

## Formation of the abundance of microfungi on the barley grain grown as pure and mixed crops in Central and North Estonia

Elina Akk<sup>a,b,✉</sup>, Heino Lõiveke<sup>a</sup>, Liina Edesi<sup>a,b</sup>, Mary-Liis Kütt<sup>c</sup>,  
Enn Lauringson<sup>b</sup>, and Veiko Kastanje<sup>a</sup>

<sup>a</sup> Department of Plant Sciences, Estonian Research Institute of Agriculture, Teaduse 13, 75501 Saku, Harjumaa, Estonia

<sup>b</sup> Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 5, 51014 Tartu, Estonia

<sup>c</sup> Department of Food Processing, Faculty of Chemical and Materials Technology, Tallinn University of Technology, Ehitajate tee 5, 19806 Tallinn, Estonia

✉ Corresponding author, elina.akk@eria.ee

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**Abstract.** For centuries barley has been an important food crop for mankind. It is important to produce crops that are of good quality and safe to human and animal organisms. However, pathogenic fungi in cereals cause health problems both to humans and animals. In Estonia the microbiological quality of cereals has been studied to identify *Fusarium* species in feed cereals. Still, the relationship between the agro-ecological conditions and the total abundance of moulds and yeasts on grain has been studied little in Estonia. In 2009 and 2010, we carried out field trials in the experimental station of the Estonian Research Institute of Agriculture in North Estonia (59°18'N, 24°39'E) and in the experimental station of Olustvere School of Service and Rural Economics in Central Estonia (58°33'N, 25°34'E). The variants of the experiment were barley in pure crops with added ammonium nitrate 120 kgN ha<sup>-1</sup>, barley in pure crops with added ammonium nitrate 60 kgN ha<sup>-1</sup>, barley in pure crops with no added ammonium nitrate, and barley–pea mixed crop. After harvesting, the grain was dried to 14% of moisture and grain samples were taken from each trial variant. The abundance of moulds, yeasts, and *Fusarium* spp. was determined in grain samples using the dilution method. The impact of the levels of nitrogen, location of the trial site, and year (weather conditions) on the abundance of moulds, yeasts, and *Fusarium* spp. was studied. The common genera of moulds identified were *Cladosporium*, *Acremonium*, and *Fusarium*. According to our results, yeasts were the most common fungi on barley grains. In North Estonia the abundance of moulds was lower compared to Central Estonia. The abundance of fungi was not affected by different levels of nitrogen or whether barley had been grown as a pure crop or a barley–pea mix. The weather conditions had the greatest impact on the abundance of microfungi.

**Key words:** cereal, moulds, yeasts, *Fusarium* spp., quantitative method.

## INTRODUCTION

For centuries barley has been an important food crop for mankind. Today most of the barley grown is used in the beer and malt industry, but also as animal and human food. In general, about two-thirds of the world's barley production has been used for feed, one-third for malting, and 2% for the food industry (Newman & Newman, 2006).

Environmental variations, temperature, and humidity have an important impact on the yield and quality of cereals (Šarić et al., 1997; Lõiveke et al., 2003; Ingver et al., 2010). Furthermore, the agronomic practices may influence the yield and microbiological quality of the crop. The effect of tillage practices, fertilizer treatments, cropping system, and crop protection on *Fusarium* spp. and on levels of mycotoxins has been studied mainly on wheat (Champeil et al., 2004; Fernandez et al., 2008; Lori et al., 2009; Vujanovic et al., 2012). Previous studies on barley have focused on the resistance of varieties to ear blight (Legzdina & Buerstmayr, 2004), concentration of mycotoxins (Mankevičienė et al., 2006; Edwards, 2007; Ibáñez-Vea et al., 2012), and the effect of *Fusarium* spp. infection on the malting process (Wolf-Hall, 2007).

