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Impact of *Alternaria* spp. and *Alternaria* Toxins on Quality of Spelt Wheat

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Abstract

There is an increasing consumer demand for alternative cereals nowadays. Spelt wheat (*Triticum aestivum* ssp. Spelta) appears to be a future source for the agriculture and food sector. *Alternaria* spp. infections might become a serious danger to the worldwide grain industry, resulting in yield losses and reduction of end-use quality, with potential harmful effect of *Alternaria* toxins on human and animal health. This paper presents the first assessment of the impact of *Alternaria* infection and its toxins to quality of spelt wheat. The results showed that fungal contamination significantly reduced both trade and technological quality parameters. Volume weight, thousand kernel weight and wet gluten were significantly decreased, while protein content was significantly higher in *Alternaria* inoculated treatments. Although with slight decrease, falling number was not significantly affected by fungal contamination. The most negative impact of alternariol (AOH) was registered on volume weight and thousand kernel weight (-0.847; -0.898), while highly significant positive correlation was found between AOH and protein content (0.758). *Alternaria* spp. additionally destroyed spelt gluten structure, resulting in reduction of dough energy and baking quality with no significant influence of mycotoxins (AOH and AME) on technological quality parameters.

Keywords: *Alternaria* spp., Spelt wheat, trade, technological quality

1. Introduction

Driven by consumer demand for more "natural and healthier" sustainably produced food, spelt wheat (*Triticum aestivum* ssp. Spelta) has been attracted increasing popularity on the market nowadays (Schober, Clarke, & Kuhn, 2006). Spelt is recognized as one of the most suitable cereals for organic farming. Due to high tolerance to environmental factors, spelt can be grown in rough ecological conditions, with no pesticides use (Bavec & Bavec, 2006). It is considered that natural protection provided by the hulls makes spelt more resistance to pathogens and their toxic metabolites (Bodroža-Solarov et al., 2010, Vučković, et al., 2013). Spelt kernels are also recognized by valuable nutritional properties, making spelt based products easy digested with a higher protein content compared to common wheat (Bonafaccia et al., 2000; Kohajdová & Koravičová, 2008; Bodroža-Solarov, Mastilović, Filipčev, & Šimurina, 2009; Pasqualone et al., 2011; Escarnot, Jacquemin, Agneessens, & Paquot, 2012; Filipčev, Šimurina, Bodroža-Solarov, & Brkljača, 2013). On the other side, due to inferior gluten performances, spelt species is often crossed with winter wheat to improve the quality of flour (Schober, Clarke, & Kuhn, 2002).

Alternaria spp. are cosmopolitan saprophitytic and phatogenic fungi reported on various crops causing diseases on cereals (Zur, Shimoni, Hallerman, & Kashi, 2002; Mercado Vergnes, Renard, Duveiller, & Maraite, 2006; Bensassi, Zid, Rhouma, Bacha, & Hajlaoui, 2009; Tóth et al., 2011; Andersen, Nielsen, Pinto, & Patriarca, 2015), but also fruits (Serdani, Kang, Andersen, & Crous, 2002; Tournas & Katsoudas, 2005; Magnani, De Souza, & Rodrigues-Filho, 2007; Ntasiou, Myresiotis, Konstantinou, Papadopoulou-Mourkidou, & Karaoglanidis, 2015; Andersen et al., 2015), and vegetables (Noser, Schneider, Rother, & Schmutz, 2011; Edin, 2012; Zheng, Zhao, Wang, & Wu, 2014; Andersen et al., 2015). The importance of *Alternaria* spp. is not only limited to yield losses, but even more significant is post-harvest decay and quality reduction of final products (Kosiak, Torp, Skjerve, & Andersen, 2004; Ostry, 2008; de Miranda et al., 2006). During the fungal infection process, *Alternaria* spp. very often prouduce toxic secondary metabolites, where the the most important are alternariol (AOH), alternariol monomethyl ether (AME), altenuen (ALT), tenuazoic acid (TEA) and altertoxins (ATX-I, II, III) (Logrieco, Moretti, & Solfrizzo, 2009). The EFSA reported they frequent occurrence in small grains and cereal-based products and highlighted their potential health risks for humans and/or livestock (EFSA, 2011).

