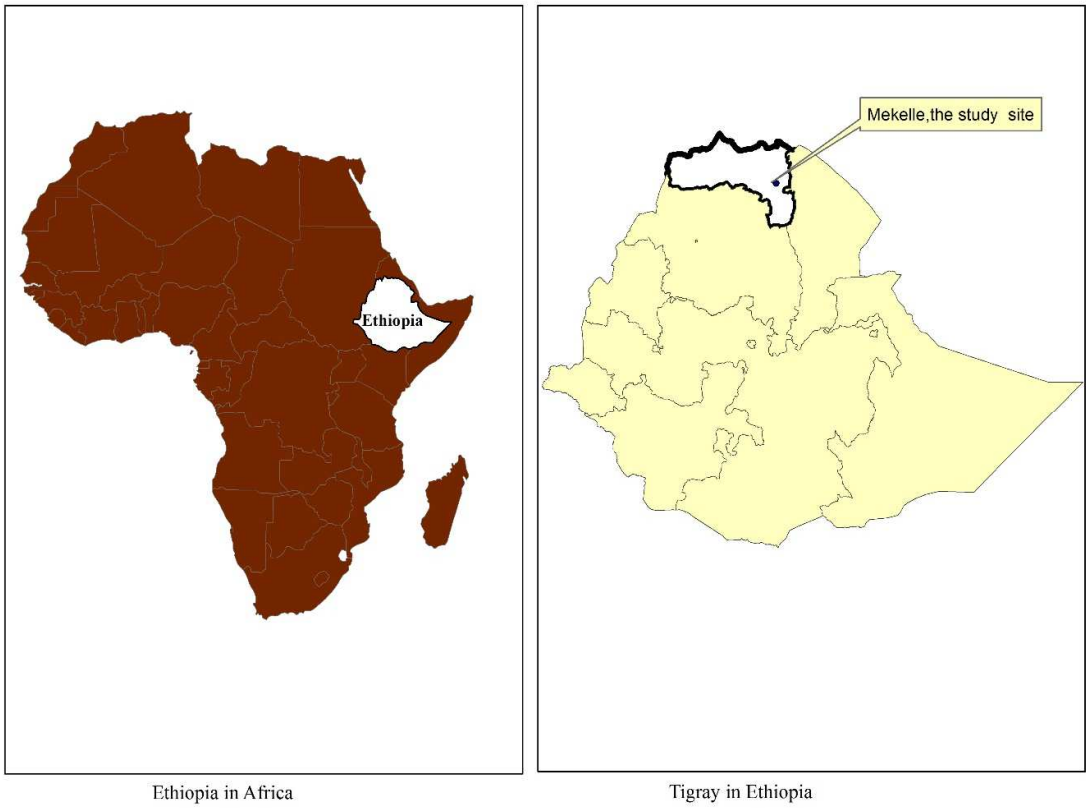

Fig. 1 Map of Ethiopia and Mekelle the study site for the field experiment.

The experiment was conducted using randomized complete block design with three replications. Each cultivar was assigned to three plots that were 3 m long and 4.5 m wide with 5 rows per plot (spacing 0.75 m between rows and 0.25 m between plants). At the time of planting, two seeds were placed per planting hole and two week after emergence, plants were thinned to one plant. In total, there were 55 plants per cultivar per plot. The trials were fertilized with Phosphorous (P_2O_5) and Nitrogen (N) fertilizers at a rate of 46 kg ha^{-1} and 64 kg ha^{-1}, respectively. All the P and one-third of the N were applied at planting; and the rest of N was applied as top dressing two times, approximately at 4 and 6 weeks after crop

emergence. Supplemental irrigation was applied once per week via furrow irrigation at the end of the growing season when the rain declined, to provide sufficient soil moisture to allow the plants to come to a complete development stage.

Table 1 List of the maize cultivars evaluated for resistance to *Fusarium* ear rot and fumonisin accumulation, and some of their agronomic characteristics

Cultivar	Days to Maturity §	Seed color	Grain texture	Type
Melkassa-2	130	white	Semi-dent	OPV
Melkassa-3	125	white	Semi-dent	OPV
Melkassa-4	105	white	Semi-dent	OPV
Melkassa-5	125	white	flint	OPV
Melkasa-6Q	120	white	Semi-flint	OPV
Melkassa-7	115	yellow	Semi-flint	OPV
MH-130	120	white	Semi-dent	Hybrid
MHQ-130	140	white	Semi-dent	Hybrid
BH-140	145	white	Dent	Hybrid
BH-540	145	white	Dent	Hybrid
BHQP542	138	white	Semi-dent	Hybrid
Gibe-1	145	white	Semi-dent	OPV
Gibe-2	116	white	Semi-flint	OPV
Berihu	90	yellow	Semi-flint	Local landrace
NSCM-411881(32)	145	white	Semi-flint	Inbred line

§: data from national average, OPV= open pollinated cultivar

Preparation of fungal inoculum and inoculation of maize ears

Plants were inoculated with inoculums prepared from culture of *F. verticillioides* (SR-6952) to ensure better infection and disease development. This isolate was obtained from infested grain of maize in the Sire area, central-western Ethiopia and it was chosen for its ability to produce highest amounts of fumonisin under *in vitro* testing. The isolate was grown on Mung bean agar plates for good spore production (Dill-Macky *et al.*, 2003). Cultures were incubated at 23°C±2 ºC under alternating 12 h of near ultraviolet/white light and darkness (12 h) for 10

days. The inoculum were prepared by washing conidia from the surface of the agar media using sterile distilled water, and the resulting spore suspension was filtered through two layers of cheesecloth. The obtained spore suspension was counted using a Kova glasstic slide and spore concentration was adjusted to 1×10^6 conidia mL^{-1} using sterile distilled water amended with Tween 20 surfactant (polyoxyethylenesorbitan monolaurate; Sigma-Aldaich, St. Louis Missouri) at a rate of 0.2 mL liter $^{-1}$. Ten plants were randomly selected in each plot, and the primary ears of the plants were inoculated with 4 mL conidial suspension through the silk channel at the blister (R_2) growth stage, using a hypodermic needle and syringe (Afolabi *et al.,* 2007; Clements *et al.,* 2003). After inoculation, maize ears were sprayed with sterile distilled water, and covered with paper bags to maintain high humidity and to protect the inoculum from being washed off by rain or dried by high daytime temperature.

Disease assessment and collection of other agronomic data

At the time of harvest, the inoculated ears were handpicked separately, husk-leaves carefully removed, and evaluated immediately for severity of ear rot symptoms. The severity of *F. verticillioides* infection was visually assessed by determining the percentage (0 - 100 %) of each ear surface covered by visible symptoms of fungal infestation such as kernels displaying rots, discolorations (darkened, brown, pinkish stains) and white mycelial growth on kernels (Clements *et al.,* 2004; Small *et al.,* 2012). After rating, ears were dried naturally to approximately 15 % grain moisture content, hand-shelled and bulked by plots. From this bulk samples, subsamples of 100 g grains from each plot were retained for subsequent fumonisin analysis.

The number of days from planting to 50 % silking was measured based on the registered number of days between planting and silk emergence on half of the plant population in a plot. Besides, agronomic parameters such as plant height, seed weight and grain yield was recorded per each plot.

Climatic data

Climatic data such as rainfall and temperature (minimum, maximum) were obtained from the nearest Meteorological station to the experimental field (Mekelle University, about 300 m

away). Summary of the monthly climatic data for the study site during 2013 and 2014 is presented in Fig. 2.

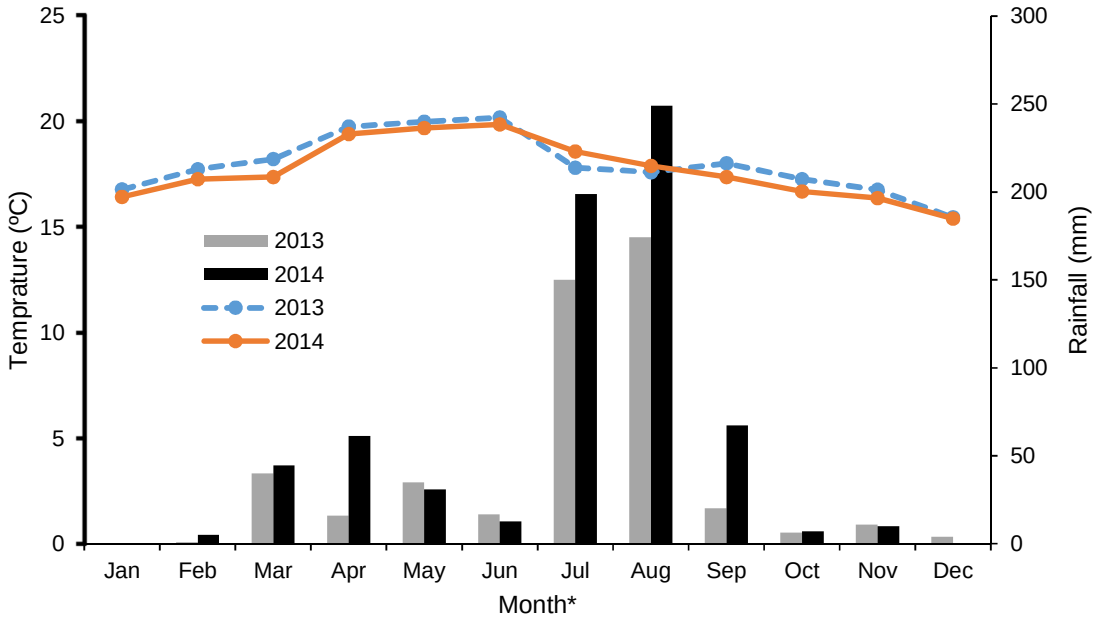

Fig. 2 Weather data (annual rainfall, mean temperature) recorded from the meteorological station near the study site in 2013 and 2014. Bars represent annual rainfall and lines represent mean temperature. * Month; Jan: January, Feb: February, Mar: March, Apr: April, Jun: June, Jul: July, Aug: August, Sep: September, Oct: October, Nov: November, Dec: December.