In Estonia precipitation exceeds evaporation. Strongest rainfalls occur from July to November (Estonian Second National Report..., 1998). Therefore, in most years the periods of anthesis, grain filling, and harvesting of cereals coincide with the rainy period. In Estonia spring barley cultivation is prevalent because winter barley is not cold resistant in our climate (Bothmer et al., 2003).

Nowadays increasingly more attention is paid to food and feed safety and the quality of crops. However, microorganisms, such as moulds, may cause the spoilage of grain, which then becomes hazardous for humans and animals (Richard, 2007). A high amount of moulds in malt can cause toxicity of raw material in the brewing industry (Medina et al., 2006). Therefore, investigation of pathogenic moulds deserves high attention, and early discovery of infestation is important (Placinta et al., 1999; Siegel & Babuscio, 2011). Several researchers have studied the microbiological quality of barley focusing on moulds such as *Alternaria* spp., *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. and their toxins (Rabie et al., 1997; Yli-Mattila, 2011).

In Estonia the microbiological quality of cereals has also been studied to identify *Fusarium* species in feed crops. Lõiveke et al. (2003) reported that *F. avenaceum*, *F. ventricosum* (synonym of *F. solani*), *F. sporotrichioides*, and *F. poae* are the most common *Fusarium* species on feed cereals. They rated 14–46% of the barley crop samples infected. However, the relationship between the agro-ecological conditions and the total abundance of moulds and yeasts on barley grain has been little studied in Estonia.

The general method applied for analysing grain quality is the assessment of the total abundance of moulds and yeasts on cereals. The results of the quantitative assessment show whether the food or feed is contaminated by microfungi (Schmidt-Lorenz, 1980; Baumgart & Firnhaber, 1993).

Although the capacity of well-equipped laboratories to identify and quantify mould species on grain is high, the general abundance of moulds and yeasts on crop fields has been little studied. The aim of this study was to identify, using a quantitative method, the impact of the level of nitrogen, field location, and year (weather conditions) on the abundance of moulds, yeasts, and *Fusarium* spp. on barley grain.

## MATERIALS AND METHODS

The field experiments with barley (*Hordeum vulgare* L.) were carried out in 2009–2010. In North Estonia the field trials were performed in the Saku experimental station of the Estonian Research Institute of Agriculture (59°18'N, 24°39'E), where Cambisols (CM) are the prevailing soil type (FAO, ISSS, ISRIC, 1998). In Central Estonia the field experiments were made in the experimental station of Olustvere School of Service and Rural Economics (58°33'N, 25°34'E); there the prevailing soil type is Podzoluvisols (PD) (FAO, ISSS, ISRIC, 1998). In both locations the field trials were sown in the first 10 days of May and harvested in the last 10 days of August. Spring barley variety Anni and pea (*Pisum sativum* L.) variety Clarissa were used in both experiments.

During the sowing of barley pure crops and barley–pea mixed crops the complex fertilizer Skalsa with a low nitrogen content was added at a rate 250 kg ha<sup>-1</sup> (nutrients N10–P<sub>2</sub>O<sub>5</sub>50–K<sub>2</sub>O63 kg ha<sup>-1</sup>). At the stem stage of barley, the pure crop received an additional ammonium nitrate fertilizer. The variants of the experiment with ammonium nitrate were (1) N120: barley pure crops with added ammonium nitrate 120 kgN ha<sup>-1</sup> (quantity of fertilizer 320 kg ha<sup>-1</sup>); (2) N60: barley pure crops with added ammonium nitrate 60 kgN ha<sup>-1</sup> (quantity of fertilizer 145 kg ha<sup>-1</sup>); (3) N0: barley pure crops without ammonium nitrate; and (4) BP: barley–pea mixed crop without ammonium nitrate. However, it was taken into account that the soil of barley–pea mixed crop contains about 40–50 kg of free N per ha (Freyer, 2003). For weed control the crops (stem stage of barley and 3–9 leaves stage of pea) were treated with the herbicide Butoxone (1520 g ha<sup>-1</sup> MCPB in active ingredient), dosage 3.8 L ha<sup>-1</sup>.