Considering the fact that spelt is usually cultivated in low input system, with reduced use pesticides, continuous monitoring of pathogens and their impact on food safety and quality is of high importance. The latest literature overview showed that the predominant mycobiota on spelt wheat are *Fusarium* spp. (Hudec & Lacko-Bartošova, 2012) and *Alternaria* spp. (Kurowski, Damszel, & Wysocka, 2012), which are reducing the yield, but there is no data about impact of fungal contamination on technological parameters of spelt quality and food safety.

This research was carried out to assess the influence of *Alternaria* spp. to trade and technological quality of spelt wheat and to evaluate the correlation between *Alternaria* toxins and quality parameters.

2. Method

2.1 Field Experiment and Artificial Inoculation

The filed trial was set up in randomized complete block design with four replicates in mid-October 2013. Each plot was consisted of 10 rows, 10 cm apart and 5 m long (the harvested area was 5 m^2) with seedling density of 500 seeds m^2. Inoculation of three spelt wheat genotypes (Nirvana, Austria and Ostro) was performed on the full flowering stage by spraying spikes with 200 mL suspension of *Alternaria* spp. infection material (in a concentration of 1×10^6/mL). In parallel, fungicide treatment (prochloraz 267g/L + tebuconazol 133g/L) in a concentration of 0.2% was applied.

Alternaria spp. isolates, derived from the contaminated kernels of spelt wheat in northeastern Serbia, were previously identified by PCR assays. The sequence of the primers, PCR reaction mixture, and the conditions used for detecting were identical to those described by Bensassi et al. (2009).

Multiplication of isolates was done on a potato dextrose agar followed with 14 days incubation at 25 °C in the dark. The inoculum was prepared by mixing infectious material in a blender with the addition of distilled water. Filtered suspension was adjusted to a concentration of 1×10^6 infective particles mL^{-1} using a haemocytometer.

Inoculated spikes were immediately covered with polyethylene bags. After 24 h the bags were removed. Inoculation treatments were replicated after two weeks. In the full ripeness stage spikes were cut by hands and dehulled using laboratory tresher MDF1, RePietro, Gaggiano, Italy.

2.2 Alternaria Mycotoxins Determination

Determination of alternariol (AOH) and alternariol monomethyl ether (AME) was carried out on a 1260 series HPLC system with a DAD detector (Agilent Technologies, USA). Milled and homogenized spelt samples were extracted with acetonitrile 4% KCl (9:1, 75 mL) by shaking on a rotary mechanical shaker (185 rpm) at room temperature (22-24 °C) for 30 min followed by the addition of 1 N HCl (15 mL). After filtration, 45 mL of the filtrate (equal to 7.5 g of wheat sample) was purified with 90 mL of 0.05 M lead acetate. The filtration is repeated and 75 mL filtrate was extracted three times with 20 mL of dichloromethane. The organic phases were joined, evaporated to dryness, and dissolved in 1 mL of methanol for AOH and AME analyses by HPLC. The analytical column was Agilent Eclipse XDB-C18 (4.6 × 50 mm, USA) thermostated at 40 °C. Injection volume was 5 µL. A methanol/water mobile phase was used in a gradient regime (solvent A:H20 with 385 mg L^{-1} amoniumacetate, solvent B:methanol) at a flow rate of 0.5 mL min^{-1}. Initially mixture of the mobile phase was held 0.5 min, successively elevating the concentration of methanol linearly to 100% within 9.5 min. By maintaining of these conditions for 3.5 min, the concentration of methanol was converted back to the initial composition. The acquisition of chromatograms was completed after 20 min. The detector operated at an excitation wavelength of 257 nm with quantification limit of 1.25 µg mL^{-1} for both examinated *Alaternaria* toxins. Detection and quantification of the toxins were carried out by the Software Agilent Chem Station for LC 3D Systems Rev.B.02.01SR1 (260).

