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Fungal Diversity of Maize (*Zea Mays* L.) Grains

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Abstract. Maize is becoming more and more important crop for dairy farming as forage and as substrate for biogas production. The mycotoxin producing fungi can spoil feed, reduce cattle productivity and cause health problems. The aim of this research was to study the mycoflora of maize grains in order to clarify the fungal composition and verify the presence of potential mycotoxin producing fungi. The grain samples were collected from different maize hybrid performance trial in Research and Study farm "Vecauce" of Latvia University of Agriculture in 2014. The fungi from 14 genera were isolated from surface sterilized grains. The most abundant were *Alternaria*, *Fusarium* and *Penicillium* spp. Mycotoxin producing fungi are present in maize grain mycoflora, and there is a risk that maize production can contain mycotoxins.
Key words: mycoflora, mycotoxins, *Fusarium*, ear rot, kernels.

Introduction

In Latvia, maize is important source for forage, mostly silage, some farmers are successful to harvest maize for grain despite the temperate climate. Significant part of maize production is used as substrate for biogas. According to data of Central Statistical Bureau of Latvia, the total area of maize in Latvia has increased rapidly during the last decade from less than 5 thousand to 20 thousand hectares. Maize is potentially high quality forage, but mycotoxin producing fungi can spoil the production. Mycotoxin contamination in feed can cause serious health and productivity problems to livestock, which can lead to significant losses for farmers. Also, mycotoxin residues from contaminated feed may appear in milk (Bennett & Klich, 2003). Mycotoxins were produced by different fungi, including causal agents of ear rot.

In Europe, ear rot is most economically important maize disease, it can be caused by different fungi from *Aspergillus*, *Penicillium*, *Diplodia*, *Nigrospora*, *Botryosphaeria*, *Cladosporium*, *Rhizoctonia* and other genera (Medić-Pap, Maširević, & Šofhauzer, 2011). However, the most important and harmful pathogens belong to *Fusarium* spp. (Czembor, Stępień, & Waśkiewicz, 2014). There is inconsequence in the systematic of genus *Fusarium*, in some cases names of anamorph, but in other names of teleomorph were used. In this study names were used accordingly to Index Fungorum (http://www.indexfungorum.org), but some important synonyms also were mentioned.

Gibberella zeae (*F. graminearum*) have been found common for maize and small grain cereals. In Latvia, *G. zeae* has been found by Treikale, Priekule, Javoisha, & Lazareva in 2010 in wheat, and it can become a serious problem when maize is grown in crop rotation with wheat (Asran & Buchenauer, 2003).

In Latvia, fungal mycoflora of maize grain or ears has not been studied before. The aim of this research was to study the mycoflora of maize grains in order to clarify the fungal composition and verify the presence of potential mycotoxin producing fungi under climatic conditions and agricultural technologies of Latvia.

Materials and Methods

Grain samples were collected in 2014 from different maize hybrid performance comparison trial in Research and Study farm "Vecauce" of Latvia University of Agriculture (latitude: N 56°28′, longitude: E 22°53′). In this trial, 26 different maize hybrids were arranged in randomized blocks with 4 replications, plot size 16.8 m^2 (4 rows, 70 cm apart). Soil type in trial site was *Calcaric Luvic Epigleyic Phazeozem* (pH KCl - 6.9, P_2O_5 - 583 mg kg^{-1}, K_2O - 219 mg kg^{-1}, organic matter - 3.0%). Pre-crops in trial site were maize in 2013, sunflower in 2012 and maize in 2011 and 2010. Conventional soil tillage was used, crop residues were

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very well incorporated. Seeds were drilled on 6th May, seed density - 83000 seeds ha^{-1}. The fungicides were not applied in this trial. Maize was harvested for silage on 6th October, average dry matter content was 33.5% and average yield 18.51 t ha^{-1}.