The field trials were harvested with a combine harvester. The grain was dried to 14% of standard moisture, sorted, and a 1.5-kg average sample was taken per every field experiment variant. Microbiological analyses were carried out in the Laboratory of Plant Health and Microbiology at the Agricultural Research Centre, Estonia. The preparation of samples was performed according to the standard EVS-EN ISO 6887-1:2001 (Estonian Centre for Standardisation, 2001). The dilution method was used to determine the abundance of moulds, yeasts, and *Fusarium* spp. in each sample. The moulds and yeasts were grown on Czapek agar (ICC Standard No. 146, 1992) and *Fusarium* spp. fungi on Nash and Snyder selective medium (Booth, 1971; Gerlach & Nirenberg, 1982). The abundances of moulds, yeasts, and *Fusarium* spp. were presented as the number of colony-forming units per 1 g of dry grain (CFU g<sup>-1</sup> of dry grain).

The microbiological quality of barley grain was evaluated using the indicators for food and feed established by Schmidt-Lorenz (1980) and Baumgart & Firnhaber (1993). According to these indicators, on grain of normal quality the abundance of moulds or yeasts must not exceed  $3 \times 10^4$  CFU g<sup>-1</sup> of dry grain in food grain and  $4 \times 10^4$ – $8 \times 10^4$  CFU g<sup>-1</sup> of dry grain in feed grain (Lõiveke et al., 2004).

The meteorological data were obtained from the observation points of the Estonian Meteorological and Hydrological Institute.

The statistical analyses were performed using Microsoft Excel 2003. ANOVA test was used to compare the differences between variants at the 95% probability.

## RESULTS AND DISCUSSION

### Weather conditions

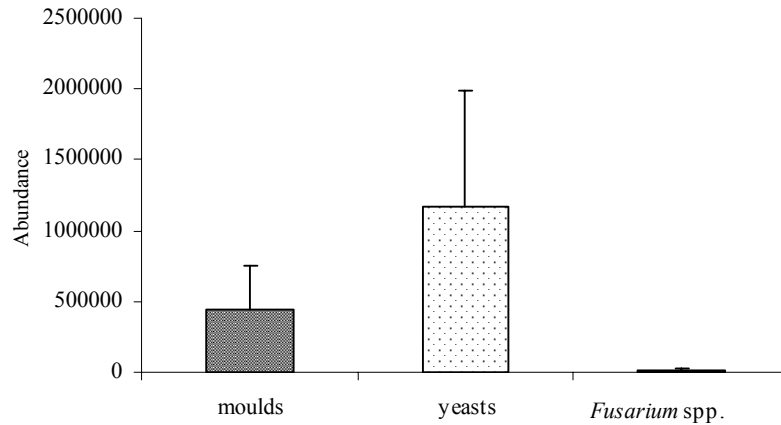
During the two vegetation periods the air temperatures differed significantly but both trial years were rich in precipitation. During the growing season of 2009 the air temperatures in North and Central Estonia were similar and favourable for plant growth. In 2010 the growing season was warm. The mean air temperature was 3.3°C above the region's long-term average (14.4°C) in Central Estonia and exceeded the region's long-term average (13.8°C) by 2.1°C in North Estonia.

The major difference between the two regions during the study period was the registered precipitation amounts. In Central Estonia the amounts of precipitation during the vegetation seasons of 2009 and 2010 were 120% and 114%, respectively, of normal long-term average (277 mm). The precipitation amounts for North Estonia were 74% and 67%, respectively, of normal long-term average (268 mm). Besides, the temporal distribution of precipitation was different in North and Central Estonia.

