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Characterizing effects of fermentation and baking on the deoxynivalenol content of rolls

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Summary

The effects of fermentation and baking on the deoxynivalenol (DON) contamination level of rolls prepared with contaminated wheat flour were studied. Contaminated wheat was obtained from a field experiment where wheat heads were inoculated at anthesis using a *Fusarium graminearum* macroconidial suspension. The DON contamination level of dough decreased linearly by 45 % within a fermentation (32°C, 75 % relative humidity) period of 90 min. The decrease of the DON content in the dough due to fermentation was not related to yeast, starch or gluten. The DON contents of flour/water mixtures were decreased under fermentation conditions, whereas no effect was observed if fermentation was omitted. After the centrifugation of flour/water mixtures, the effect of fermentation was observed in the supernatant, but not in the sediment, suggesting that a yet unidentified water-soluble compound of the flour was responsible for the reduction during fermentation. The DON content of rolls decreased almost linearly with baking time (0 – 20 min) but it was not significantly affected by baking temperature (220 vs. 250 °C). The DON content of the rolls decreased during baking at an average rate of 0.35 ± 0.05 mg DON kg^{-1} dry mass min^{-1}. Fermentation and baking decreased the DON content of the rolls to a similar extent, but a combination of both processes (fermentation with subsequent baking) did not result in a better reduction than one of the steps alone. The latter result suggests that the compound(s) responsible for the decrease of the DON content during fermentation is/are not heat stable. The DON contents of crust, dough and whole rolls did not differ significantly and the distribution of the DON within the rolls was not affected by yeast.

Introduction

DON (vomitoxin) is one of the toxic 12, 13-epoxytrichothecenes produced by various plant pathogenic fungi of the genus *Fusarium*, particularly by *F. graminearum*. Wheat plants are infected by *Fusarium* spores at anthesis if relative humidities are sufficiently high to allow spore germination (BEYER et al., 2004; BEYER and VERREET, 2005). DON is among the mycotoxin contaminants most frequently found in cereal crops such as wheat, corn and barley (TRUCKSESS et al., 1996). Health risks associated with the consumption of cereal products contaminated by DON and/or other *Fusarium* mycotoxins are recognized worldwide and depend on the extent to which they are consumed in a diversified diet (TRUCKSESS et al., 1995; 1996; PACIN et al., 1997; YOSHIZAWA and YIN-ZHE, 1998; SCHOLLENBERGER et al., 2002). Wheat products represent the major source of human DON intake (SCHOTHORST and VAN EGMOND, 2004).

In 2004, maximum tolerable levels of 0.5 mg kg^{-1} for cereals and 0.35 mg kg^{-1} for bread were implemented for DON in Germany. DON is slightly soluble in water and heat stable at least up to 120 °C (WOLF and BULLERMAN, 1998). Furthermore, it is stable at neutral and weakly acidic pH, but stability decreased with increasing pH in alkaline solution (WOLF and BULLERMAN, 1998). Due to the physico-chemical properties of the molecule, changes in DON levels can be expected during food processing in particular at high temperatures or high pH. Furthermore, relocation or leaching may be expected if water is involved in food processing steps.

HAZEL and PATEL (2004) reviewed literature on the effects of food processing on trichothecene levels and concluded: "The effects of baking, brewing and extrusion on DON levels is process dependent, with yeast (and other microorganisms present during fermentation) having effects not yet fully understood." It was the purpose of this study to (1) evaluate in how far DON levels are reduced during the preparation of rolls when following a (German) standard protocol for assessing wheat flour quality (PELSHENKE et al., 1978), (2) test if the DON reductions occur during fermentation or baking or both and (3) characterize the role of yeast for DON levels during fermentation.

Materials and methods

Ingredients and chemicals

Wheat (*Triticum aestivum* L. cultivar Ritmo) contaminated by DON was obtained from a field experiment carried out near Kiel, Germany (latitude 54° 21' north, longitude 10° 28' east). Plants were infected artificially at anthesis by overhead application of a *Fusarium graminearum* macroconidial suspension of 6 isolates. *Fusarium* isolates were obtained from M. Goßmann (Humboldt University Berlin, isolates 1, 2, 3 and 5) or J. Lepschy (LBP Freising, isolates 4 and 6). Crops were grown according to the current recommendations of integrated pest management except that fungicide applications against *Fusarium* species were omitted. The harvest was carried out using a commercial combine harvester and the dry grain was milled to flour (type 550). The flour was used in the following fermentation and baking experiments.