Fumonisin analysis

Grain subsamples (100 g) from inoculated ears of each plot were grinded, using a laboratory mill (IKA®-Werke, GmbH & Co. KG., Germany), and passed through a 1 mm mesh sieve. Fumonisin was extracted from 5 g powder samples using 25 mL of 70 % methanol with shaking (1000 rpm) for 3 min. The extracts were filtered through a Whatman no. 1 filter paper and fumonisin content in the samples was determined using an Enzyme Linked Immunosorbent Assay (ELISA) kit (RIDASCREEN®Fumonisin, R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instruction. The absorbance was measured using a microplate reader at 450-nm, and fumonisin content of samples were evaluated using the RIDA®SOFT Win software (Art. Nr. Z9999, R-Biopharm AG, Darmstadt, Germany). The minimum detection limit of fumonisin concentration in the kit was 0.025 mg kg^{-1}. If the samples exceeded the highest detection limit (> 2 mg km^{-1}) they were further diluted with the solvent, and the results were obtained by multiplying with the dilution factor.

All samples were analysed in duplicates and the A450-nm readings in the duplicates were averaged.

Statistical analysis

Severity of *Fusarium* ear rot, fumonisin content in grains and data on agronomic performance of maize cultivars were analysed using the MIXED procedure of Statistical Analysis System (SAS) software (SAS institute, Cary, NC), with cultivars as fixed effects and blocks as random effects. When there was no significant interaction between traits of interest measured on cultivars and the year in which cultivars grown, combined analysis were performed and results for both years presented together. Differences between cultivars were determined with Fisher's protected least significance (LSD) test. Data on ear rot severity, and fumonisin content were natural log transformed to achieve variance homogeneity and normal distribution of residuals. Pearson's correlation coefficients were determined for the relationship between ear rot severities, days to 50 % silking, grain yield and fumonisin concentration based on the non-transformed treatment means.

Results

The disease severity of *Fusarium* ear rot on different maize cultivars inoculated with a fumonisin producing *F. verticillioides* strain in 2013 and 2014 is summarized in Fig. 3. The *Fusarium* ear rot severity was significantly influenced by year ($p < 0.012$) and maize cultivars ($p < 0.0001$). *Fusarium* ear rot severity was significantly lower in 2014 than in 2013. In 2013, ear rot severity ranged from 4.9 % to 60.3 %, with mean ear rot of 30 %; while in 2014, maize ear rot severity ranged from 2.8 % to 40.2 % (mean of 20 % rot on the cobs). Cultivars Berihu, Melkassa-7, Melkassa-2, Melkassa-4 and MHQ138 had consistently lower disease severity (< 20 % rot on ear) over the two years trial (Fig. 3).

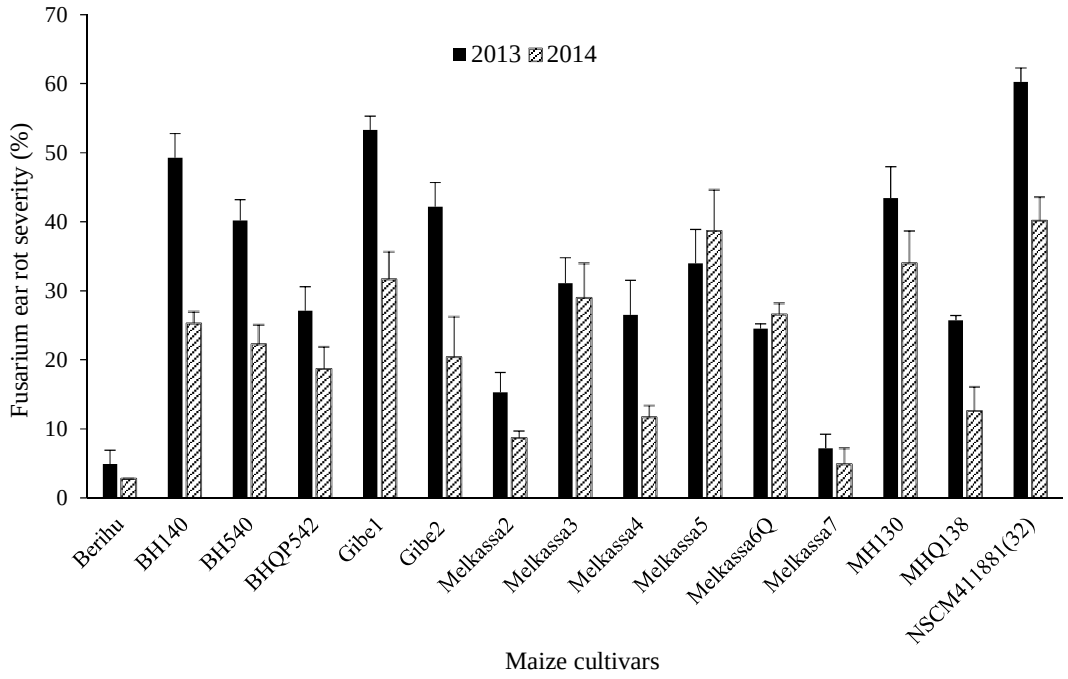

Fig. 3 *Fusarium* ear rot disease severity in maize cultivars in the experimental field at Mekelle, in 2013 and 2014; vertical bars represent standard error of the mean.

The total fumonisin content of maize cultivars inoculated with *F. verticillioides*, in 2013 and 2014 is illustrated in Fig. 4. Fumonsin content was affected significantly by year ($p < 0.018$) and maize cultivars ($p < 0.0001$). Fumonisin content in maize grains ranged from 2.7 to 67.7 mg kg $^{-1}$ in 2013 with a grand mean of 33.2 mg kg $^{-1}$, and from 1.8 to 52.7 mg kg $^{-1}$ in 2014 with a grand mean of 23.1 mg kg $^{-1}$. Maize cultivars with low level of fumonisin in both years include the local landrace Berihu, Melkassa-2, Melkassa-7, Melkassa-4, BHQP542 and MHQ-138 with mean concentrations of 2.3 to 17.3 mg kg $^{-1}$ (Fig. 4). The majority of cultivars that showed best resistance to *F. verticillioides* are early maturing type, except BHQP542, which is a late maturing type. Ranking order of some maize cultivars differed between years in their responses to ear rot and fumonisin contamination. In 2013, highest amount of fumonisin accumulated in grains of cultivar Gibe-1 (76.3 mg kg $^{-1}$), NSCM-411881(32) (67.8 mg kg $^{-1}$) and BH-140 (58.7 mg kg $^{-1}$). While in 2014, the highest fumonisin content was recorded on cultivar Melkassa-5 (52.7 mg kg $^{-1}$), followed by MH-130 (51.4 mg kg $^{-1}$) and NSCM-411881(32) (48.9 mg kg $^{-1}$) (Fig. 4).

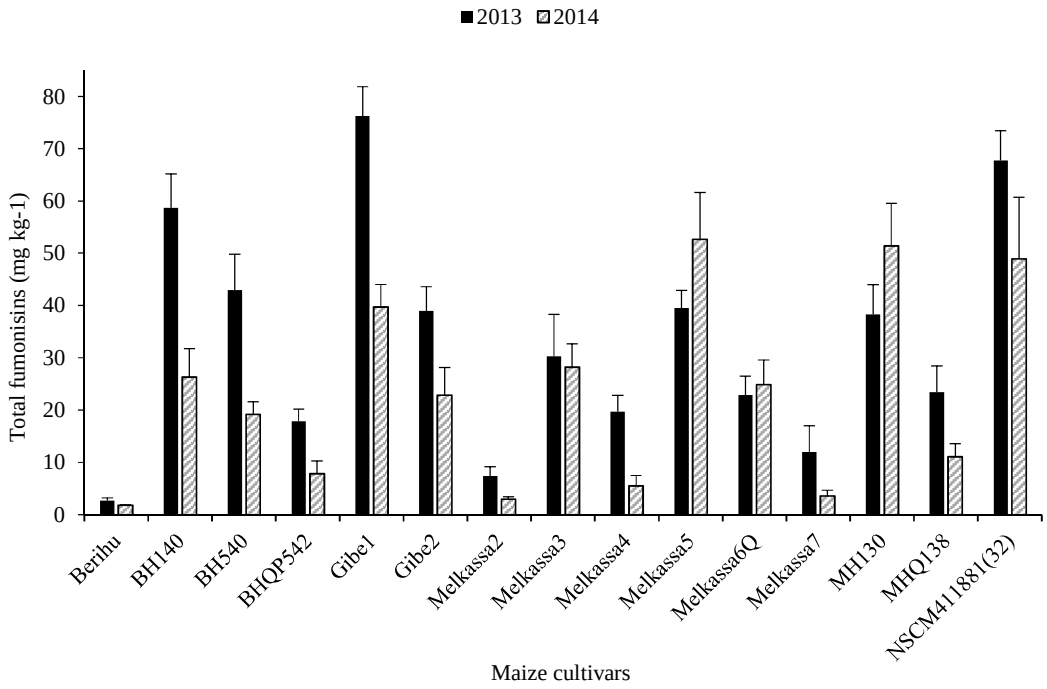

Fig. 4 Total fumonisin contamination in grains of different maize cultivars inoculated with *Fusarium verticillioides* in Mekelle, in 2013 and 2014; vertical bars represent standard error of the mean.

Agronomic performance data from the two years were combined, and analysis of variance (ANOVA) indicated highly significant variation among cultivars for earliness (days to 50 % silking), plant height, thousand seed weight and grain yield. In terms of earliness, the open pollinated cultivars Berihu, Melkassa-7 and Melkassa-3 were selected as the best cultivars for the testing area. While maize cultivars Gibe-1, BHQP-542, BH-540 and BH-140 were late in terms of days required to reach 50 % silking and physiological maturity (Table 2). Hybrids BHQP-542, BH-140 and BH-540 were more superior in grain yield compared to other cultivars, with mean grain yield of 7.73 tonnes ha^{-1}, 7.72 tonnes ha^{-1} and 7.64 tonnes ha^{-1}, respectively (Table 2). These cultivars could be recommended for irrigated agriculture in the study area.

