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Effect of Fungicide Treatment on *Fusarium culmorum* and *Tri* Genes Transcription in Barley Malt

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Abstract

Pavel J., Vaculová K., Faltusová Z., Kučera L., Sedláčková I., Tvarůžek L., Ovesná J. (2015): **Effect of fungicide treatment on *Fusarium culmorum* and *Tri* genes transcription in barley malt**. Czech J. Food Sci., 33: 326–333.

Malting barley grains are essential components in the beer production. *Fusarium* infection can have severe effects on malt and beer, because it may inhibit the enzymatic activity in malt and may induce the occurrence of gushing and changes in the colour and flavour of the finished beer. We examined the growth of the filamentous fungi *Fusarium culmorum* in artificially infected and non-infected barley malting grains during the first steps of the malting process and under the effects of fungicide pretreatment (Hutton and Prosaro 250 EC) of barley plants. Our study focused on the fungi growth in two distinct barley malting cultivars Bojos and Malz. *Fusarium* growth was investigated by quantitative real-time PCR using TagMan MGB probes. Furthermore, we focused on the *Tri5* and *Tri*6 genes because they play the most important roles in trichothecene biosynthesis. Surprisingly, the higher transcription activity of the *Tri* genes was found in the fungicide-treated cultivar Malz as compared with untreated cultivars.

Keywords: realtime PCR; barley malting cultivar; fungicide pretreatment; trichothecene biosynthesis

Barley, *Hordeum vulgare* L., is one of the most worldwide spread and economically significant crops, mainly used for the beer production and animal feed and, to some extent, as food for direct human consumption. Similar to other plants, barley is also susceptible to fungal diseases. The fungi *Fusarium* spp. are a group of serious pathogens that colonise plants and produce small toxic molecules that contaminate agricultural products, rendering them unsuitable for consumption (Menke *et al*. 2013). These filamentous fungi cause disease called Fusarium head blight (FHB), which significantly reduces the yield and quality and results in the contamination of grain with mycotoxins (Scherm *et al*. 2013). *F. graminearum* and *F. culmorum* are the *Fusarium* species most often found in the Czech Republic (Kmoch *et al*. 2012; Matusinsky *et al*. 2013). Creppy (2002) reported that the species *F. graminearum* and *F. culmorum* are primarily associated with the occurrence of deoxynivalenol (DON). As the most destructive is considered *F. graminearum*, which causes the disease in wheat and barley and often infests grains with harmful trichothecene mycotoxins (Menke *et al*. 2013). *F. culmorum* is well adapted to colder regions, and infects cereals at temperatures between 10–14°C (Rajčáková 2006). A high *F. culmorum* infection level in barley grains results in substantial malt loss, changes in enzymatic activity, kernel ultrastructure deterioration, and deoxynivalenol accumulation (Oliveira 2012; Oliveira *et al*. 2013). Garda-Buffon *et al*. (2010) reported that trichothecenes interfere with the performance of hydrolytic enzymes, which were described by Khattak *et al*. (2013) to play an important role in the malting process.

The occurrence of FHB may be reduced by the application of fungicides, of which the most frequently

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used are triazoles, but their effectiveness depend on the active ingredients, the terms of application, meteorological conditions, virulence of the pathogenic strain, fungi species involved etc. (Kmoch *et al*. 2012). The efficacy of FHB control using fungicides is variable in different cereals and often unsatisfactory, less than 50–60% (Mesterházy *et al*. 2003). The azole group (prothioconazole, tebuconazole, etc.) have an inhibitory effect on the biosynthesis of ergosterol, which is necessary for the formation of pathogen cell membranes (Fan *et al*. 2013), but they act differently with other active ingredients. For instance, with spiroxamine they block the enzyme reductase, thus inhibiting sterol biosynthesis in fungal pathogens (Gaurilčikienė *et al*. 2010).

Genes associated with trichothecene biosynthesis have been described in several studies. The majority of the trichothecene genes are arranged into so-called clusters in the mold genome (Seong *et al*. 2009). Most *Tri* genes have been found within the *Tri5* cluster (Kimura *et al*. 2003). Two trichothecene genes have also been found in a two-gene minicluster (*Tri1* cluster), while another gene, *Tri101*, has been identified at a separate locus (Kimura *et al*. 1998). The *Tri6* and *Tri10* genes have been shown to be regulators capable of influencing the gene transcription of other trichothecene biosynthetic genes (Seong *et al*. 2009). Trichothecene production is driven by the transcription of the *Tri5* gene (encoded trichodiene synthase) (Gardiner *et al*. 2010; Beccari *et al*. 2011).