2.3 Standard Trade Technological Quality Parameters

Samples were analyze by standard methods for assessing trading and technological wheat quality: crude protein content (CP) in grain dry matter (NIT analyzer ‘‘Infratec 1241’’), wet gluten content (WG) in grain dry matter (ICC Standard No. 106/2) and gluten index (GI) using the apparatus Glutomatic Perten (ICC Standard No. 155), falling number – FN (ICC Standard No 107/1), volume weigh (VW) – ISO7971-2, and TKW (thousand kernels weight).

2.4 Rheological Characteristics

2.4.1 Mixolab®

Spelt samples were milld on Bühler mill automat MLU 202 (Bühler, Uzwill, Switzerland). Protein and starch parameteres of the spelt flour (dough development, protein weakening, starch gelatinization, diastatics activity, and anti-stalling effect) were detected on Mixolab (Chopin, Tripette et Renaud, Paris, France) following Mixolab protocol Chopin+ (Mixolab appl. Handbook, 2008). A typical Mixolab curve, presented in Figure 1, is devided to the five stages showed by five (C1-C5) points.

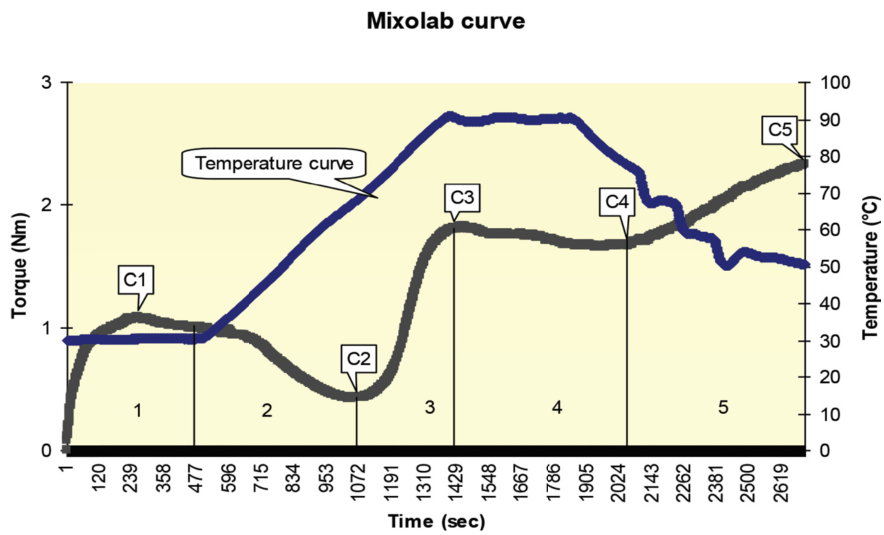

Figure 1. Standard Mixolab curve

2.4.2 Extensograph

Extensograph measurements on dough were made using standard method ICC 114/1 on Brabender Extensograph (Brabender, Duisburg, Germany). The recorded load-expansion curve is used to survey the general quality parameters of flour such as the resistance of the dough to the extension, the degree to which it can be extended before breaking, energy and the ratio between resistance and an extent (R/E).

2.5 Statistical Analysis

The evaluation of results was done by the analysis of variance (ANOVA) followed with Tukey's HSD test of the statistical significance at the level $p < 0.05$ and $p < 0.10$. The calculation was done in Statistica 12.0 (StatSoft, Inc., 2012). Principal Component Analysis (PCA) was applied to characterize the samples according to obtained results and to differentiate these samples in order to discover the possible correlations among measured parameters.

3. Results and Discussion

3.1 Content of Alternaria Toxins and Trade Quality Parameters

The average levels of *Alternaria* toxins in spelt grains are summarized in Table 1.