Unless specified otherwise, dough was prepared using 500 g wheat flour, 290 ml water, 25 g fresh yeast, 5 g sugar and, 5 g fat, 7.5 g salt and 10 ml ascorbic acid solution (0.1 % [w/v]). A kneader (model KM 800, Kenwood, Neu-Isenburg, Germany) was used for mixing the ingredients and kneading the dough. The dough was fermented in a universal incubator (model UE 400, Memmert, Schwabach, Germany) at 32°C and 75 % relative humidity. The relative humidity in the incubator was adjusted using saturated NaCl solution.

Deoxynivalenol (3a, 7a, 15-trihydroxy-12, 13-epoxytrichothec-9-en-8-one; Sigma-Aldrich, Steinheim, Germany) having a chemical purity of 98 % was used for the artificial contamination of previously uncontaminated dough, starch and gluten in some experiments (details see below).

Experiments

A two-factorial experiment was conducted to study the effects of different fermentation and baking (220°C) times on the DON content

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of rolls. Factor levels for fermentation and baking were 0 (control), 50, 70, 90 min and 0 (control), 10, 20 min, respectively. All baking experiments were carried out using an H-330 E-KAT oven (Miele, Gütersloh, Germany). Unless specified otherwise, baking experiments were performed following the national standard protocol by PELSHENKE et al. (1978).

In a second experiment, the role of yeast for the DON content of the dough was examined by preparing dough with or without yeast. The dough was separated into two pieces and one piece of each group was fermented for 100 min whereas the other (control) piece was not fermented.

The effects of starch and gluten on the DON content during fermentation were studied. In this experiment, dough was prepared using commercial wheat flour to obtain material with a low contamination level. The dough was separated into two pieces and the gluten of one of the pieces was extracted by washing the dough thoroughly under a stream of deionised water. The dough, the starch (Walter GmbH & Co. KG, Kiel, Germany) or the gluten were artificially contaminated using DON (Sigma-Aldrich, Steinheim, Germany). DON (powder) was dissolved in deionised water and the dough, the starch and the gluten were contaminated to a final DON contamination level of 10 mg kg^{-1} fresh mass. Non-contaminated samples served as controls. Samples for DON determination were taken before and after fermentation (100 min). The DON content of the commercial wheat flour was determined as well.

The effect of incubating flour/water mixtures under fermentation conditions on the DON content was studied. One kg of artificially contaminated wheat flour was mixed with 2 l of water. Samples for DON determination were taken. The mixture was centrifuged (Suprafuge 22, Heraeus Sepatech, Osterode, Germany) at 10,000g for 15 min. Samples for DON determination were taken from supernatant and sediment. The samples were subdivided into two groups. In group 1, the DON content was determined before fermentation and in group 2 the DON content was determined after the samples were incubated under fermentation conditions for 90 min. Liquid samples were cleaned up prior to HPLC using Mycosep columns as described below.

The effects of baking time (0, 10, 15 and 20 min) and baking temperature (220 and 250°C) on the DON content of rolls prepared with contaminated flour obtained from the field experiment were studied. Furthermore, the DON content of rolls prepared with or without yeast was measured in the crust, in the dough and in the whole rolls.

DON standard was dissolved in HPLC gradient grade MeOH / water (12.5 : 87.5 [v/v]). The mass of aluminium caps was determined using a micro balance (model MC5, Sartorius, Göttingen, Germany). Afterwards, the DON solution was transferred into the caps and the solvent was allowed to evaporate. Subsequent weighing revealed that the caps contained between 0.10 and 0.13 mg DON. The aluminium caps loaded with DON were incubated in an oven (model U 40, Memmert, Schwabach, Germany) at 80, 100, 120, 140, 160, 180, 200 and 220°C for 30 min. After each incubation period, the mass of the caps was determined on the microbalance. The number of replicates was n = 3.