In our study, real-time PCR was used to evaluate the effects of the fungicide treatment on the pathogen content and *Tri* genes transcriptional activity. Because there are not many studies dealing with the synthesis of *Fusarium Tri* genes during the malting process, we focused on measuring the transcription of the *Fusarium culmorum Tri5* and *Tri6* genes in artificially infected barley malting grains.

MATERIAL AND METHODS

Two spring barley cultivars (Bojos and Malz) were used in our study. The experiments were set up under field conditions in Kroměříž in 2011 under natural and artificial infection conditions with the defined, highly pathogenic strain *Fusarium culmorum* (W.G. Smith) Saccardo, listed in the collection of the Agricultural Research Institute Kroměříž, Ltd. under the name KM16902 (DON chemotype) (Matusinsky *et al*. 2013). The agronomic management applied was standard and optimal for spring barley. Herbicide preparations Lintur 70 WG (150 g/ha) and Puma Extra (0.8 l/ha) were used against weeds in the stands, and then the stands were treated in the defined phenological development stages with fungicides directed against FHB. Plant growth stages were identified according to the BBCH scale (Biologische Bundesanstalt, Bundessortenamt and Chemical Industry) (Phenological growth stages 1997). The variants were treated with the fungicides Hutton (0.8 l/ha in flag leaf stage, BBCH 39; 25.5.2011) and Prosaro 250 EC (0.75 l/ha in the full flowering vegetation phase (BBCH 65; 6.6.2011), while control variants were untreated. Inoculation with *F. culmorum* was performed according to Tvarůžek *et al*. (2012) by spraying at an appropriate growth stage (BBCH 61–64) when 50% of plants were at the beginning of anthesis (concentration of 0.5 million conidia of *F. culmorum* in 1 ml of inoculum, spray dose of 200 l/ha).

Harvesting was done at full maturity (BBCH 99; 5.8.2011), and grain samples from all experimental variants (each containing two repetitions) for malting and mycotoxin analysis were identified (in the amount of 600 g per repetition) using a laboratory divider for grain samples according to the methodology of ES No. 401/2006. The samples were malted in the micromalting plant M-3BX (RAVOZ; Agrotest Fyto, Ltd., Kroměříž, Czech Republic) according to the standard procedures. Laboratory samples for the genetic and mycotoxin analyses were first taken after steeping, then in germination stage I (after 24 h of germination stage), and finally in the germination stage II (after 72 h of germination stage) in three biological replicates. In all steps micromalting observed, the temperature was 14.5°C. The samples for RNA analysis were stored at –80°C until processing.

The mycotoxin deoxynivalenol (DON) content was analysed by ELISA using R-Biopharm AG kits (Darmstadt, Germany). A combination of the RIDASCREEN® DON and RIDACREEN® FAST DON kits was used. The limit of quantification (LOQ) for DON was 40 µg/kg. The analyses were performed according to the guidelines provided by the manufacturer. The DON content in µg/kg was measured for each variant (Table 1).

Nucleic acids were isolated from 0.1 g tissue of malting barley grains homogenised in liquid nitrogen. Total RNA was isolated with TRIzol® Reagent (Invitrogen, Grand Island, USA) following the manufacturer's protocol. The isolated RNA was purified

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Table 1. Characterisation of experimental variants and the deoxynivalenol content in dry grain and samples from the malting process of the barley cultivars Bojos and Malz

Cultivar	Inoculation *F. culmorum*	Treatment Hutton + Prosaro 250 EC	Deoxynivalenol (µg/kg)			
			barley	after steeping	germination stage II	malt
Bojos	inoculated	untreated	67 362	26 960	105 374	68 080
		treated	39 793	25 053	62 321	62 708
	non inoculated	untreated	798	314	434	849
		treated	42	114	< 40	356
Malz	inoculated	untreated	44 755	43 189	62 550	64 063
		treated	17 327	12 808	24 969	33 873
	non inoculated	untreated	732	110	113	394
		treated	49	95	< 40	49

using the RNeasy Mini Kit and RNase-Free DNase Set and stored at –80°C. The DNeasy Plant Mini Kit (both Qiagen, Hilden, Germany) was used for DNA isolation, and it was stored at –20°C according to the manufactures instructions. The quantity and quality of the isolated RNAs and DNAs were determined with spectro Nano-Photometer™ Implen (Implen GmbH, Munich, Germany) by measuring the absorbance at the 260, 230, and 280 nm wavelengths. RNA integrity was verified by evaluating the signal quality of the large and small ribosomal subunits in electrophoretograms.