From each plot 10 cobs were collected, from each cob 5 grains without visible fungal contamination were sampled. Surface of grains was sterilized with 1% sodium hypochlorite for one minute, rinsed three times in sterile distilled water. Grains placed onto petri dishes with streptomycin and penicillin enriched potato-dextrose agar (PDA). Plates were incubated at +20 °C for 7–10 days, subsequently, the obtained fungi were transferred to a new PDA or a specific medium. Fungi were identified either directly on the isolation plates (by the color and texture of mycelium and pigmentation of medium) or microscopically (by morphological characteristics of the spores and spore-bearing structures). Obtained isolates were divided in morphologically similar groups and examples of each group were sequenced to confirm identification, all together 40 samples were sequenced.

The results of morphological identification were confirmed by sequencing of the ribosomal RNA gene Internal transcribed spacer (ITS) region and subsequent BLAST analysis of acquired DNA sequences against NCBI nucleotide database. The DNA extraction from fungal isolates was carried out by employment of NucleoMag® 96 Plant kit according to manufacturer's instructions (Macherey-Nagel, Germany). Prior extractions ~10µg of fungal material were homogenized for 2×60 sec. using FastPrep®-24 instrument and Lysing Matrix D (MP Biomedicals, USA) and supernatant phase of acquired lysate underwent phenol and chloroform treatment.

The amplification of ITS region by PCR was carried out for 40 cycles (95 °C-30 sec, 57 °C-30 sec, 72 °C-30 sec, GeneAmp PCR System 9700 (Applied Biosystems, USA) in total volume of 12 µl. The reaction mixture was comprised of following components: 0.1 u of HOT FIREPol® DNA Polymerase (Solis Biodyne, Estonia), 1×BD reaction buffer (Solis Biodyne, Estonia), 2.5 mM Mg2Cl (Solis Biodyne, Estonia), 0.2 mM dNTP each (Thermo Fisher Scientific, USA), 0.3 µM ITS4 (5'-TCCTCCGCTTATTGATATGC-3') - primer, 0.3 µM ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') primer (White et al, 1990) and 1µl of fungal DNA.

The success of the ITS region amplification was verified through the inspection of PCR products by agarose gel electrophoresis.

Positive reaction mixtures were cleaned up from excess of dNTPs and primers through employment of Exonuclease I (0.5 µl) (Thermo Fisher Scientific, USA) and Shrimp Alkaline Phosphatase (2 µl) (Thermo Fisher Scientific, USA) (incubated for 40 min at 37 °C and inactivated at 95 °C for 20 min). Further, 1µl of cleaned up fragment solution was transferred to BigDye® Terminator v3.1 Cycle Sequencing reaction mixture which was prepared according to the manufacturer's instructions (Applied Biosystems, USA). Both DNA strands of every PCR product were sequenced using ITS4 and ITS5 as primers and sequencing products were analyzed on 3130xl Genetic Analyzer (Applied Biosystems, USA).

All molecular biology related activities were carried out at "Genome center"– a genetic analysis core facility of Latvian Biomedical Research and Study center.

The Relative Density (RD) of fungal species and genera were calculated according to the method suggested by Broggi *et al*., 2007 and Tadych *et al*., 2012.

$$RD(\%) = \frac{ni}{Ni} \times 100,$$

ni – the number of genus or species isolated,
Ni – the total number of isolates.

Results

In total 2196 isolates were obtained. More than 50 morphologically different forms of fungi were isolated. In total 14 fungal genera were found.

The most abundant was fungi from genus *Alternaria*, the RD for this genus was 38% (Figure 1).

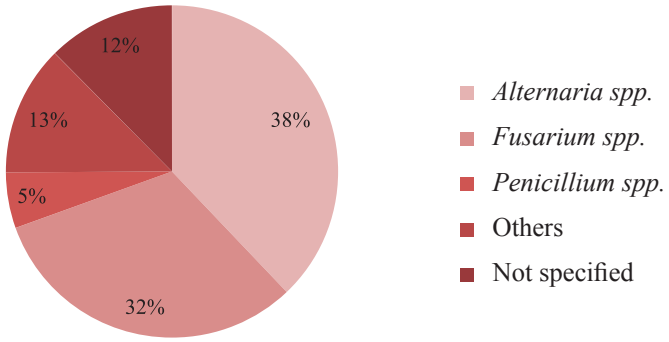

Figure 1. Proportion of fungal species in the mycoflora of maize grain.
(Others – fungal spieces with RD≤3%; Not specified – unidentified isolates)