Table 2 Agronomic performance of maize cultivars tested in Mekelle, combined data for 2013 and 2014

Maize cultivars	Plant height (cm)	Days to midsilk (DAP)	1000 seed weight (g)	Yield (tonnes ha^{-1})
Melkassa-2	164.3d	100.5d	344.7e	4.80d
Melkassa-3	161.2de	92.0e	364.4cd	4.5de
Melkassa-4	161.3de	99.0d	345.5e	4.26ef
Melkassa-5	163.8d	105.8c	360.0de	4.10f
Melkasa-6Q	162.0de	109.5c	308.5f	4.61de
Melkassa-7	153.7f	81.7f	301.0f	3.96f
MH-130	157.3ef	106.0c	354.5de	5.96c
MHQ-138	165.8d	108.0c	352.4de	6.22bc
BH-140	211.5b	123.7a	398.0b	7.72a
BH-540	210.3b	125.0a	407.5ab	7.64a
BHQP-542	206.3b	126.0a	365.0cd	7.73a
Gibe-1	220.0a	127.8a	421.6a	6.48b
Gibe-2	198.0c	117.0b	377.8c	6.25b
Berihu	144.0g	72.8g	302.0f	3.26g
NSCM-411881(32)	200.0c	115.0b	397.0b	6.56b
Mean	178.6	107.4	360	5.55
$LSD_{0.05}$	6.1	4.47	15.78	0.398
CV (%)	2.97	3.6	3.8	1.3
p-value	0.0001	0.0001	0.0001	0.0001

Within each column, means followed by the same letter do not differ significantly according Fisher's least significant difference test (LSD) at 0.05 probability level; CV: coefficient of variation

Fumonisin content in grains was significantly correlated with *Fusarium* ear rot severity both in in 2013 and 2014 (Fig. 5). Correlations between fumonisin content and silking date, ear rot severity and silking date, were small to medium for combined data over two years (Table 3).

Table 3 Pearson correlation coefficients (r) between *Fusarium* ear rot severities (FER), days to 50 % silking and fumonisin contamination in maize cultivars inoculated with *F. verticillioides* (combined data for 2013 and 2014)

	FER[a]	50 % silking	Fumonisin
FER	1		
50 % silking	0.627**	1	
Fumonisin	0.918**	0.519**	1

[a]*Fusarium* ear rot severity, ** significant at $p < 0.001$

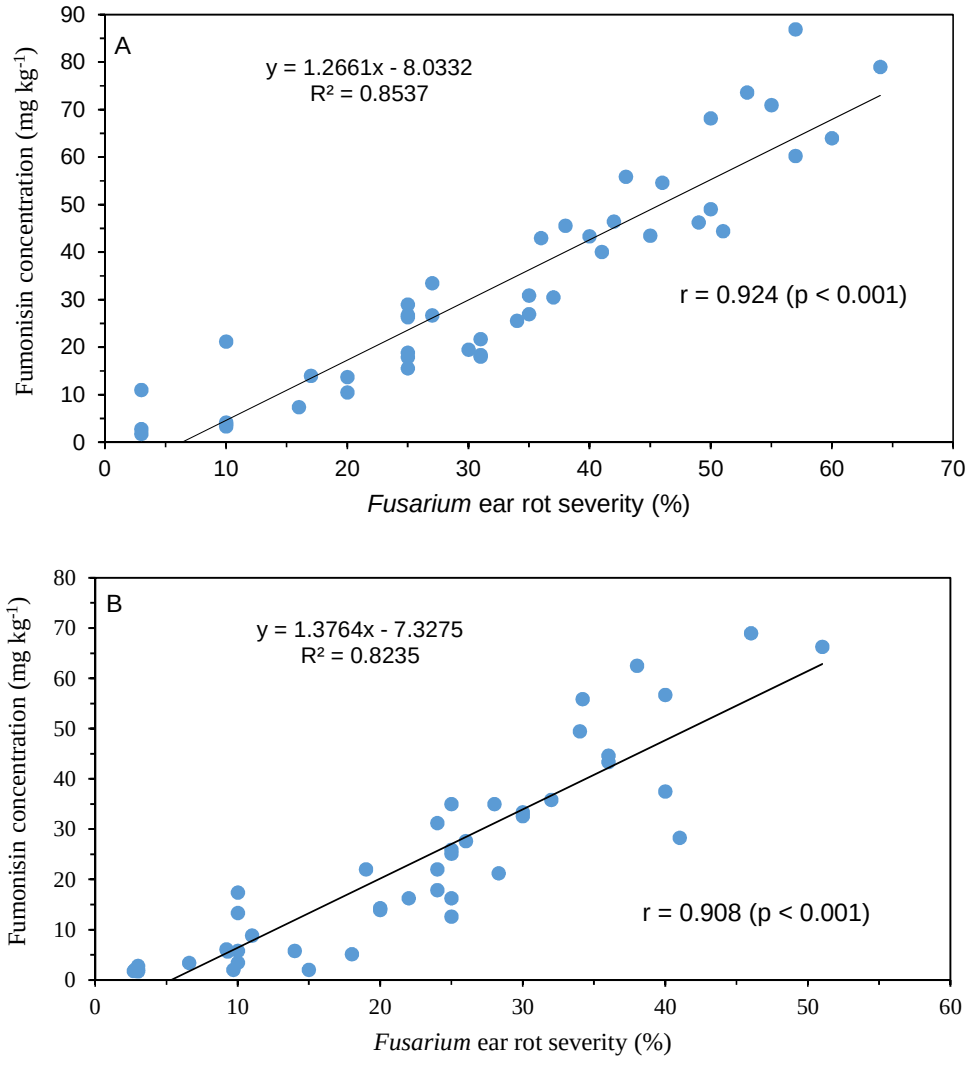

Fig. 5 *Fusarium* ear rot severity and associated fumonisin concentration in 15 maize cultivars inoculated with *F. verticillioides* in 2013 (A) and 2014 (B), in Mekelle.

Discussion

In the present study, maize cultivars commonly grown in the medium rainfall to moisture-limited areas of Ethiopia were evaluated over two years, with the aim to identify cultivars that are resistant to *Fusarium* ear rot and fumonisin accumulation. Maize cultivars with consistently low or high ear rot disease severity and fumonisin accumulation in grains were identified in the two years of inoculation trials. Maize cultivars Berihu, Melkassa-7, Melkassa-2, Melkassa-4, MHQ-138, BHQP-542 and Melkassa-6Q had low ear rot disease severity and subsequently low fumonisin levels. In fact, these cultivars display 59 % to 96 % lower fumonisin levels than the most susceptible line (NSCM-411881(32)). This indicates the existence of potential resistance genes against *F. verticilloides* and the toxin it produces, in the maize germplasms of Ethiopia. These cultivars may be important as sources of resistance to *F. verticillioides* for introduction into advanced breeding programs, as this pathogen is the most common fungal pathogen associated with maize grains in Ethiopia (Ayalew, 2010; Wubet and Abate, 2004). Resistance to *Fusarium* ear rot is a polygenic in nature, quantitatively inherited with additive gene action (Clements *et al.*, 2004), with moderate to high heritability (Robertson *et al.*, 2006). Thus, it is possible that alleles for resistance can be transferred from donor populations into maize elite materials (Clements *et al.*, 2004). Eller *et al.* (2010) has demonstrated the usefulness of backcross breeding for transferring quantitatively inherited disease resistance traits from an agronomical poor line (GE 440), into an elite commercial inbred line (FR 1064) without significantly affecting the yield potential of the latter.

Fusarium verticillioides established infection in all maize cultivars studied and none of the cultivars tested were free from fumonisin contamination. Some cultivars (8 out of 15) tested in this study exhibited consistently moderate to high ear rot severity (mean 30.1 - 50.3 %) and fumonisin content (mean 29.2 - 58.4 mg kg^{-1}) across the two years of inoculation experiment. This may indicate that several high yielding maize cultivars widely grown in Ethiopia do not possess high level of resistance to *F. verticillioides* infection and fumonisin contamination. Particularly some of the improved (high yielding) maize cultivars including Gibe-1, Melkassa-5 and MH-130 displayed larger amount of fumonisin compared to the susceptible line (NSCM-411881(32)) in at least one of the years, indicating that these cultivars are susceptible to fumonisin accumulation. The observed broad range of variation among maize cultivars for resistance to *Fusarium* ear rot and fumonisin accumulation agree well with

previous studies by Small *et al.* (2012) in South Africa, Afolabi *et al.* (2007) in Nigeria and Clements *et al.* (2004) in US.

This study indicates that late-maturing maize cultivars are generally more susceptible to *Fuarium* ear rot and fumonisin contamination than early-maturing maize cultivars. This is in agreement with findings of Battilani *et al.* (2008) and Löffler *et al.* (2010) who also observed higher susceptibility to fumonisin accumulation in late-maturing maize cultivars compared to early types. This may be caused by a more slow reduction in grain moisture content in late cultivars as water availability plays an important role for fungal growth (Fandohan *et al.*, 2004). Furthermore, fumonisin contamination is cumulative, so delayed or extended period required to reach physiological maturity, can aggravate the problem of infected ears, even when some resistance to contamination is present in late maturing cultivars (Battilani *et al.*, 2008). The significant positive correlation between silk date and fumonisin levels indicates that maturity plays some role in resistance.

Under natural condition, fumonisin contamination of maize grains is believe to be influenced by phenotypic traits such as physical barriers to initial ear infection and further spread of the pathogen inside the ear. Morphological characters of the ear, such as husk covering and tightness (Butron *et al.,* 2006; Warfield and Davis, 1996), as well as silk channel length (Bolduan *et al.,* 2009) have been associated with ear rot resistance and fumonisin contamination. Cultivars with good husk cover characteristics and/or ear tips that are fully cover by husk leaves often have reduced insect damage. Reduced insect damage prevents fungal entry and consequently minimize ear rot disease and fumonisin contamination of maize grains (Munkvold, 2003; Parsons and Munkvold, 2012). While cultivars with bare tip and loose husk cover may harbor great number of insect pests, and are therefore more prone to ear rot and fumonisin contamination (Fandohan *et al.,* 2004; Mesterházy *et al.,* 2012). Physical properties of the grain pericarp such as intact kernel, pericarp thickness and wax content are associated with low fumonisin contamination (Sampietro *et al.,* 2009). Maize hybrids with flinty endosperm characteristics have been considered more resistant to *Fusarium* ear rot and fumonisin accumulation than hybrids with dent (softer) endosperm (Desjardins *et al.,* 2005). However, there are contradictory reports to this and Löffler *et al.*, (2010) reported that maize inbred lines with flint endosperm characteristics are more susceptible to fumonisin contamination than dent types. Preliminary observation in this study indicates that maize cultivars with larger spacing between rows of kernels in the cobs, and

cultivars with loose husk cover appears to create suitable condition for easy spread of the fungus down to the bottom parts of the inoculated cob.