Pathogen quantification was performed with qPCR using TagMan MGB (minor groove binder) probes and two specific primers as described by Leišová *et al*. (2006).

The isolated and purified RNA was reverse-transcribed using TaqMan® Reverse Transcription Reagents in a thermal cycler Verity® (Applied Biosystems, Inc., Waltham, USA). The reaction mixture and thermal cycling conditions were prepared according to the manufacturer's instructions. Each 50 µl reaction mixture contained 1 µg total RNA, 1× RT buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 1.25 µM oligo $d(T)_{16}$, 1.25 µM random hexamers, 0.4 U/µl RNase inhibitor, 1.25 U/µl MultiScribe™ Reverse Transcriptase and Nuclease-Free Water (Ambion, Inc., Austin, USA) up to the total volume. The temperature profile was as follows: incubation at 25°C (10 min), reverse transcription at 48°C (30 min), inactivation of reverse transcriptase at 95°C (5 min), and cooling at 10°C (∞). The cDNA samples were stored at –20°C.

Thermal gradient PCR was used to define optimal annealing temperature. The reaction mixture contained 1 µl cDNA corresponding to 20 ng of total RNA, 1× Power SYBR® Green PCR Master Mix (Applied Biosystems, Inc., USA), 200 nM of each primer and Nuclease-free water up to the final volume of 20 µl. Nuclease-free water was used as a negative control, and the isolated and diluted (10 ng/µl) genomic DNA from *F. culmorum* was used as a positive control. The amplification was performed in a StepOnePlus™ thermal cycler (Applied Biosystems, Inc., USA). The thermal profile was as follows 95°C for 10 min followed by 42 cycles of 95°C for 30 s, the temperature gradient of 57, 58, and 60°C for 30 s/min, and dissociations steps including 95°C for 15 s, 60°C for 1 min, and 95°C for 15 second. The selected annealing temperatures for each primer combination were used for subsequent RT-qPCR reactions. Each PCR amplification was performed in triplicate for each of the three biological replicates.

The expression was measured with RT-qPCR using the StepOnePlus Real-Time PCR System (Applied Biosystems, Inc., USA) and primers (Table 2). Real-time PCR data were collected and processed using StepOne™ Software v. 2.1 (Applied Biosystems, Inc., USA). Quantification was based on the threshold cycle (C_T). The experimental data were processed using the MS Excel program (Microsoft Corporation, Redmond, USA). The mathematical model for relative quantification published by GeNorm manual was used for the data evaluation. Because a suitable reference gene is needed for measuring the gene transcription (Pfaffl 2001), we used the *Fusarium ubiquitin* (*UBC*) gene, which was previously reported to be a suitable gene for normalisation of infected barley caryopsis (Havránková *et al*. 2011). We verified the amplification efficiencies of the sequences of interest (*Fusarium* specific) and the reference gene (*UBC*) by a dilution curve.

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Table 2. Oligonucleotide primer sequences used in this study and their amplification efficiency

Primers	Oligonucleotide primer sequences (5'→ 3')	Annealing temperature (°C)	Efficiency
UBC	F: TCCCCTTACTCTGGCGGTGTC R: TTGGGGTGGTAGATGCGTGTAGT	57	2.077
Tri5	F: GTGGGCACTTGTCAACGA R: ACCCAAACCGTTCATACGAC	60	1.900
Tri6	F: CGGTGGATTCAACCAAGACT R: AACTAGGGAATGGGCATTTG	58	1.905

RESULTS AND DISCUSSION

The mycotoxins contaminations constitute a serious problem for the malting and brewing industries as mycotoxins can enter the food chain and endanger the consumers.

The immediate effects of severe pre-harvest barley infections by species of the FHB complex are the reduced seed germination and grain functionality. Further quality problems arise during malting and brewing with severely infected malts being associated with the occurrence of gushing and/or changes in the colour and flavour of the finished beer (Oliveira *et al*. 2012).

We used two micromalted cultivars, Bojos and Malz, to quantify the pathogen content and *Tri* genes transcriptional activity during the first steps of malting.