The RD for second most widespread fungal genus *Fusarium* was 32%. Relative density for genus *Penicillium* was much lower - 5%. RD for all other 11 fungal genera together was almost 13%. In this group RD was from 0.05% up to 3%. But 12% (273 isolates) haven't been identified yet. The RD of endophytic fungal genera *Sarocladium* and *Trichoderma* was 3% and 2%, respectively. The RD for *Microdochium bolleyi* was 2%. RD for fungi from *Epicoccum* spp. was 1.5%. The RD 1% and below was for *Bipolaris* spp., *Chaetomium* spp., *Cladosporium* spp., *Sclerotinia* spp., *Nigrospora* spp., *Trichothecium* spp., *Lecanicillium* spp.

Five species of *Fusarium* were identified. In this study, the majority (38%) of all *Fusarium* isolates was *G. zeae* (Figure 2).

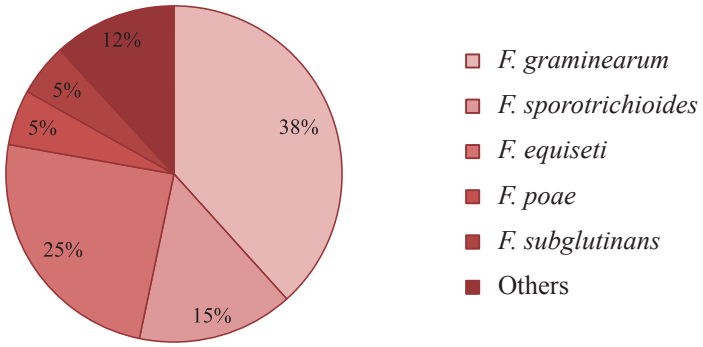

Figure 2. Proportion of *Fusarium* spp. Isolates.

The second most widespread fungi was *Gibberella intricans* (*F. equiseti*), 25% of *Fusarium* isolates belonged to this genus. Smaller share in proportion was for *F. sporotrichioides*, *F. poae* and *Gibberella fujikuroi* (*F. subglutinans*).

Discussion

Four major mycotoxin producing genera associated with maize are *Alternaria*, *Fusarium*, *Penicillium* and *Aspergillus* (Mansfield, 2005).

Fusarium spp. are the most frequently occurring fungi in temperate climates. Pathogens from *Fusarium* spp. are common for maize and small grain cereals. In Latvia, the maize is often grown in crop rotation with small grain cereals. Therefore, there is a risk that infection level of *Fusarium* spp. can become high in maize and in the whole crop rotation (Asran & Buchenauer, 2003).

In maize grains a variety of *Fusarium* species is even greater than in cereals. In this current study the relative density for five different species of *Fusarium* was higher than 5%. The most abundant was fungi from species *G. zeae* (RD 38%). These fungi have been found on wheat in Latvia, too. In studies carried out in Czech Republic, Switzerland and Italy the *G. zeae* wasn't so abundant. In Czech Republic and Switzerland (silage), 19.8% and 16% respectively of isolated *Fusarium* spp. was *G. zeae*. (Eckard et al., 2011; Nedělník, Lindušková, & Kmoch, 2012).

The second most widespread fungi in this study *G. intricans* (25%) is not between most frequently isolated fungi in other studies. For instance, in the study carried out in Italy 8% of all *Fusarium* isolates were F. *equiseti* (Logrieco *et al.*, 1995). *F. equiseti* was between less widespread fungi in the study carried out in Switzerland, but in Czech Republic it was not reported at all. (Vogelgsang, 2011; Nedělník, Lindušková, & Kmoch, 2012).

In this study, RD for F. *sporotrichioides* was 15%, which is much more than reported in Czech Republic on maize grains (1.3%). The *F. sporotrichioides* has been reported to be more widespread on maize silages. (Cheli, Campagnoli, & Dell'Orto, 2013) For example, in Switzerland where *Fusarium* fungi were isolated from silage, 16% were *F. sporotrichioides*. (Wettstein, Forrer, & Vogelgsang, 2011).