The content of AOH and AME was determinated at several times higher concentrations within inoculated kernels in the range of 227-428 µg/kg with significant difference compared to kernels treated with fungicide (81-153 µg/kg). Different treatments significantly influenced both mycotoxins content, with significant differences between the spelt wheat genotypes in AME contamination, treated with *Alternaria* spp. These results are confirmed in previous research indicating significant protective impact of spelt hulls on *Alternaria* spp. contamination and their mycotoxins (Vučković et al., 2013, Đisalov et al., 2015).

Table 1. Content of *Alternaria* mycotoxins in inoculated spelt wheat kernels and treated with fungicide

Treatment	Genotype	AOH	AME
Alternaria spp.	Nirvana	322±16.18[b]	428±30.8[bc]
	Austria	331±30.34[b]	398±28.55[bc]
	Ostro	227±12.79[b]	277±17.97[c]
Fungicide	Nirvana	84±8.23[a]	153±13.41[a]
	Austria	89±13.75[a]	120±11.76[a]
	Ostro	81±5.49[a]	113±10.81[a]

Note. Different letters in the column show statistically significant values at $p \leq 0.05$, according to Tukey's HSD test; AOH-alternariol, AME-alternariol monomethyl ether.

Alternaria spp. influenced significant reduction of volume weight (VW) and thousand kernel weight (TKW) in all inoculated samples with exception in Ostro (Table 2). This was expected since kernels affected by *Alternaria* spp. may become shriveled, directly resulting the reduction of VW and TKW. On the other hand, protein content (CP) was significantly higher in *Alternaria* spp. inoculated spelt genotypes with high significant differences between treatments at all three genotypes. The similar findings reported (Šarić, Stojanović, Škrinjar, & Menkovska, 2008; Malaker, Mian, Bhuiyan, Reza, & Mannan, 2009) in evaluation of the impact of *Alternaria* spp. on the same quality parameters of common wheat.

Levels of wet gluten were variable depending on treatment with statistically significant differences between genotypes with exception at genotype Austria, indicating that *Alternaria* inoculation additionally decreased gluten properties. Falling number was not significantly affected by *Alternaria* spp. treatment, showing levels over 350 s at all three genotypes. Nevertheless, inoculation influenced degradation of starch and higher alpha-amylase activity and falling number was slightly decreased.

Table 2. Trade quality parameters of spelt wheat genotypes

Treatment	Genotype	VW (kg/hL)	TKW (g)	CP (%)	FN (s)	WG (%)	GI
Alternaria spp.	Nirvana	74.2±0.19[b]	41.45±0.53[ac]	18.21±0.10[b]	373.75±12.69[a]	38.8±0.43d	85
	Austria	73.95±0.12[b]	39.6±1.70[c]	17.70±0.18[c]	361.25±6.10[a]	45.7±0.8[e]	30
	Ostro	74.65±0.10[c]	42.45±1.00[ab]	18.56±0.05[a]	353.25±14.10[a]	49±0.45[b]	26
Fungicide	Nirvana	75.65±0.16[c]	42.9±1.00[a]	16.79±1.00[e]	374±9.93[a]	42.7±0.16[c]	66
	Austria	74.85±10.16[a]	44.10±1.40[b]	17.20±0.10[d]	373.50±8.74[a]	46.5±0.36[e]	23
	Ostro	74.8±0.10[a]	43.85±0.82[ab]	16.00±0.13f	363.75±5.85[a]	50.6±0.22[a]	29

Note. Different letters in the column show statistically significant values at $p \leq 0.05$, according to Tukey's HSD; VW: volume weight; TKW: thousand kernels weight; CP: crude protein content in the grain dry matter; FN: falling number; WG: wet gluten content in the grain dry matter; GI: gluten index.

Abovementioned results were additionally affirmed by correlations between *Alternaria* toxins and assessed quality parameters (Table 3), where the statistically significant negative correlation was found between *Alternaria* toxins content and VW and TKW. AOH the most negatively effected VW and TKW (-0.847; -0.898), while highly significant positive correlation coefficient was detected between AOH and crude protein content (0.758).