Initially, we investigated whether the causal agent *Fusarium culmorum* continued to grow during the first steps of the malting process. The *Fusarium* content measured using qPCR gave different results for the inoculated and naturally contaminated samples. In the non-inoculated fungicide-untreated samples (NFU) and non-inoculated fungicide-treated samples (NFT), the *Fusarium* content was only in the picogram scale (Figure 1), while in the inoculated fungicide-untreated samples (IFU) and inoculated fungicide-treated samples (IFT), the values reached the nanogram scale (Figure 2). Mycelium resulting from the natural infection by *Fusarium* was detected in the NFU and NFT samples (Figure 1). These data indicate that, in the NFT samples, the fungicide treatment protected the spikes against the natural infection (Figure 1B). A significant increase in the fungi content during the first steps of malting was detected in both of the IFU cultivars. A higher increase in the fungi content was detected in cv. Bojos (5.3-fold), whereas in cv. Malz, only a 3.7-fold increase after germination stage II was measured (Figure 2A). *Fusarium* growth was also found in IFT samples, which ranged from a three-fold increase after steeping in germination stage I to a six–fold increase in germination stage II in cv. Malz. *Fusarium* was growing significantly, after steeping in germination stage I – 18-fold and it stayed at similar level after germination stage II in ITF cv. Bojos (Figure 2B).

Fungicide treatment decreased the *Fusarium* content from 50 ng DNA in both IFU cultivars to 15.5 ng DNA in cv. Bojos and 4.7 ng DNA in cv. Malz. The greatest effect of fungicides (Hutton and Prosaro 250 EF) in suppressing the infection by *F. culmorum* was observed in the inoculated cv. Malz. However,

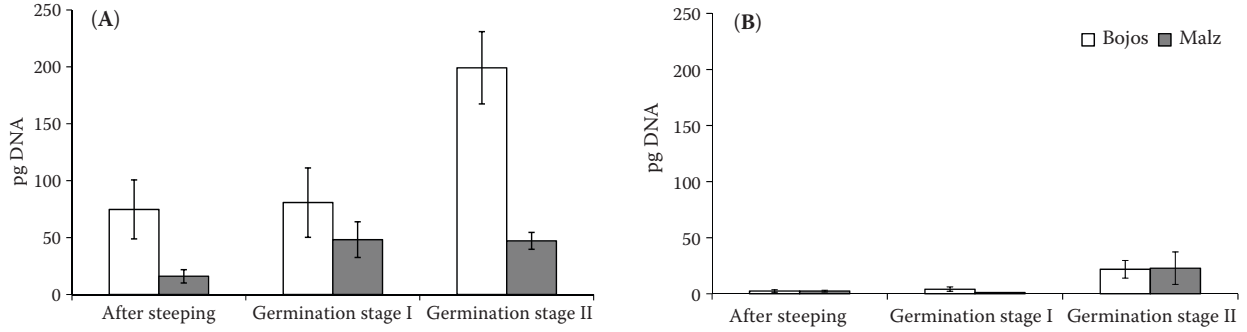

Figure 1. Relative amount of DNA on the pg scale during the dynamic growth of *Fusarium culmorum* infections at various stages of the malting process with confidence interval: (A) non-inoculated, fungicide untreated (NFU) samples and (B) non-inoculated, fungicide treated (NFT) samples

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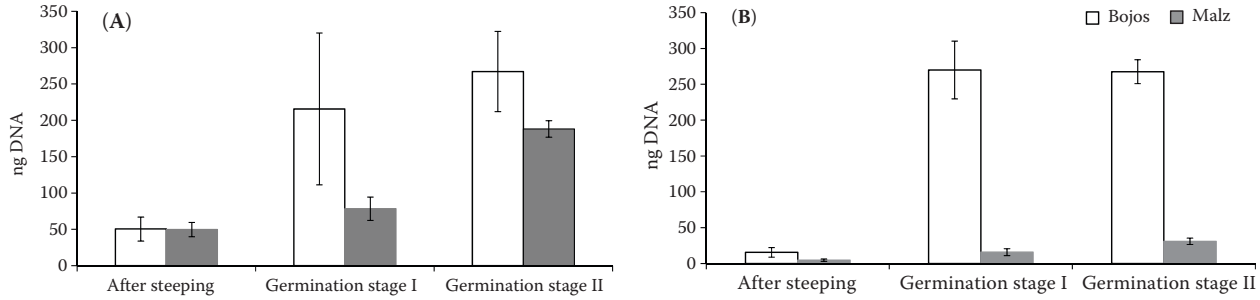