In this study, although primary ears of all plants studied were injected with the same quantity of inoculum, the year of inoculation had a significance influence on disease severity and fumonisin concentration. In 2013 mean ear rot was 32.3 % and mean fumonisin concentration 33.2 mg kg^{-1}, while in 2014 mean ear rot was 21.8 % and mean fumonisin concentration 23.1 mg kg^{-1}. This could be due to variation in weather factors. The lower level of ear rot and fumonisin in 2014 may be caused by the fact that the weather conditions were not as favorable to the pathogen growth as in 2013. *Fusarium verticillioides* infection and fumonisin contamination may be limited under wetter and cooler condition around pollination (Cao *et al.,* 2014). As indicated in Fig. 2 temperature was consistently higher in 2013 than 2014 through the months of September to December, while precipitation was very low in 2013. This indicates the prevalence of stressful weather condition after inoculation in 2013 than in 2014 for the plant in the field. Environmental conditions that result in plant stress favors growth and pathogenicity of *F. verticillioides* (Oren *et al.,* 2003). Stress conditions such as high temperature and low precipitation (doughtiness) during pollination and grain-filling period have been associated with increased *F. verticillioides* infection and fumonisin contamination of maize grains (Cao *et al.,* 2014; Munkvold 2003; Parsons and Munkvold, 2012). This suggests that the interaction between the plant and environmental factors could be important in determining resistance to *Fusarium* ear rot and fumonisin contamination. Shelby *et al.,* (1994) who evaluated the response of maize hybrids to fumonisin accumulation across 17 different locations in the US, reported a significant hybrid by location interaction, in which hybrids grown outside their adaptation range were more susceptible to fumonisin accumulation and they detected an increase in fumonisin level as latitude of the location decreased. Differences in longitude, maturity class and length of the vegetative period (growing weeks) are among the key risk factors associated with fumonisin contamination in maize (Battilani *et al.,* 2008). Low adaptation of cultivars to physiological stress, may favor growth of the pathogen. Thus, evaluations aiming at obtaining resistant cultivars should be carryout over several years and over multiple environments to expose cultivars to a wide range of climatic conditions.

The relationship between ear rot severity and fumonisin concentration is documented in the scatter plots measured on individual field plots (Fig. 5 A and B). A trend of simultaneous

increase in ear rot severity and fumonisin content is noticed. The strong association between ear rot severity and fumonsin content suggests that selection for resistance to ear rot may eventually result in resistance to fumonisin contamination in maize grains. This is in agreement with previous studies that detected strong association between visible ear rot severity and fumonisin content (Bolduan *et al.,* 2009; Clements *et al.,* 2003; Robertson *et al.,* 2006). Therefore, visual rating of ear rot disease severity might be used as initial selection criteria to exclude cultivars accumulating high levels of fumonisin, because visual ear rot rating is easier, less expensive and large number of maize accessions can be considered for evaluation across different locations or years. However, significant amount of fumonisin may also be produced in symptomless or in kernels with minimal ear rot severity (Munkvold *et al.,* 1997). Thus, cultivars selected on the bases of ear rot rating should further evaluated for fumonisin accumulation analysis to avoid cultivars with minimal ear rot but greater fumonisin concentration.

In conclusion, the present study has shown a high level of variation in resistance to *Fusarium* ear rot and fumonisin accumulation among the Ethiopian maize cultivars tested. The results document the presence of certain sources of resistance to *Fusarium* ear rot and fumonisin contamination within the Ethiopian maize germplasms. The cultivars that display very low ear rot disease severity and fumonisin level may be useful for the development of resistant maize cultivars in the maize breeding program. Growing the most resistant cultivars may substantially reduce fumonisin contamination of maize grains in areas where ear rot disease is most prevalent. Since the materials included in the present study are limited, further extensive evaluations should continue by including locally adapted public landraces and hybrids currently grown in Ethiopia. This will increase the chances for selecting maize material with higher resistance levels. As artificial inoculation compromises the natural barriers to fungal infection and increased ear rot severity in maize cultivars, it may also be advisable to perform the initial selection process under natural infection in hot spot areas for the disease.

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Paper V

Biocontrol potential of native *Trichoderma* species against *Fusarium verticillioides* and fumonisin contamination in field-grown maize

Hadush Tsehaye [1,4,*], Anne Marte Tronsmo [1], Leif Sundheim [1,2], Arne Tronsmo [3]

[1] Norwegian University of Life Sciences, Department of Plant Sciences, P.O. Box 5003, NO-1432 Ås, Norway

[2] Norwegian Institute for Bioeconomy Research, Biotechnology and Plant Health Division, P.O. Box 115, NO-1431 Ås, Norway

[3] Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science, P.O. Box 5003, NO-1432 Ås, Norway

[4] Mekelle University, Department of Dryland Crop and Horticultural Sciences, P.O. Box 231, Mekelle, Tigray, Ethiopia

* Corresponding author: Hadush Tsehaye, email address: hadush.beyene@nmbu.no; had031@yahoo.com

Abstract

The biological control potential of native *Trichoderma* species was assessed against a *Fusarium verticillioides* under *in vitro* and field condition. The antagonistic *in vitro* potential was assessed in dual culture interactions. The most effective isolates under *in vitro* antagonism were selected, and evaluated over two years under field condition using seed bioprimed for their effectiveness in controlling *F. verticillioides* and fumonisin contamination of maize kernels. *Trichoderma* species showed a wide range of variation (8.8 - 53.9 %) in inhibiting the growth of the *F. verticillioides* isolate within 4 days after incubation. Ten days after incubation, 67 - 96 % reduction in mycelial growth territory of the pathogen was observed compared to the free growth in control plates. Maize seeds treated with *Trichoderma* isolates reduced *F. verticillioides* colonization of kernels by 38.3 - 86.4 % in 2013 and by 36.8 - 69.6 % in 2014 compared to plots that received *F. verticillioides* treatment alone. Reduction in fumonisin contamination level of 44 - 89.8 % in 2013 and 48.6 - 88.8 % in 2014 were observed in *Trichoderma*-treated plots compared to the control. An increase in plant growth and development was also recorded when seeds treated with spore suspension of *Trichoderma* isolates. Overall results of this study indicates that some *Trichoderma* strains have the potential to control *F. verticillioides* and fumonisin contamination of maize kernels.

Keywords: *Fusarium verticillioides, Trichoderma* species, fumonisin, maize

Introduction

Maize (*Zea mays* L.) is one of the most important staple crops in Ethiopia, ranking first in total grain production (7.2 million tons) and second in area coverage, > 2 million ha per year (FAOSTAT, 2014). *Fusarium verticillioides* is the most frequently encountered fungal pathogen of maize in Ethiopia (Ayalew, 2010 Wubet and Abate, 2004). This pathogen is a common soil and seedboren fungus, which causes a number of diseases including root rot, seedling blight, stalk rot, ear and kernel rots in maize (Bacon *et al.,* 2008; Munkvold 2003). *Fusarium verticillioides* is responsible for significant losses in both grain yield and grain quality (Presello *et al.,* 2008) and the pathogen can also produce a number of mycotoxins such as fumonisins, moniliformes, fusaric acid and fusarins (Bacon *et al.,* 2008; Darnetty and Salleh, 2013). Almost all of the strains are capable of producing fumonisins at different concentrations during the pre-harvest and postharvest periods (Bacon *et al.,* 2008). Contamination of grains by fumonisins is of great concern in food and feed safety, as fumonisin presents serious and harmful effects on the health of humans and certain domestic animal species. Consumption of fumonisin contaminated maize have been associated with leukoencephalomalacia in horses (Kellerman *et al.,* 1990), pulmonary oedema in pigs (Harrison *et al.,* 1990), oesophageal cancer (Rheeder *et al.,* 1992) and neural tube defects in humans (Missmer *et al.,* 2006).

Management of *F. verticillioides* is not easy, as the fungus can disseminate vertically through seed infection or from soil to roots and then to above ground parts of the plant (Bacon *et al.,* 2008). Infection can also occur by fungal propagules reaching the plant surfaces horizontally via insect vectors, rain splash and wind (Munkvold, 2003). Fungicide treatments are not sufficiently effective to control the pathogen (Bandyopadhyay *et al.,* 2003). The use of fungicides is also being discouraged due to the high cost for small-scale farmers as well as growing concern for environmental and safety issues. Management with host plant resistance is not yet possible, because there are no maize varieties with sufficient level of resistance to control this fungal pathogen. Biological control could be a potentially sustainable solution for managing soil and seedborne pathogens such as *F. verticillioides*. Biological control can easily integrate with good agricultural practices including maintaining optimal condition for crop growth and minimizing environmental stresses.

Some of the major biocontrol agents getting great attention due to multiple action to suppress fungal pathogens in various crops are the filamentous fungi in the genus *Trichoderma* (Howell, 2003). *Trichoderma* species have been widely used as biocontrol agents, because they are fast growing, producers of antifungal substances such as hydrolytic enzymes and antibiotics to suppress plant pathogenic fungi (Vinale *et al.,* 2008). These groups of fungi also have the ability to parasitize other fungi, as well as competing for space and nutrients with plant pathogens (Harman *et al.,* 2004; Howell, 2003). In addition, certain strains can induce systemic and localized resistance to several plant pathogens (Harman *et al.,* 2004; Vinale *et al.,* 2008), and they may enhance plant growth and development (Howell, 2003; Vinale *et al.,* 2004). *Trichoderma* species are cosmopolitans in nature free living in the soil and root ecosystem. *Trichoderma harizanum, T. viridae, T. hamatum,* and *T. koningii* are among the species that are most often used for biological control of fungal pathogens (Anees *et al.,* 2010; Akrami *et al.,* 2011; Dubey *et al.,* 2007; Hajieghrari *et al.,* 2008).

In biological pest control, in addition to selecting the appropriate isolates, the way to produce and deliver the biological control agent may influence the effectiveness under field condition. One of the popular and effective methods for introducing the biological control agent into a plant to control soilborne pathogens is seed treatment. Most of the previous works on antagonistic and biocontrol ability of *Trichoderma* species have been carryout under controlled environmental conditions, which may not exactly fit the complex field condition. An isolate that presents antagonism under *in vitro* may not be effective in field condition, thus testing in the actual field situation is necessary to select the most efficient strain. The first important condition for development of biological control strategy is the identification and deployment of highly effective strains. The potential of *Trichoderma* spp. to control *F. verticillioides* infection has not tested yet in maize in Ethiopia.