In July 2009 North Estonia suffered from drought and normal rainfall came only in August. In Central Estonia it rained a lot in both months. The July of 2010 was very rainy in North Estonia while in Central Estonia rainfalls were scanty. In August 2010 the situation was the opposite: it was dry in North Estonia while in Central Estonia the amount of precipitation reached almost the double monthly norm. During the two experimental years the rainfall in July and in August made up 88% of the monthly norm in North Estonia and 129% in Central Estonia.

### Microbiological composition

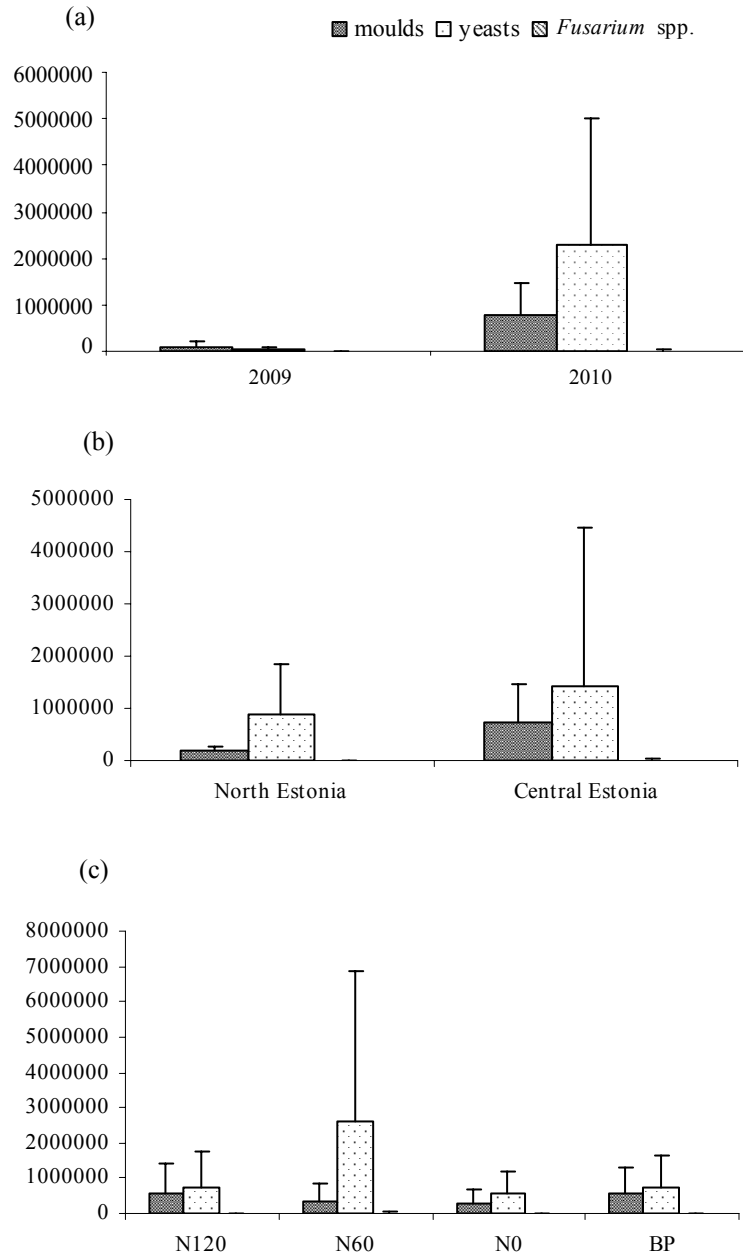
Our results of microbiological analysis of grain showed that the microbiological composition differed greatly by the groups of microfungi (Fig. 1). Olstorpe et al. (2010) also detected that the microbial flora in barley grain varies considerably and the microbial population from the field changes during storage. In our study the common genera of moulds were *Cladosporium*, *Acremonium*, and *Fusarium*. *Cladosporium* spp. are among the most common saprophytes on cereals. *Fusarium* spp.