Relative density in this study for *F. poae* was 5%, and it corresponds to results in other studies. In the study carried out in Czech Republic, it was 9.3%. In the study carried out in Switzerland, 4.3% of isolated fungi from silage were *F. poae*.

RD in this study for *G. fujikuroi* was the same as for *F. poae* - 5%, but in the study carried out in Czech Republic *G. fujikuroi* were the most abundant fungi (40.4%) (Nedělník, Lindušková & Kmoch, 2012). In the study carried out in Italy, the share of *G. fujikuroi* was from 6 to 27% depending on a year and farming system. (Lazzaro, *et al.*, 2015).

It is interesting to note that one of the dominant species in other studies *F. verticillioides* was not found in this study. (Eckard *et al.*, 2011; Nedělník, Lindušková, & Kmoch, 2012; Lazzaro *et al.*, 2015).

Most *Fusarium* spp. found in this study also has been found in other studies in other countries. Abundance of *Fusarium* species is variable between sites

and years even in one region. (Eckard *et al.*, 2011; Lazzaro *et al.*, 2015). If compared this to other trials, it is possible to find common tendencies in proportion of fungal composition with this study (Eckard *et al.*, 2011; Nedělník, Lindušková, & Kmoch, 2012).

In this study, the most frequently isolated were fungi from *Alternaria* spp. Fungi from genera *Alternaria* has been reported to produce a wide range of mycotoxins; however, *Alternaria*, spp. includes also endophytic fungi (Ostry, 2008). As *Alternaria* toxins are supposed to be less harmful than toxins produced by *Fusarium* and *Aspergillus* species they haven't been studied so much (Ostry, 2008; Logrieco, Moretti, & Solfrizzo, 2009).

Penicillium spp. is often isolated from maize, and it was also present in current study. *Penicillium* spp. has been considered as storage fungi; however, in many cases it is present on maize before harvest (Mansfield, Jones & Kuldau, 2008). In the study carried out in Italy, the RD for *Penicillium* in organic maize was lower than 2%. In this current study, the RD for *Penicillium* spp. was 5%. (Lazzaro, Moretti, Giorni, Brera, & Battilani, 2015).

Less frequently isolated in the current study was fungi from *Epicoccum* and *Sarocladium* spp. *Epicoccum* spp. includes both endophytes and pathogens and fungi from *Sarocladium* spp. has been reported as endophytes, which can deter the growth of maize pathogens such as *F. verticillioides* and *Aspergillus flavus* (Summerbell & Scott, 2015)

Aspergillus spp. are worldwide distributed and able to produce the most toxic mycotoxins aflatoxins. Recently the presence of *Aspergillus* spp. in maize grain mycoflora has been found in European countries where it had not been a problem before, e.g. Croatia, Serbia, Slovenia, Romania and Northern Italy (Giorni, Magan, Pietri, Bertuzzi, & Battilani, 2007; Szőke et al., 2013). In Italy, in the study carried out by Lazzaro, Moretti, Giorni, Brera, & Battilani (2015) in 2010 and 2011 the RD for *Aspergillus* was low or it was not found at all. In this study, the Aspergillus spp. was not found.

The results obtained in this study correspond to results from other studies around Europe. Other studies reveal that fungal mycoflora is influenced by the site and year; therefore, it is important to continue the current research and monitor the situation. (Logrieco, Moretti, Ritieni, Bottalico, & Corda, 1995; Eckard, Wettstein, Forrer, & Vogelgsang, 2011; Nedělník, Lindušková, Kmoch, 2012).

Conclusions

The fungi from *Alternaria*, *Fusarium* and *Penicillium* spp. were most frequently isolated from maize grain in this study. These three genera are potential mycotoxin producers. The majority of all *Fusarium* isolates was *G. zeae* (38%), the second most widespread fungi was *G. intricans* (25%), a smaller share in proportion was for *F. sporotrichioides*, *F. poae* and *G. fujikuroi*. The potential mycotoxin producing fungi were present in maize grain samples without visible symptoms of fungal contamination collected from different maize hybrid performance comparison trial in Research and Study farm "Vecauce" of Latvia University of Agriculture in 2014. There is a risk that maize production can contain mycotoxins. Therefore, it is important to continue research.

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