Figure 2. Relative amount of DNA on the ng scale during the dynamic growth of *Fusarium culmorum* infections at various stages of the malting process with confidence interval: (A) inoculated, fungicide untreated (IFU) samples and (B) inoculated, fungicide treated (IFT) samples

the cv. Bojos showed no appreciable change in this case (Figure 2). Fungicide control of cereals against *Fusarium* spp., which has been the subject of a large number of studies, concluded that fungicide effectiveness depends not only on the virulence of pathogens (Maier *et al.* 2006), but particularly on the timing and frequency of application, the active ingredient (Homdork *et al.* 2000; Ioos *et al.* 2005) and dose (Mauler-Machnik & Zahn 1994). The application of tebuconazole was found to be the most effective in the inoculated wheat stands (Homdork *et al.* 2000; Mesterhazy *et al.* 2003), while the effect of propiconazole did not differ from that of tebuconazole. In contrast, Kmoch *et al.* (2012) observed distinct varietal sensitivity to *F. culmorum* infection and found only low fungicide effects after the application of the fungicides Hutton and Prosaro 250 EC (the occurrence of *Fusarium* spp. was lower by only 4–18% in comparison with the untreated control variant). These fungicides possess the best suppression effects against FHB infection when applying the fungicide combination Hutton (BBCH 39) + Zantara (BBCH 65), which reduced the occurrence of *Fusarium* spp. by 41.8% in the non-inoculated variants and by 31.4% in the variants inoculated by *F. culmorum*. In our experiment, the efficacy of fungicides has been demonstrated by the decrease in the DON content in the barley grain after treatment.

Nicolaisen *et al.* (2009) reported that the *F. culmorum* and *F. graminearum* DNA content correlates well with the DON content in wheat and maize. In our study, the grains collected from the inoculated spikes of cv. Bojos contained 67 362 µg/kg DON, and fungicide treatment reduced this mycotoxin content to 39 793 µg/kg. The cv. Malz contained DON in the inoculated grains at 44 755 µg/kg, and after fungicide treatment at 17 327 µg/kg (Table 1). In the case of IFU samples, the DON content was found to range from 43 189 µg/kg in the stage after steeping to 62 550 µg/kg in germination stage II for cv. Malz and from 26 960 µg/kg in the stage after steeping to 105 374 µg/kg in germination stage II for cv. Bojos. In addition, increases in DON content after fungicide treatment were detected. Cv. Bojos contained 25 053 µg/kg DON in the stage after steeping and 62 321 µg/kg DON in germination stage II. Cv. Malz contained 12 808 µg/kg DON in the stage after steeping and 24 969 µg/kg DON in germination stage II. Generally, a lower DON content was observed in cv. Malz in comparison with cv. Bojos. Moreover, in a comparison of the DON content and amount of the fungi, a lower *Fusarium* content occurred in cv. Malz. In our study, an increase in DON content during the first steps of the malting process was detected (Table 1). Comparable results were observed by Oliveira *et al.* (2012) who showed that the DON concentration steadily increases with the mycotoxin content as malting progresses. In another study cited by Belochová *et al.* (2015), it was reported that during malting, mycotoxin levels decline; however, their production may increase during germination because the warm and humid environment of malthouses is suitable for mold growth.

Furthermore, we compared the increase in DNA content measured above and ubiquitin RT-qPCR quantity, which served as a reference gene for the normalisation of the *Tri* genes transcription. A statistically significant correlation between two datasets measured for cv. Bojos was found (r = 0.773–0.907; $P < 0.05$). For the cv. Malz (r = 0.836; $P < 0.05$), a statistically significant correlation coefficient (r = 0.836) was found only with the IFU sample. No statistically significant correlation coefficient was found for the IFT samples of cv. Malz, which had a lower *Fusarium* content and a higher standard variation.