**Fig. 1.** Average total abundance of moulds, yeasts, and *Fusarium* spp. (CFU g<sup>-1</sup> of dry matter) on barley grain in 2009 and 2010.

are pathogens, which produce mycotoxins on cereal grains (Suproniene et al., 2011). *Acremonium* spp. appear in soil and associate with plants (Domsch et al., 2007; Vujanovic et al., 2012). In 2009 moulds were the most common microfungi group on barley grain while in 2010 yeasts (species not determined) dominated (Fig. 1). The overall abundance of yeasts on barley grain was higher compared to the overall abundance of moulds and *Fusarium* spp. Yeasts increase the pH and harm the storage stability of cereals (Middelhoven & van Baalen, 1988). However, yeasts may also have positive effects, inhibiting the growth of moulds on the moist cereal grains (Passoth & Schnürer, 2003; Druvefors & Schnürer, 2005) and enhancing the nutritional value of animal feed (Bui & Galzy, 1990). Yeasts are useful and necessary for the fermentation processes of cereals, especially in cereal silage and in the beer industry (Fleet, 2007; Olstorpe et al., 2010).

Compared with the abundance of moulds and yeasts that of *Fusarium* spp. was relatively low, constituting only 1.6% of the total abundance of moulds and yeasts. Krysinska-Traczyk et al. (2007) found that *Fusarium* strains have been very rarely compared to the common species of moulds and yeasts. The reason may be that some yeasts and *Acremonium* spp. inhibit the growth and progression of *Fusarium* spp. on the grain (Vujanovic et al., 2012). However, in 2010 the abundance of moulds, yeasts, and *Fusarium* spp. was higher compared to the 2009 trial (Fig. 2a). An increase of precipitation in July during the time of anthesis and seed formation may significantly raise the abundance of moulds, yeasts, and *Fusarium* spp. on the grains. Furthermore, a high precipitation amount during the growing season, particularly during the pre-harvest period, could increase the abundance of moulds on the grain (Sutton, 1982). This means that weather conditions are extremely important for the development of various microfungi (Torp & Nirenberg, 2004; Uhlig et al., 2007; Tirado et al., 2010; West et al., 2012). The development of yeasts was favoured by 2–3 degrees higher than average air temperatures in 2010. It was also observed that an increase of yeasts could cause



**Fig. 2.** Comparison of the abundance (CFU g<sup>-1</sup> of dry grain) of moulds, yeasts, and *Fusarium* spp. between (a) 2009 and 2010, (b) trial locations, and (c) different amounts of nitrogen applied at the stem stage of barley (N120 = 120 kgN ha<sup>-1</sup>, N60 = 60 kgN ha<sup>-1</sup>, N0 = no ammonium nitrate added, BP = barley-pea mixed crop).

a decrease of moulds. Despite the erratic precipitation in both habitats during 2010, a high abundance of yeasts was registered in both test locations.

The results a two years of trial showed that the average abundance of moulds in barley samples from Central Estonia was four times higher and the abundance of yeasts two times higher compared to the samples from North Estonia (Fig. 2b). As we used the same sowing time, fertilization, varieties, crop protection, and harvesting time in different locations, this indicates that the weather conditions in the growth location were more important for the formation of the abundance of moulds and yeasts than agronomic practices. In addition, the formation of the microbial composition on grain is significantly affected by interactions between moulds and yeasts (Fernandez et al., 2008; Vujanovic et al., 2012).