The first objectives of the present study were to assess the antagonistic potential of native *Trichoderma* species in suppressing the growth of a *F. verticillioides* pathogen under in vitro assays, and to select the most efficient strains for field-testing. The second objective of this study was to evaluate the effectiveness of selected *Trichoderma* isolates as biocontrol agents to reduce *F. verticillioides* colonization and fumonisin contamination of maize grains following seed biopriming under field conditions.

Material and methods

Isolation and identification of *Trichoderma* species

Trichoderma species were isolated from soil collected from the rhizosphere of growing maize plants and from post-harvest maize grains. For isolation of *Trichoderma* species from the rhizosphere of maize plants 21 soil samples were collected from different maize growing areas of Ethiopia during 2012 cropping season. Soil samples were brought to the laboratory on sterile polyethylene bags and air-dried at room temperature (23±2 °C) for 3 days. The samples were then mixed thoroughly, grinded using mortar and pistil, and sieved using 200 micron mesh-size. The soil dilution plate method was used to isolate the *Trichoderma* species; with 10 g from each of the ground soil sample were suspended in 100 ml sterile water and mixed well by stirring. Then from this stock soil suspension, 1 ml suspension was taken, serially diluted with sterile distilled water from 1:10 to $1:10^5$ dilutions. Finally, aliquots of 1 ml from each dilution were transferred to 90 mm petri plates containing *Trichoderma* selective medium (TSM) (Elad *et al.,* 1981). Plates were incubated at 23±2 °C under alternating 12 h of near ultraviolet/white light and darkness (12 h) for 7 days. The culture plates were examined daily, and individual colonies appearing on the plates were counted, then picked up and transferred onto a new potato dextrose agar (PDA, Difco, Madison, USA). Further purification was performed by the single spore isolation method (Leslie and Summerell 2006). Additional *Trichoderma* species were obtained from maize kernels in the process of isolation of *Fusarium* species.

Trichoderma species were identified by morphological characteristics using interactive identification keys provided by (Chaverri *et al.,* 2003; Gams and Bissett, 1998; http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm) and comparing with cultures of formerly identified strains. For this, single spore isolates were grown on PDA and SNA (Nirenberg, 1976) at 22 °C for about 7 to 10 days. Culture characteristics, such as growth rate and mycelia (pustules) distribution pattern, colour and changes in medium over time for each isolate were examined. The macroscopic and microscopic morphological characteristics (shape and size of conidia, shape and size of conidiophores, presence or absence of sterile hairs) were performed from slide mounts prepared by the tape touch method (Harris, 2000) in a drop of lactofuchsin.

Dual culture interaction

The *Trichoderma* isolates were evaluated against a *F. verticillioides* isolate following the dual culture method (Dubey *et al.,* 2007; Hajieghrari *et al.,* 2008) to select the most efficient antagonists. Plates (90 mm) containing PDA were inoculated with a 6 mm diameter mycelial disc from a 7 day old culture of *F. verticillioides* and *Trichoderma* species at equal distance (1 cm) from the periphery. Tree replicates of each treatment were made, and inoculated petri dish plats were incubated for 15 days at 22 ℃ under alternating 12 h of photoperiod. The radial growth of *F. verticillioides* isolate was measured 4 and 7 days after incubation. Ten days after incubation, plate area covered by free mycelial growth of the *F. verticillioides* isolate in dual culture with no visible overgrowing with the antagonistic *Trichoderma* isolates were measured to compute the reduction in territory occupied compared to the plate area covered on control plates. PDA plates inoculated with the *F. verticillioides* isolate without the antagonists were served as controls, and percent inhibition of radial growth of the *F. verticillioides* pathogen was calculated (Dennis and Webester, 1971). The growth inhibition was determined using the following equation:

$$Growth\ inhibition\ (\%) = \frac{(GC-GT)*100}{GC}.$$

In the equation, GC is radial growth measurement of the pathogen in control and GT is the mycelial growth of the pathogen from the point of inoculation in the direction of the antagonistic *Trichoderma* isolates.

The plates were examined for any possible antagonistic effect between the pathogen and *Trichoderma* isolates by measuring the inhibition zone formed between them after 4 days of pairing. Total number of days required to entirely cover the plate including overgrowing the *F. verticillioides* colony by *Trichoderma* isolates were recorded at the end of incubation period. Mycelial samples were taken from the interaction region of both fungi and the presence of mycelial coiling structures (hyperparasitism) were examined with a microscope as described by Dennis and Webester (1971). The coiling frequencies were estimated and categorized as: less, medium or high frequent.

Field experiment

Study site

The study was conducted at the experimental field site of Mekelle University main campus, northern Ethiopia. The site is located at the 13º 29' N latitude and 39º 30' E longitudes, and at an altitude of 2130 m above sea level. The soil type of the experimental site was Cambisols with silt-clay textural class and the area is characterized by semi-arid (limited moisture) climatic conditions (Araya *et al.*, 2011).

Experimental design and field management

Field experiment were conducted for two consecutive years during the rainy season of 2013 and 2014. The experimental design was randomized complete block design with thirteen treatments and three replications for each treatment. Each plot was 13.5 m^2, with 3 m length and 4.5 m width. Spacing was 0.75 m between rows and 0.25 m between plants. Spacing between blocks and between plots were 1.5 m and 1.0 m, respectively. Maize variety Melkassa-4 which is one of the recommended cultivars for the study area was used. All crop management techniques were according to recommended practices for maize production in the region. Thus, DAP (Di-ammonium phosphate) and urea fertilizer were applied at a rate of 100 kg per ha each (64 kg N and 46 kg P per hectare). Nitrogen (N) was applied three times: one third at sowing and the remaining, two times, 4 and 6 weeks after crop emergence. To provide adequate soil moisture to allow the plants to come to a complete development stage, supplemental irrigation was applied, once per week via furrow irrigation at the end of the growing season when the rain ceased.

Fungal inoculum preparation and seed treatment

The best performing isolates were selected based on *in vitro* antagonism determined against *F. verticillioides* isolate in the dual culture experiment. A total of 11 *Trichoderma* isolates belonging to *T. harzianum* (5 isolates), *T. hamatum* (5 isolates), and *T. viride* (1 isolate) were tested using seed biopriming under field condition. A *F. verticillioides* strain (SR6952) previously isolated from infected maize kernels collected in Ethiopia was used for the field experiment. This strain was selected for its potential to produce fumonisin, as it was one of

the highest fumonisin producing strains among the field-collected isolates tested on autoclaved maize cultures (Tsehaye *et al.,* unpublished).

For spore production, the *F. verticillioides* strain was grown on Mung Bean Agar (MBA) (Dill-Macky *et al.,* 2003), at 23±2 ℃ for 7 to 10 days under alternating 12 h of near ultraviolet/white light and darkness (12 h) (Leslie and Summerell, 2006), whereas *Trichoderma* species were grown on PDA in the same conditions. Spore suspension was prepared by washing conidia from the surface of the agar media with sterile distilled water, and the resulting propagule suspension was filtered through two layers of cheesecloth. Conidial concentration was determined using Kova glasstic spore counting chamber and adjusted to a concentration of 10^7 conidia ml^{-1} using sterile distilled water, amended with Tween 20 (polyoxyethylenesorbitan monolaurate; Sigma-Aldaich, St. Louis Missouri) a surfactant, at a rate of 0.2 ml $liter^{-1}$. Maize seeds were treated by submerging them in the conidial suspension of the *Trichoderma* species in Erlenmeyer flasks. Fifty grams of maize seeds were bioprimed using spore suspension of the different isolates, and after soaking overnight at room temperature seeds were removed from the spore suspension and used for planting immediately without air-drying. Conidial suspension of the *F. verticilloide*s pathogen was applied onto autoclaved maize seeds in separate flasks. Three autoclaved seeds colonized with the *F. verticilloides* strain and two viable maize seeds treated with the *Trichoderma* isolates were placed closely together per planting hill in the field in all cases. Seeds treated with a fungicide recommended for protection of seedborne and soilborne pathogens in Ethiopia, Apron Star 45 WS at the rate of 2.5 g kg^{-1}, was also included in the treatments. Other seeds soaked in Erlenmeyer flasks containing sterile distilled water without any biological control agent but planted together with *F. verticillioides*-colonized autoclaved seeds were used as fungal control.

Assessment of kernel infection level and collection of other agronomic data

On the 10^{th} day after sowing total number of emerged plants breaking the soil surface per plot was recorded to calculate percentage germination. The final plant stand was expressed as the percentage of emerged plants divided by the expected number based on the planted seeds. At 45 days after emergence, shoot height was recorded on 10 randomly selected plants per plot by measuring the distance from soil up to the base of last leaf with visible collar. Then seedling vigour index (VI) was calculated as: $VI = average\ shoot\ length\ (cm) *$

$germination\ percentage$ and the germination percentage of each plot was computed as: $Germination\ percentage = \frac{number\ of\ seedlings\ emerged * 100}{Total\ number\ of\ seeds\ planted}$.

When plants reached physiological maturity, all cobs were harvested per individual treatment and replica. Peeled cobs from each plot were collected together, and by manual threshing the grains were separated from the cobs. After threshing and drying to a moisture level of about 15 %, kernels per plot weighed separately to determine grain yield. Thousand seed weight was also determined for each treatment-replica. Samples of grains (200 seeds per plot) were taken randomly and used for assessment of fungal contamination level using the deep-freeze blotter method (ISTA, 2003). Initially seeds were disinfected in 70 % ethanol for 1 min followed by soaking in 3 % hydrogen peroxide solution for 2 min, then washed twice in distilled water. Then replicates of 25 seeds were plated in 18 cm diameter petri dish plats containing three layers of water soaked blotters. Plats containing the seeds were incubated at room temperature (23±2 °C) for 24 h and then 24 h in a freezer at - 20 °C. This was followed by incubation at 23±2 °C under 12 h alternative cycles of near ultraviolet/white light and darkness for 9 days. Infected and healthy seeds were counted to calculate proportion of seeds contaminated by fungal agents. Identification of fungal species was performed based on morphological structures according to Leslie and Summerell (2006).

Fumonisin analysis

For fumonisin analysis, 100 g maize grains from each plot were ground fine, using a laboratory mill (IKA®-Werke, GmbH & Co. KG., Germany), to pass through 1 mm mesh sieve. Fumonisin contamination in samples was determined using Enzyme Linked Immunosorbent Assay (ELISA) kits (RIDASCREEN®Fumonisin, R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions. The absorbance was measured, using a microplate reader at 450-nm, and fumonisin concentration of samples evaluated using RIDA®SOFT Win software (Art. Nr. Z9999, R-Biopharm AG, Darmstadt, Germany). The minimum detection limit of the kit was 0.025 ppm and all samples were analysed in duplicate.