Extraction of deoxynivalenol

Unless specified otherwise, freeze-dried samples were milled in a laboratory mill (model MF 10 basic, IKA-Werke, Stauffen, Germany) and 10 g of the milled sample were extracted using 50 ml of MeOH + H_2O (84 + 16 [v/v]) in Erlenmeyer flasks and shaken for 1.5 h at room temperature (≈ 20°C). Extracts (10 ml) were filtered and 6 ml of filtered extract were cleaned up using Mycosep columns (Coring System Diagnostix GmbH, Gernsheim / Rhein, Germany). Two ml of cleaned extract were transferred to round bottom flasks and evaporated to dryness (evaporator model R 110, Büchi Labortechnik AG, Flawil, Switzerland). The residues were redissolved in 500 µl aqueous 12.5 % MeOH, transferred to 0.8 ml glass vials and stored at -70°C until use.

DON analysis by HPLC

Deoxynivalenol contents of the extracts were determined by comparison with standards (Supelco, Bellefonte, PA, USA) using a high performance liquid chromatography (HPLC) gradient system consisting of two constaMetric 3500 pumps (ThermoQuest Italia, Milan, Italy), a photodiode array detector (Spectro Monitor 5000, LDC Analytical, Riviera Beach, FL, USA) and a CI-10B integration system (Milton Roy, Ivyland, PA, USA). Twenty µl of sample extract were manually injected onto a LiChroCart 250-4 HPLC cartridge column RP-18 (Merck Eurolab, Darmstadt, Germany) at a flow rate of 1.5 ml min^{-1} at 20°C. Elutions were monitored at 220 nm and the gradient elution was performed with MeOH and H_2O. The gradient program was described by BEYER et al. (2005). Recovery rates for samples contaminated artificially by DON ranged from 93 to 101 % in previous experiments and from 97 to 116 % (treatments that were not effective in reducing the DON contamination level, Tabs. 2, 3) in the present study when the extraction protocol and the HPLC settings described above were used. The detection limit of this procedure was 0.1 mg DON kg^{-1} (LUDEWIG, 2003).

Statistics

Data were analysed using the procedure GLM (general linear model) of the Statistical Analysis System software package (version 8.02; SAS Institute Inc., Cary, N.C., USA). Comparison of means was performed using Tukey's studentized range test at $P \leq 0.05$. Curve fitting was carried out using linear or sigmoid models. Unless specified otherwise, data are presented as mean ± standard error of the mean (SE) of duplicates.

Results

The DON contamination level of dough decreased linearly with fermentation time from 11.56 ± 1.23 mg kg^{-1} to 6.37 ± 1.35 mg kg^{-1} within a fermentation period of 90 min (filled circles, Fig. 1).

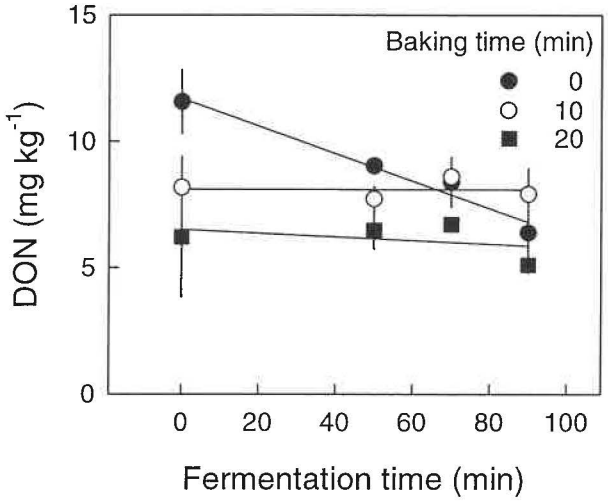

Fig. 1: Deoxynivalenol (DON) content of rolls prepared with contaminated wheat flour as affected by fermentation and baking time. Data are presented as mean ± SE of duplicates.

Tab. 1: Effect of fermentation on the deoxynivalenol (DON) content (mean ± SE) of dough prepared with or without yeast.