The differences between the abundances of microfungi groups from trials with different nitrogen levels were not statistically significant (Fig. 2c). The highest abundance of yeasts was observed in the N60 variants whereas the highest average abundance of moulds was shown by the BP and N120 variants. In the trial variant N60 the highest percentage of yeasts (87%) and the lowest percentage of moulds (12%) were observed. In the trial variants where the percentage of yeasts in the microbiological composition was lower (55–65%), the percentage of moulds was higher (about 33–44% of the total fungal abundance). This indicates a potential positive effect of yeasts in reducing moulds in the microbiological composition of barley grain. Consequently, the effect of soil nitrogen content on the abundance of microfungi groups was less significant than the impact of the weather. Apparently, the use of different dosages of nitrogen in the cultivation of barley as a pure crop or a mixed crop with pea did not have a direct impact on the microbiology of barley grain in Central and North Estonia. The effect of soil nitrogen content and abundance of moulds, particularly *Fusarium* spp., and plant diseases has been studied by several researchers. In some cases the reports show that a low level of nitrogen tends to promote the infection by *Fusarium* spp. while a high level of nitrogen reduces the grain contamination with *Fusarium* spp. (Yang et al., 2010). However, in another study exactly the opposite conclusion was drawn: the high levels of nitrogen were found to favour the contamination of grain with *Fusarium* spp. (Champeil et al., 2004).

Another important aspect is agro-ecological conditions, which may also affect the content of moulds, especially *Fusarium* spp., on cereals. Through the implementation of crop rotation, moderate fertilization, and use of pesticides the development of the *Fusarium* fungi as well as the emergence of mycotoxins could be reduced (Edwards, 2004). In our study the abundance of *Fusarium* spp. was not affected by the level of nitrogen. We found that the effect of weather conditions during the growing season in the growth location was more important for the abundance of microfungi than the effect of fertilization with nitrogen: we did not observe any significant impact of soil nitrogen content on the abundance of moulds, yeasts, and *Fusarium* spp. on barley grain.

In Estonia no guidelines have been established for counting moulds and yeasts on grain. To assess whether the microbiological quality of barley grain is suitable for food or feed the indicators of Schmidt-Lorenz (1980) and Baumgart & Firnhaber

**Table 1.** Suitability of barley from the experiment for microbiological quality of food and feed according to Schmidt-Lorenz (1980) and Baumgart & Firnhaber (1993) indicators for cereal crops (CFU g<sup>-1</sup> of dry grain)

Location	2009		2010	
	Moulds	Yeasts	Moulds	Yeasts
Central Estonia	$5.9 \times 10^3$	$6.56 \times 10^4$	$13.7 \times 10^5$	$28.2 \times 10^5$
North Estonia	$14.9 \times 10^4$	$3.5 \times 10^4$	$19.4 \times 10^4$	$17.5 \times 10^5$

Indicator for food  $3 \times 10^4$ ; for feed  $4 \times 10^4$ – $8 \times 10^4$ .

(1993) are used. To meet the quality standards of food grain and feed grain the abundance of moulds or yeasts must not exceed  $3 \times 10^4$  CFU g<sup>-1</sup> of dry matter and  $4 \times 10^4$ – $8 \times 10^4$  CFU g<sup>-1</sup> of dry matter, respectively (Lõiveke et al., 2004). According to microbiological quality indicators, in 2010 the barley grain did not correspond to the Schmidt-Lorenz (1980) and Baumgart & Firnhaber (1993) indicators for feed and food. In 2009 the barley grain grown in Central Estonia met the requirements for feed but not for food (Table 1).

The saprophytic and pathogenic fungi on barley kernels induce various symptoms. Although seed damage begins before harvest, it may increase if grains are stored under moist or wet conditions or they are harvested late (Wiese, 1987). At harvest and storage of cereals, spore containing material and humidity combined with temperature are factors affecting mould and yeasts formation in cereals (Schrödter, 2004).

## CONCLUSIONS

The trials of this study were conducted under contrasting weather conditions; therefore we cannot draw any definitive conclusions on the basis of the two-year results. Still, our results showed that the most frequent fungi on the analysed barley grain were yeasts. The abundance of moulds on barley was lower in North Estonia than in Central Estonia. The total abundance of microfungi was most affected by the weather conditions during the vegetation season in both locations. Growing barley at different soil nitrogen levels in pure crops and mixed crops with pea did not have any impact on the amount of microfungi groups in the barley yield.

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