Statistical Analysis

Data were analysed with analysis of variance (ANOVA) for complete randomized block design, using procedure of Statistical Analysis System (SAS) software (SAS institute, Cary, NC). Data on percentage of maize kernels colonized by fungal agents and fumonisin concentration were log-transformed before statistical analysis (Araṅkacāmi and Rangaswamy, 1995). Differences between treatments within years were determined with Fisher's protected least significance (LSD) test and a $p < 0.05$ significance level was used throughout.

Results

Isolation and identification of *Trichoderma* species

From a total of 21 soil samples, collected from different maize growing areas of Ethiopia, *Trichoderma* species were isolated from 9 samples. Among 200 maize kernel samples assessed, *Trichoderma* species were recovered from 7 samples. Totally, 18 *Trichoderma* isolates were recovered, 7 from maize kernels and 11 from soil samples. Based on culture characteristics and morphological criteria, the *Trichoderma* species belong to three different species, namely *T. harzianum* (7), *T. hamatum* (9) and *T. spirale* (2) (Table 1).

The antagonistic effect of *Trichoderma* strains against the mycelial growth of *F. verticillioides* in the *in vitro* test was as shown in Table 1. All the *Trichoderma* isolates tested inhibited the growth of the *F. verticilloides* isolate, but differed significantly ($p < 0.0001$) in the magnitude of suppressing the pathogen (Table 1). The inhibitory effects of the *Trichoderma* isolates against *F. verticilloides* were in the range of 8.8 - 53.9 % 4 days after pairing. The highest inhibitory effect on radial growth of the *F. verticillioides* pathogen was achieved by *T. harzianum* isolates Thr5 (53.3 %) and Thr6 (53.9 %). The lowest radial growth inhibitory effect of the *F. verticillioides* pathogen was observed using the Tsp1 isolate (8.8 %) 4 days after incubation. *Trichoderma harzianum* isolates were growing faster than others *Trichoderma* species, while the least reduction in radial growth of the pathogen was obtained in *T. spirale* isolates (Table 1).

Distinct inhibition zones was noticed before contact, when the different *Trichoderma* isolates grown in dual culture with the *F. verticillioides* isolate (Table 1). *Trichoderma spirale* isolates (Tsp1 and Tsp2), and *T. hamatum* isolates (Thm8 and Thm9) gave the least inhibition zone (< 7 mm) compared to others (Table 1). At the end of the incubation period a clear zone of inhibition without or with little physical contact between the fungal colonies were observed in *T. hamatum* isolate Thm1 and *T. harzianum* isolate Thr7 (Fig. 1 F) compared to others.

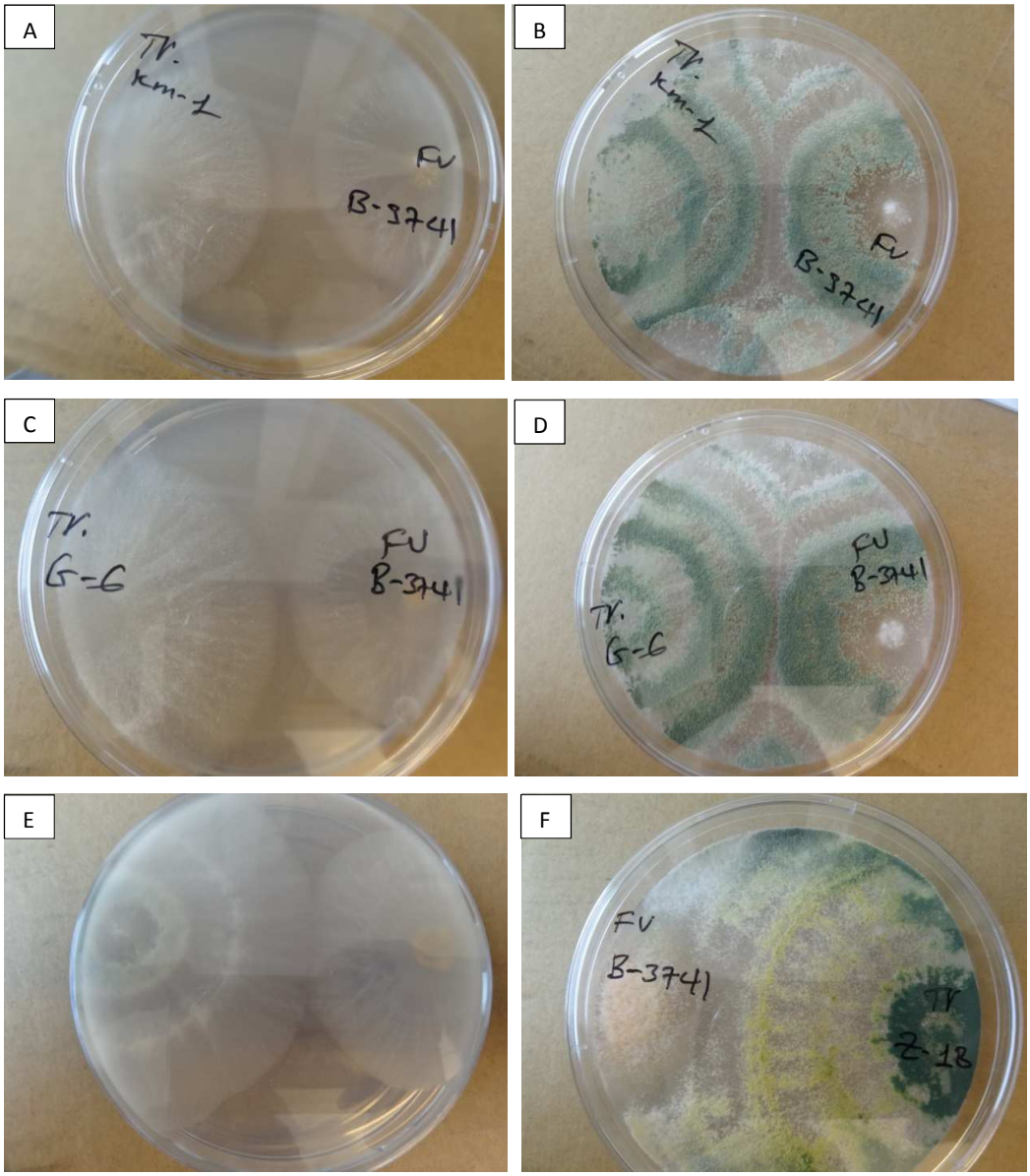

Fig. 1 Dual culture interaction of *Trichoderma* species with a *F. verticillioides* isolate. *Trichoderma* isolates retard the growth of the pathogen 4 days after pairing (A, C & E), and *Trichoderma* isolates growing over the pathogen 7 to 10 days after incubation at 22 °C (B & D), but not crossing the inhibition zone or little hyphal interaction (F).

Table 1 Antagonistic potential of *Trichoderma* species, isolated from soil samples in maize rhizosphere and from maize kernels collected from different growing areas of Ethiopia, against *F. verticillioides* pathogen

Trichoderma isolates [a]	District of origin	Source material	Growth inhibition (%)[b]		Reduction in plate coverage (%), 10 days [c]	[d] Zone of inhibition	Number of days to fully cover plate [e]	[f] Mycelial coiling frequency
			4 Days	7 Days				
T. hamatum (Thm1)	Gibe	Soil	35.43de	55.56e	77.78f	++	> 15	-
T. hamatum (Thm2)	Gibe	Soil	49.47c	65.28c	92.22c	+++	12	High
T. hamatum (Thm3)	Halaba	Soil	50.67b	73.61b	92.23c	+++	12	Very high
T. hamatum (Thm4)	Mekelle	Soil	34.53f	51.37gh	73.34gh	++	15	Medium
T. hamatum (Thm5)	Jimma	Seed	36.87d	65.59c	93.34bc	++	13	High
T. hamatum (Thm6)	Kemissie	Soil	50.10c	73.59b	94.45ab	+++	12	Very high
T. hamatum (Thm7)	Kemissie	Soil	29.93f	52.77fg	74.44g	++	15	Medium
T. hamatum (Thm8)	Kobo	Soil	22.93g	58.31d	83.32d	+	>15	Medium
T. hamatum (Thm9)	Korem	Soil	9.43h	35.67j	66.29j	-	>15	Low
T. harzianum (Thr1)	Bako	Seed	52.07b	76.11a	95.45a	++	11	Very high
T. harzianum (Thr2)	Bedele	Seed	50.97bc	77.74a	96.12a	+++	11	High
T. harzianum (Thr3)	Bedele	Seed	31.03f	48.61i	71.12i	++	13	Medium
T. harzianum (Thr4)	Jimma	Soil	30.20g	49.98hi	72.22hi	++	13	Medium
T. harzianum (Thr5)	Nekemte	Seed	53.30a	76.48a	96.11a	+++	10	Very high
T. harzianum (Thr6)	Sire	Seed	53.93a	77.78a	96.11a	+++	10	Very high
T. harzianum (Thr7)	Ziway	Soil	36.17d	57.37d	80.00e	++	12	-
T. spirale (Tsp1)	Axum	Soil	8.80h	34.45j	66.67j	-	>15	Low
T. spirale (Tsp2)	Melkassa	Seed	22.78g	54.16ef	77.76f	+	>15	Medium

[a] Thm: *T. hamatum*, Thr: *T. harzianum*, Tsp: *T. spirale*; [b] inhibition in radial growth of the pathogen; [c] Reduction in mycelial growth territory (plate coverage) of the pathogen in dual culture compared to mycelial area coverage in control (considering free mycelial coverage of pathogen with no visible overgrowth with the antagonistic *Trichoderma* isolates); [d] After 4 days – no zone of inhibition, + zone of inhibition 1-6 mm diameter, ++ zone of inhibition 7-12 mm diameter, +++ zone of inhibition 13-21 mm diameter; [e] Number of days taken for the *Trichoderma* species to over grow *F. verticillioides* colony and fully cover the plates; [f] – no hyphal coiling observed. All values are averages of three determinations; within columns, means followed by the same letter are not statistically different according to LSD (0.05).