Table 3. Correlation between mycotoxin content and the trade quality parameters of spelt wheat genotypes

	VW	**TKW**	**CP**	**FN**	**WG**	**GI**
AOH	-0.847*	-0.898*	0.758**	-0.286	-0.422	0.270
AME	-0.784**	-0.891*	0.746**	-0.183	-0.537	0.405

Note. *Correlation is statistically significant at $p < 0.05$ level, **Correlation is statistically significant at $p < 0.10$ level; AOH: alternariol; AME: alternariol monomethyl ether; VW: volume weight; TKW: thousand kernels weight; CP: crude protein content in the grain dry matter; FN: falling number; WG: wet gluten content in the grain dry matter; GI: gluten index.

3.2 Technological Quality Parameters

The ANOVA test showed statistically high significance of impact all sources of variation on examined parameters, indicating that extensographic parameters are genotype characteristic, but also indicators of dough quality (Table 4).

Table 4. Extensograph parameters and mixolab characteristics of spelt flour from different genotypes inoculated with *Alternaria* spp. and treated with fungicides

Factor		Extensograph				Mixolab
Treatment	Genotype	Extension area (cm^2)	Resistance to extension (BU)	Extensibility (mm)	Ratio (R/E)	C2 (Nm)
Alternaria spp.	Nirvana	$85±0.82^{b}$	$240±0.96^{b}$	$179±1.52^{e}$	1.31	0.32^{b}
	Austria	$26±2.16^{de}$	$100±1.63^{e}$	$186±3.56^{d}$	0.57	0.29^{c}
	Ostro	$24±1.63^{e}$	$95±0.98^{de}$	$203±2.83^{b}$	0.49	0.28^{c}
Fungicide	Nirvana	$92±2.16^{a}$	$270±1.16^{a}$	$183±2.16^{de}$	1.51	0.36^{a}
	Austria	$31±0.82^{c}$	$110±2.16^{d}$	$194±0.82^{c}$	0.59	0.32^{b}
	Ostro	$29±1.16^{cd}$	$108±1.41^{c}$	$217±1.41^{a}$	0.44	0.28^{c}

Note. Different letters in the column show statistically significant values at $p \leq 0.05$, according to Tukey's HSD test.

Alternaria spp. influenced reduction of dough energy and thus baking quality as it is shown in Table 4. The highest and statistically significant energy is detected at Nirvana genotype in both treated with fungicides (92 cm^2) and inoculated samples (85 cm^2), while Ostro has the lowest energy in the treated and inoculated samples at the levels of 24 cm^2 and 29 cm^2, respectively. Resistance and extensibility were also significantly lower in *Alternaria* spp. inoculated variants. Our results are in correlation with conclusions of Antes et al. (2001) who assessed *Aspergillus* and *Penicillium* species and concluded that fungal contamination have reduced dough resistance and extensibility influencing low bread volume. In contrary, Prange et al. (2005) reported that that *Fusarium* infection did not negatively influence baking quality highlighting concerns that fungal contamination and their mycotoxins is not evidenced through baking quality analysis which might have considerable impact on food safety.

Mixolab results confirmed that *Alternaria* spp. infection markedly demages both protein and starch quliaties of spelt wheat. The average value of C2, representing the protein weakening, was lower in artificially inoculated variants for all genotypes, with exception in Ostro. Our results confirmed findings of Papoušková et al. (2011) and Capouchová et al. (2017) where *Fusarium* spp. significantly affected protein structure at wheat grains, with a slightly better resistance of spelt wheat.

According to correlation analysis presented in Table 5, there were no statistically significant correlations between the mycotoxin content (AOH and AME) and the technological quality parameters.

Table 5. Correlation between the mycotoxin content and the rheological quality parameters

	Extension area	Resistance	Extensibility	Ratio	C2
AOH	0.100	-0.027	-0.481	0.031	-0.302
AME	0.242	0.116	-0.566	0.172	-0.184

Note. *no correlation was statistically significant.