Fermentation time (min)	Yeast	DON (mg kg^{-1})	Significance[z]
0	Yes	8.85 ± 0.26	a
0	No	10.45 ± 0.21	b
100	Yes	6.21 ± 0.20	c
100	No	6.24 ± 0.18	c

[z] Means followed by different letters are significantly different according to Tukey's studentized range test at $P \leq 0.05$.

The average reduction rate during fermentation was 0.05 ± 0.01 mg DON kg^{-1} dry mass min^{-1}. The decrease of the DON content in the dough due to fermentation did not depend on the presence of yeast in the dough, because the effect was also observed in the absence of yeast (Tab. 1). Furthermore, no effect of fermentation on the DON content of artificially contaminated starch and gluten was observed (Tab. 2).

Tab. 2: Effect of fermentation on the deoxynivalenol (DON) content of artificially contaminated dough, starch and gluten.

Treatment	DON (mg kg^{-1})[z]	Sign.[y]	Recovery (%)[x]
Contaminated dough before fermentation	11.57 ± 0.01	a	116
Contaminated starch after fermentation	10.38 ± 0.04	a	104
Contaminated gluten after fermentation	10.05 ± 0.51	a	101
Contaminated dough after fermentation	6.62 ± 1.15	b	66
Flour without artificial contamination	0.04 ± 0.04	c	-
Gluten without artificial contamination	n.d.d.[w]	-	-
Starch without artificial contamination	n.d.d.[w]	-	-

[z] DON content calculated on fresh mass basis.
[y] Means followed by different letters are significantly different according to Tukey's studentized range test at $P \leq 0.05$.
[x] Theoretical artificial contamination level = 10 mg kg^{-1} DON (fresh mass basis).
[w] n.d.d. = no deoxynivalenol detected. Detection limit = 0.1 mg kg^{-1} (LUDEWIG, 2003).

The DON contents of flour / water mixtures were also decreased under fermentation conditions, whereas no significant effect was observed if fermentation was omitted (Tab. 3). After the centrifugation of the flour / water mixtures, the reduction during fermentation was observed in the supernatant, but not in the sediment (Tab. 3).
The DON levels were decreased by fermentation prior to baking (baking time = 0, Fig. 1), but there was no relationship between the DON content and fermentation time after baking rolls for 10 or 20 min (Fig. 1). The DON content of the rolls decreased almost linearly with baking time ($P = 0.01$) but it was not significantly affected by baking temperature (220 vs. 250 °C, $P = 0.29$, Fig. 2).

Tab. 3: Deoxynivalenol (DON) content of artificially contaminated flour/water mixtures (1 kg flour in 2 l water) before (fraction "mixture") or after centrifugation (fractions "supernatant" and "sediment") for 15 min at 10,000 g as affected by fermentation.

Fraction	Fermentation conditions	DON (mg kg^{-1})	Sign.[z]	Recovery (%)
Supernatant	Yes	5.88 ± 0.03	a	59
Mixture	Yes	7.09 ± 0.14	ab	71
Supernatant	No	8.21 ± 0.92	abc	82
Mixture	No	8.86 ± 0.18	bc	89
Sediment	Yes	9.73 ± 0.30	c	97
Sediment	No	10.17 ± 0.47	c	102

[z] Means followed by the same letter are not significantly different according to Tukey's studentized range test at $P \leq 0.05$.

The average reduction rate during baking was 0.35 ± 0.05 mg DON kg^{-1} dry mass min^{-1} (Fig. 2). Fermentation and baking decreased the DON content of the rolls to a similar extent in our experiments, but a combination of both processes (fermentation with subsequent baking) did not result in a better reduction than one of the steps alone (Fig. 1).