The culture interaction showed that several *Trichoderma* isolates grows very fast, and clearly retard the growth of the pathogen after four days of pairing. After contact, most antagonist *Trichoderma* isolates grow on the top of the pathogen and mycelial growth of the *F. verticillioides* isolate was limited to a very small portion of the plate surface in the dual culture 10 days after incubation. Ten days after incubation, the highest reduction in free mycelial growth territory of the *F. verticillioides* pathogen was achieved by *T. harzianum* isolates Thr5 and Thr6 (96 % each) followed by *T. hamatum* isolates (Thm3 = 94.4 % and Thm6 = 94.8 %) compared to the control.

Most *Trichoderma* isolates tested in this study exhibited coiling structures around the hyphae of the *F. verticillioides* pathogen, which indicates the employment of hyperparasitisim for controlling growth of the pathogen. Variations in coiling frequency was also identified between *Trichoderma* species isolates. These variations were not only between species but also across isolates of the same species (Table 1). Some of the *Trichoderma* isolates (Thm1 and Thr7) displayed bigger gap, not cross the zone of inhibition, up to 10 days of pairing and no hyphal interaction in these isolates were observed (Table 1).

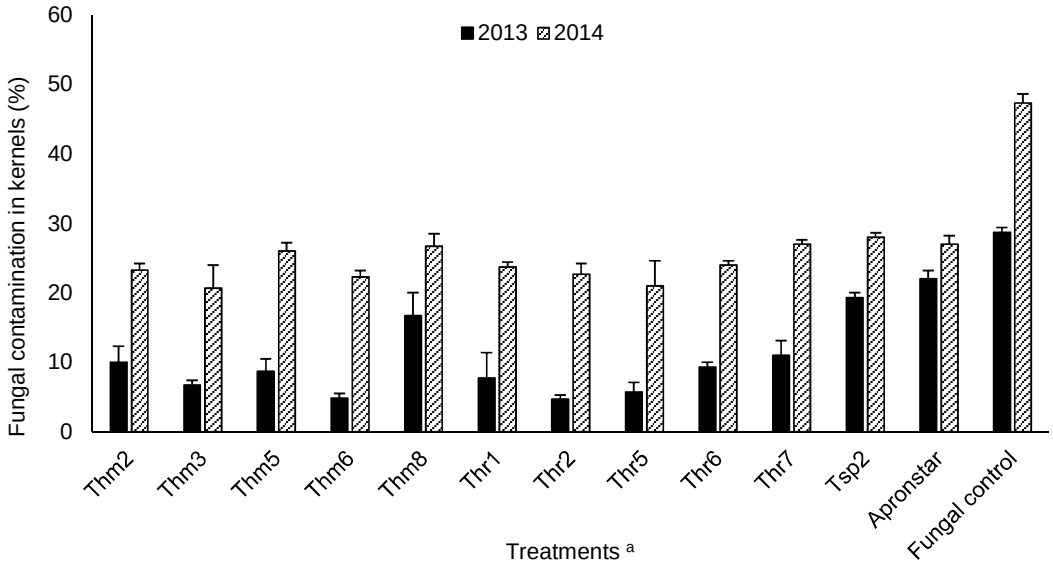

Fig. 2 Effect of seed-treatment with different *Trichoderma* isolates on total fungal (*Fusarium* and *Aspergillus* spp.) contamination of maize kernels under field condition, during 2013 and 2014, [a]=Thm: *T. hamatum*, Thr: *T. harzianum*, Tsp: *T. spirale*, Fungal control: seeds only received *F. verticillioides* treatment but with no biocontrol agent, Apron Star: fungicide recommended for seed treatment.

A significantly ($p < 0.001$) higher mean fungal contamination of kernels was observed in 2014 (26 %) than in 2013 (12 %). However, *Fusarium* spp. count on maize kernels did not significantly differ between years. The incidence of other fungal contaminants such as *Aspergllus* spp. were more abundant in 2014 than in 2013. Differences among treatments were significant for percentage of kernels contaminated with fungal agents assessed after crop harvest. *Trichoderma*-treatment reduced the proportion of maize kernels contaminated with fungi by 32.8 - 83.6 % in 2013 and 40.8 - 56.2 % in 2014 compared to plots that received *F.verticillioides* treatment alone (Fig. 2).

When maize seeds were treated with *Trichoderma* isolates, *F. verticillioides* colonization of kernels was reduced by 38.3 - 86.4 % in 2013 and by 36.8 - 69.6 % in 2014 (Fig. 3). The fungicide (Apron Star 45 WS) used in this study also reduced *F. verticillioides* colonization of kernels by 64.2 % in 2013 and by 52.6 % in 2014 compared to the untreated control (fungal control) (Fig. 3).

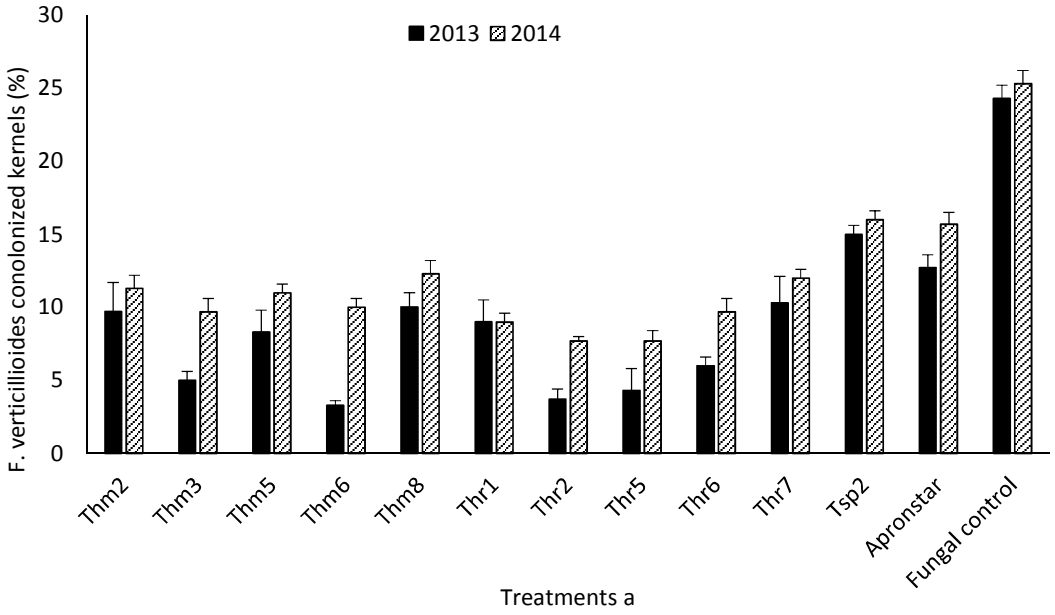

Fig. 3 Effect of seed-treatments with different *Trichoderma* isolates on *F. verticillioides* colonization of maize kernels, during 2013 and 2014, [a]=Thm: *T. hamatum*, Thr: *T. harzianum*, Tsp: *T. spirale*, Fungal control: seeds only received *F. verticillioides* treatment but with no biocontrol agent, Apron Star: fungicide used for seed treatment (positive control).

A significantly higher total fumonisin content was obtained in maize grains grown from seeds treated with fungal control (*F. verticillioides* alone) compared with *Trichoderma*-treated and

Apron Star-fungicide-treated plots (Fig. 4). Seeds treated with *Trichoderma* species reduced fumonisin contamination by 44 - 89.8 % in 2013 and by 48.6 - 88.8 % in 2014. The highest reductions of fumonisin content in maize kernels were obtained with seed treatment using *T. harzianum* isolate Thr2 (mean 89 %) followed by isolate Thr5 (87.4 %). The fungicide, Apron Star, also reduced fumonisin contamination by 38.5 % in 2013 and by 57.3 % in 2014 compared to plots received *F. verticillioides*-treatment alone (fungal control) (Fig. 4).

The *Trichoderma* strains tested in this study differed in their overall performance with respect to all the parameters measured. The efficacy of *T. hamatum* isolates (Thm3, Thm6) and *T. harzianum* (Thr2, Thr5) were better than others investigated in this study. A slight difference was also noticed in efficacy performance ranking of *Tichoderma* isolates under *in vitro* and field conditions (Table 1, Fig. 3 and Fig. 4).

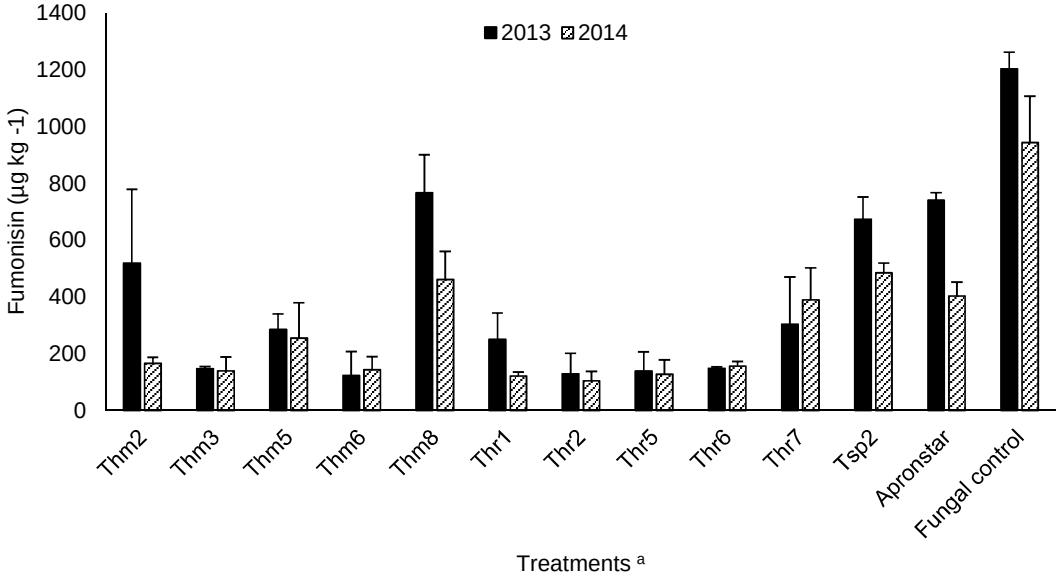

Fig. 4 Effect of seed-treatment with *Trichoderma* species on fumonisin contamination of field grown maize, during 2013 and 2014, [a]=Thm: *T. hamatum,* Thr: *T. harzianum,* Tsp: *T. spirale,* fungal control: seeds only received *F. verticillioides* treatment but with no biocontrol agent, Apron Star: fungicide used for seed treatment (positive control).