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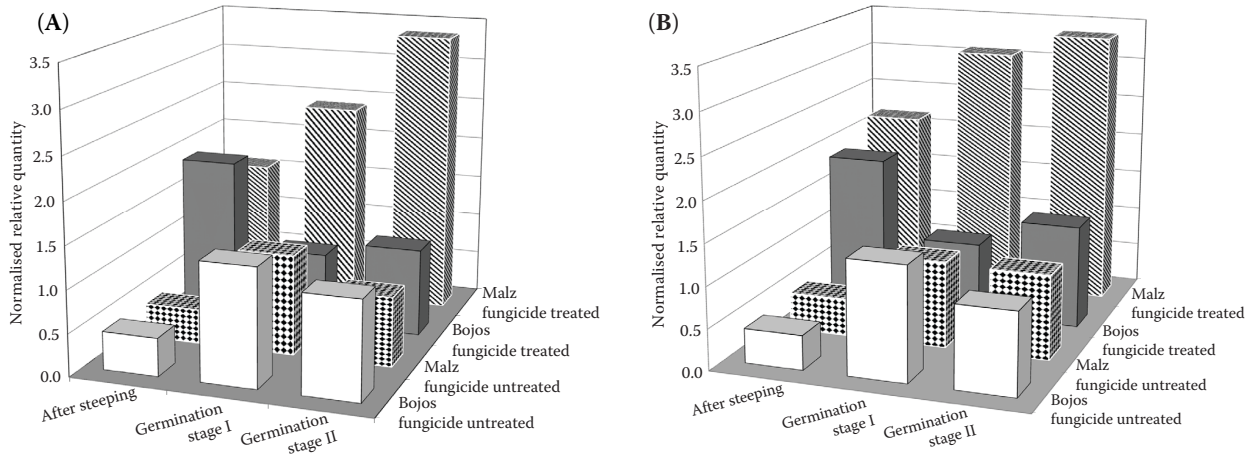

Figure 3. Changes in *Tri* gene transcriptional activity during the first steps of the malting process. Graph of the normalised relative transcription levels of the *Tri5* (A) and *Tri6* (B) genes in inoculated samples during the first steps of the malting process. Two inoculated, fungicide treated or untreated malted barley grains (cvs Bojos and Malz) were taken after steeping, in germination stage I and germination stage II

Furthermore, the transcription of the *Tri5* and *Tri6* genes associated with the trichothecene biosynthetic pathway was investigated (Figure 3). No great differences were observed between these genes. An increase in the relative quantity of *Tri* genes transcription was observed with the treated cv. Malz during the first steps of malting. However, the *Tri* genes transcription decreased after the steeping stage with treated cv. Bojos.

To our knowledge, there are only a few studies concerning monitoring *Tri* genes transcription in *Fusarium culmorum* during the malting process. Vegi *et al*. (2011) found a strong *Tri5* gene transcription in naturally infected barley during the third day of germination when compared with high-quantity and inoculated barley treatments during malting. Doohan *et al*. (1999) reported that *F. culmorum,* which had been grown in glucose-yeast extract-peptone medium, showed a high *Tri5* gene transcription on the third day after inoculation. This group observed that the transcription of *Tri5* in *F. culmorum* was increased by the fungicides prochloraz and tebuconazole *in vitro*. In our study, both genes (*Tri5, Tri6*) showed higher levels of transcription in response to *Fusarium* when fungicide treatment was used in cv. Malz (Figure 3). This finding may be explained by an increase in *Fusarium* responsiveness to the antifungal treatment. Nevertheless, additional studies are needed to confirm this hypothesis. *Tri5* gene transcription during germination precedes DON production by *Fusarium* during malting (Vegi *et al*. 2011). Comparable results were observed by Doohan *et al*. (1999) showing that the *Tri5* gene positively correlated (R^2 = 0.95) with DON production. Mycotoxin production may occur with the growth of *Fusarium* during steeping, germination, and possibly kilning in the malting process. In addition, the growth of fungi as measured by the increasing levels of ergosterol, a molecular marker for fungal biomass, was observed by Wolf-Hall *et al*. (2007) between days two and three of germination followed by deoxynivalenol production later during germination.

In summary, the results obtained in this study suggest that fungus is capable of growing during the malting process, depending on prior infection. However, when fungicide treatment was used, we subsequently observed only incomplete reduction in *Fusarium* content. *Fusarium* has suitable conditions for growth, which may also arise *de novo* during malting. This observation indicated a potential risk for malt houses; therefore, it makes sense to continue with this idea in further experiments. Surprisingly, in this study, fungicide treatment did not have any influence on the *Fusarium* content at the end of the germination stage in inoculated cv. Bojos. In the case of the transcriptional activity, we observed a higher *Tri* genes activity after using fungicide treatment in inoculated cv. Malz as compared with no treatment. This study may help in further understanding the trichothecene genes, their transcription, and fungi content reduction during the malting process.

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