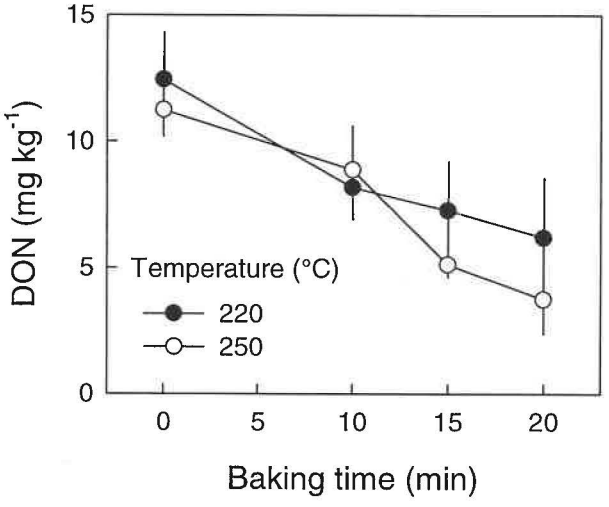

Fig. 2: Deoxynivalenol (DON) content of rolls prepared with contaminated wheat flour as affected by baking time and temperature. Data are presented as mean ± SE of duplicates.

Tab. 4: Effect of yeast on the deoxynivalenol (DON) content (mean ± SE) of rolls and DON distribution between crust and dough.

Treatment	DON (mg kg^{-1})	Significance[z]
Whole rolls without yeast	10.54 ± 0.47	a
Whole rolls with yeast	9.30 ± 0.49	a
Crust with yeast	8.02 ± 0.79	a
Dough with yeast	8.45 ± 0.90	a
Crust without yeast	9.82 ± 0.92	a
Dough without yeast	8.45 ± 1.49	a

[z] Means followed by the same letter are not significantly different according to Tukey's studentized range test at $P \leq 0.05$.

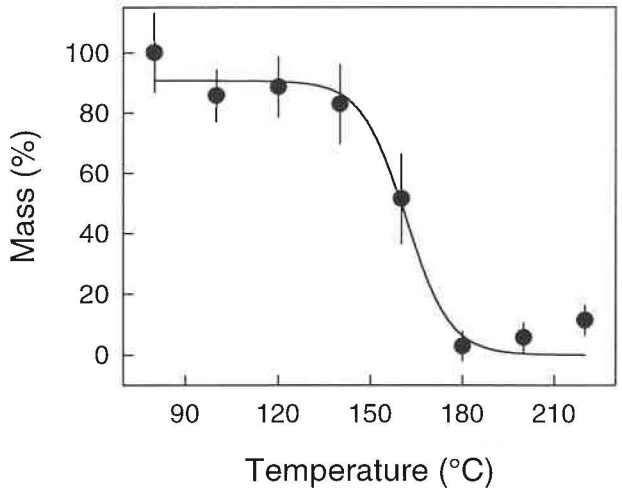

Fig. 3: Relative mass of deoxynivalenol as affected by temperature. Aluminium caps loaded with DON were incubated at the temperatures given on the x-axis of the graph for 30 min. Data are presented as mean ± SE of three replicates.

The DON contents of crust, dough and the whole rolls did not differ significantly and the distribution of the DON within the rolls was not affected by yeast (Tab. 4).

The mass of aluminium caps loaded with DON was not significantly affected by temperature up to 140°C (Fig. 3). Incubation at temperatures ≥ 160°C decreased the mass of the caps loaded with DON (Fig. 3).

Discussion

The effect of fermentation on the DON content of the dough

The DON contamination level was decreased by fermentation prior to baking, but it was independent of fermentation time after baking (Fig. 1). This result suggests that the compound(s) responsible for the decrease of DON levels during fermentation was/were not heat stable. Contradictory effects were reported for the effect of fermentation on the DON content in previous studies. NIERA et al. (1997) and SAMAR et al. (2001) observed DON reductions of about 22 – 52 % after the fermentation step of different bread types manufactured in Argentina. In contrast, YOUNG et al. (1984) observed an increase of the DON content in yeast doughnuts up to 189 and 118 % compared to the flour used. Since the yeast doughnuts were the only products in the study with a biological processing step, the authors speculated that the increase may be due to an enzymatic conversion of some precursor into DON. However, YOUNG et al. (1984) did not identify the hypothetical precursor of DON, and to our knowledge such a precursor has not been identified until today. We did not obtain any evidence for an increase of DON levels during fermentation in the present study.