Effect of seed treatment with *Trichoderma* species on agronomic performance of maize

The different seed treatments showed a significant ($p < 0.05$) difference in percentage of seed emergence assessed 10 days after sowing, and shoot height and seedling vigour index determined at 45 days after emergence. An increase in speed of germination, seedling growth

(shoot height) and seedling vigour index resulted when seeds were treated with *Trichoderma* species compared with Apron Star (fungicide) treated and with fungal control (only *F. verticillioides*-treated seeds) during the two years of field trial (Table 2). An increase in speed of seed germination (percentage of seedlings emerging at 10 days after sowing) by 16 - 17 % in 2013 and 12 - 17 % in 2014 was observed when seeds were treated with different *Trichoderma* species. Seedlings produced from *Trichoderma*-treated seeds had also greater growth (shoot length) and seedling vigour index than did seedlings from Apron Star fungicide treated and only *F. verticillioides*-treated seeds (Table 2). In *Trichoderma*-treated seeds, a significant increase was also observed for each of the other agronomic parameters measured (plant height, 1000 seed weight and grain yield), compared to plots which received *F. verticillioides*-treatment alone. In maize plants grown from *Trichoderma*-treated seeds, increments of in plant height (4.5 - 7.2 %), seed weight (5 - 14 %) and grain yield (10.5 - 23.7 %) were measured, compared to the untreated control.

Table 2 Effect of *Trichoderma* species on agronomic performance of field grown maize, in Northern Ethiopia, during 2013 and 2014

Treatments [a]	Germination (%)	Shoot height (cm)	seedling vigour index	Plant height (cm)	1000 seed weight (g)	Grain yield (tons ha^{-1})
Thm2	91.0e	25.0cde	2304ef	164abc	359.5e	4.4bc
Thm3	95.8abc	26.3abc	2526bcd	163abcd	364.8cde	4.6a
Thm5	93.3cde	26.0bcd	2424cde	162abcd	365.0cd	4.3c
Thm6	97.2a	28.0ab	2729.5a	164.5abc	368.8bc	4.7a
Thm8	91.8de	23.8def	2183fg	160.8cd	359.2e	4.4bc
Thr1	94.5abcd	27.8ab	2621abc	161.2bcd	375.8a	4.6ab
Thr2	97.3a	28.3a	2762a	165.3a	370.7abc	4.6ab
Thr5	96.5ab	28.5a	2749a	164.8ab	372.5ab	4.6a
Thr6	94.0bcde	28.3a	2660ab	162.7abcd	369.8abc	4.7a
Thr7	92.3de	25.8bcd	2386def	161.3bcd	359.7de	4.3c
Tsp2	86.5f	23.5ef	2031g	161.2bcd	346.2c	4.2c
Apron Star	87.5f	23.8def	2210fg	160d	342.3f	4.2c
Fungal control	83.0g	22.0f	1823h	154.3e	336.0g	3.8d
Mean	92.4	25.9	2414	161.9	360.08	4.4
LSD	3.08	2.17	202.4	4.4	6.2	2.1
CV (%)	2.6	7.3	7.3	2.1	1.5	4.2
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

[a] Thm: *T. hamatum*, Thr: *T. harzianum*, Tsp: *T. spirale*, LSD: Least Significant Difference, CV: Coefficient of Variation. Within columns, means followed by the same letter are not statistically different according to LSD (0.05).

Discussion

The biocontrol potential of different *Trichoderma* isolates was evaluated against *F. verticillioides* under *in vitro* and in field condition in Mekelle, Northern Ethiopia. The results of initial screening in dual culture interactions revealed that *Trichoderma* spp. isolates have the potential to restrict the growth of the *F. verticillioides* pathogen. Reduction in growth of the target fungal pathogen by the antagonists under *in vitro* condition is the first important information necessary in the selection process. The different *Trichoderma* spp. isolates demonstrated variable *in vitro* antagonistic activity, and some isolates were able to completely control the pathogen by growing fast, overgrow upon contact and diminished territory occupied by the pathogen after 10 days of pairing, while others display little inhibitory activity compared to others. Different antagonistic mechanisms were evident for different strains. Majority of the *Trichoderma* isolates tested in this study displayed hyphal coiling with variable frequency indicated that hyperparastisim played a very important role in the biocontrol mechanism to reduce the growth of the *F. verticillioides* pathogen. The inhibition zone detected before contact clearly showed that chemical compounds with capability to reduce the growth of the pathogen were being produced by the *Trichoderma* isolates.

During the field experiments, a reduction of *F. verticillioides* colonization of maize kernels by 66.5 % in 2013 and 54.6 % in 2014 as well as fumonisin contamination by 83 % in 2013 and 82 % in 2014 was observed in plants from seeds treated with the different *Trichoderma* species. These results demonstrate that the *Trichoderma* isolates tested in this study could provide control of *F. verticillioides,* which is the most common fungal pathogen of maize in Ethiopia. Results of this study are similar to previous studies in other countries, where *Trichoderma* species was used successfully to control fungal pathogens (Anees *et al.,* 2010; Akrami *et al.,* 2011; Dubey *et al.,* 2007; Hajieghrari *et al.,* 2008; Rojo *et al.,* 2007). In field experiments conducted for three years, Ferrigo *et al.,* (2014) has also observed a reduction of 58 % in fungal infection and 53 % in fumonisin contamination in maize kernels. Ŝrobárová and Eged (2005) demonstrated a significant reduction in maize root rot disease severity caused by *F. verticillioides* using an antagonistic *Trichoderma* spp.

In the present study, significant differences were noticed between *Trichoderma* isolates in suppressing *F. verticillioides* and reducing fumonisin contamination. *Trichoderma harzianum* strains (Thr5, Thr6) and *T. hamatum* strains (Thm3, Thm6) were the most efficient isolates.

Thus, these *Trichoderma* strains may be used as broad-spectrum biological control agents. Chandra Nayaka *et al.,* (2010) have also reported certain *Trichoderma* spp. strains that are highly effective in reducing *F. verticillioides* infection and fumonisin contamination in maize. Possible mechanisms of antagonism employed by *Trichoderma* species against plant pathogenic fungi includes ability to compete for nutrients and space, mycoparasitism, antibiosis or production of volatile and non-volatile compounds which have inhibitory property against various pathogens, and stimulation of plant defences (Harman *et al.,* 2004; Howell 2003; Vinale *et al.*, 2008). *Trichoderma* species secrete cell wall degrading enzymes such as cellulases, chitinases and β-1, 3 glucanases that hydrolyze the cell wall integrity of the pathogen (Howell 2003; Vinale *et al.,* 2008). Synergism between different modes of action may occur in nature for the biocontrol of fungal pathogens, and efficient biological control agents should express more than one mode of action for suppressing the pathogen. A good biocontrol agent should proliferate in the rhizoplane and rhizosphere so that it can protect the seed as well as the seedling (Harman *et al.,* 2004).

The colonization of maize kernels by fungal contaminants differed by year, in which more fungal infection was recorded in 2014 than in 2013. This was because of the high prevalence of *Aspergillus* spp in 2014 and this could be due to variation in weather factors between the two years. The amount of rainfall was lower, while temperature was higher in 2014 than in 2013 in the study area after silking. Plants were also damaged by snowfall in 2014, and the combination of these stress conditions could render plants to become susceptible to higher fungal contamination. A number of abiotic (soil temperature, soil pH and water potential) and biotic (microbial activity of the soil) environmental factors may also have an influence on the biocontrol efficacy of *Trichoderma* isolates. *Trichoderma* spp. are most favoured under acidic condition and most strains are mesophilic (Kredics *et al.*, 2003). When temperature is higher, the activity of antagonistic bacteria might be higher (Ahmad and Baker, 1987). Therefore, further evaluation for biological control potential of *Trichoderma* species may give good results, if screening has been performed under different environmental conditions.

Results of the present study shows that seed treatment with *Trichoderma* spore suspension have various positive effects on the maize plant growth including increasing speed of germination and seedling vigour index. *Trichoderma* strains also significantly increased seed weight and grain yield, which is an additional advantage, compared to other pest management options such as pesticides that frequently causes phytotoxicity. The observed high and

uniform percentage of seed germination as well as increased in seedling vigour may contribute positively for increased grain yield. In addition to their potential of biocontrol activities, *Trichoderma* species have been observed to promote plant growth and grain yield (Dubey *et al.,* 2007; Entesari *et al.,* 2013). Several explanations have been suggested for the improved plant growth and yield including enhanced root growth and development, enhancing resistance to biotic stress, solubility of insoluble plant nutrients and improving nutrients uptake (Entesari *et al.,* 2013; Mastouri *et al.,* 2010). Many biocontrol agents can compete for infection sites on the root and trigger plant defence reactions, inducing systemic resistance. Interaction of *Trichoderma* strains with plant roots also results in controlled activation of carbohydrate metabolism and enhanced photosynthesis, providing the plant with more energy and carbon source for their growth (Shoresh and Harman 2008). In maize the plant growth promotion effect is genotype specific, as some inbred lines have been observed to respond negatively to different *Trichoderma* strains (Vinale *et al.,* 2008).

In many cases, *Trichoderma*-treated plots have shown better performance than the fungicide treated. This could be because the fungicide is effective for short time, and it may not spread through the soil and plant system like that of the biological antagonists. This is similar to previous observations that have reported higher efficacy and longer persistence of *Trichoderma* species than fungicides against soilborne fungal pathogens (Chandra Nayaka *et al.,* 2010). Since public awareness is increasing regarding the potential environmental and health impact of agrochemicals, the use of biological control agents for disease management is likely to increase in the future. Because, use of *Trichoderma* products could also create opportunities for farmers to reduce costs in addition to reducing risks related to health and environmental damage due to over usage of fungicides.

In conclusion, results of the present study demonstrated that seed treatment with *Trichoderma* isolates have a potential in reducing *F. verticillioides* colonization and fumonisin contamination of maize kernels. Maize grains obtained from plants treated with the *F. verticillioides* pathogen alone with no antagonists displayed higher levels of fungal infection and higher fumonisin content. The results of the present study also indicates that *Tichoderma* isolates enhances plant growth parameters and grain yield. The use of *Trichoderma*-based products is not only safe for the farmers and consumers, but also it is good for the environment. However, much further studies need to be conducted to find techniques for

mass multiplication, to achieve appropriate formulations and delivery systems of the biocontrol agents.

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