DON reductions during bread making may occur due to thermal decomposition of the toxin, but also as a result of other processes during yeast fermentation (SAMAR et al., 2001). Selected yeast strains of technological relevance did not metabolize DON in liquid culture (BOSWALD et al., 1995; HAZEL and PATEL, 2004) but the same authors pointed out that yeasts used in bakery processing are not monocultures, and that *Lactobacillus* species may metabolize DON. EL-NEZAMI et al. (2002) observed removal of DON from liquid culture by some *Lactobacillus* strains.

Since DON was removed by viable as well as by heat-killed cells, EL-NEZAMI et al. (2002) concluded that the mechanism of DON removal was a binding of DON to the cells rather than metabolic processes in this case. But, however, the reduction of DON during fermentation was independent of yeast in the present study (Tab. 1). Because the compound(s) responsible for the decrease of DON levels during fermentation was/were not heat stable, fermentation with subsequent baking did not result in a better reduction than baking alone (Fig. 1). Furthermore, the compound(s) responsible for the decrease of DON levels during fermentation is/are water soluble (Tab. 3) and not related to starch or gluten (Tab. 2). Possible DON derivatives, which may have caused the reduction in DON during fermentation, are DON glucosides. Together with fatty acid esters, DON glucosides have been suggested to be the most likely DON metabolites in grain (SAVARD, 1991). POPPENBERGER et al. (2003) described a DON glucoside formation catalysed by a glucosyltransferase isolated from *Arabidopsis thaliana* in wheat germ extract and, furthermore, DON glucosides have recently been identified in naturally contaminated wheat (BERTHILLER et al., 2005). Glycosyltransferases are involved in the synthesis of cell wall components in plants (TAIZ and ZEIGER, 2000), and hence their occurrence can also be expected in wheat flour. Mycotoxin glycosides escape traditional analytical techniques, but the toxins can be regenerated in the digestive tract of humans and animals (POPPENBERGER et al., 2003). Hence, a decrease of mycotoxin concentrations in food is not necessarily an indication of a permanent detoxification.

The effect of baking on the DON content of the rolls

Baking at 220 or 250°C significantly lowered the DON content of rolls in the present study. Since water evaporates from the surface of dough during baking, an accumulation of water soluble non-volatile compounds may be expected at the surface of the crust of rolls. The mass of rolls decreased linearly with baking time due to water loss (KLINGLER, 1995) and so did the DON content (Fig. 2). Since the effect of fermentation did not seem to be heat stable (Fig. 1), the reduction in DON content during baking must be attributed to another mechanism than the DON reduction during fermentation. WOLF and BULLERMAN (1998) reported that temperatures higher than 120°C are required to destroy DON in an aqueous environment. Temperatures inside of bread or rolls do not exceed 100°C within reasonable baking times (KLINGLER, 1995) because of the presence of water. Thus, the decrease of DON levels in the dough cannot be attributed to thermal destruction. In contrast, temperatures at the surface of the crust exceed 100°C, the extent depending on baking time. At 170°C, WOLF and BULLERMAN (1998) observed rapid destruction of DON and, hence, thermal destruction of DON may have occurred at the surface of the crust. Alternatively, some DON may have evaporated. This hypothesis is supported by the loss of mass of aluminium caps loaded with DON at temperatures ≥ 160°C (Fig. 3), which is above the melting point of DON (151°C, BUDAVARI et al., 1996). A scenario that is in agreement with the information currently available would be a relocation of DON by water during baking from the dough into the crust and a partly thermal destruction or evaporation at the surface of the crust. WEIDENBÖRNER (2001) recently summarized that Japanese studies showed a 50% reduction in DON levels during baking compared to the original concentration, a result that is in general agreement with our observations.

Our results showed a significant decrease of DON concentration during fermentation of dough and baking of rolls. The effect of fermentation was not related to yeast, starch or gluten and it was not detectable after baking any more. Baking decreased the DON content of rolls at an average rate of 0.35 mg kg^{-1} dry mass min^{-1} at baking temperatures of 220 or 250 °C. If following studies confirm the rapid decrease of DON levels during baking, future research should focus on (1) transport of DON within and at the surface of the products during baking and (2) the identification and the toxicity of potential DON derivatives.

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