3.3 Principal Component Analysis (PCA)

The similarity of observed samples is indicated by the close vicinity of the points which are representing them in the PCA graphic. The variables are presented by vectors in factor space, while the correlation between two variables is indicated by the angle between vectors (the high correlation corresponds to small angle between vectors) (Krulj et al., 2016). Having this in mind, it can be observed that high positive correlation exists between AME and AOH content ($r = 0.990$, $p < 0.001$), and also the strong negative correlation to trade quality parameters VW and TKW. The statistically significant correlations between trade quality parameters were not observed. WG was negatively correlated to rheological parameters: extension, resistant and ration ($r = -0.887$; $r = -0.823$ and r =

-0.860, respectively, statistically significant at $p < 0.05$ level) and positively correlated with extensibility ($r = 0.910$, $p < 0.05$), while GI was positively correlated to extension, resistant and ration ($r = 0.979$; $r = 0.929$ and $r = 0.917$, respectively, $p < 0.01$). FN was correlated to C2 ($r = 0.822$, $p < 0.05$).

The PCA of the presented data explained that the first two components accounted for 87.85% of the total variance (52.04 and 35.81%, respectively) in the thirteen variables space. Considering the map of the PCA performed on the data, WG (which contributed 13.7% of total variance, based on correlations) and extensibility (10.9%) exhibited positive scores according to first principal component, whereas falling number (7.7%), gluten index (13.0%), C2 (9.8%), resistance (13.1%), ratio (13.8%) and extension (13.6%), showed a negative score values according to first principal component (Figure 2). The positive contribution to the second principal component calculation was observed for: volume weight (17.2% of total variance, based on correlations) and thousand kernel weight (16.1%), while negative scores on second principal component calculation was observed for AOH (20.3%), AME (18.6%) and protein content (12.5%).

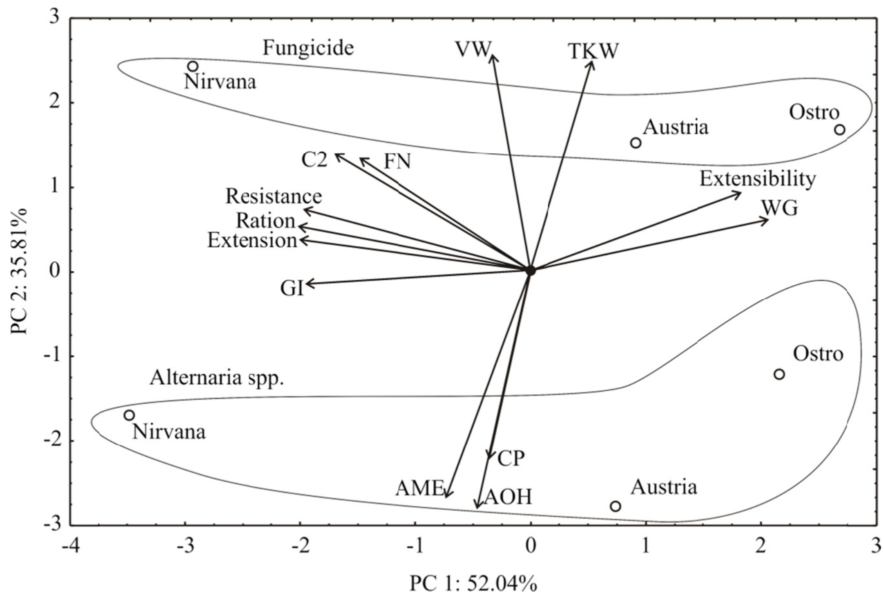

Figure 2. Principal Component Analysis ordination of observed variables based on correlations

The map of PCA graphic showed that the first principal component described the differentiation among the wheat cultivars, according to rheological properties and some trade quality characteristics (WG, FN and GI), while the second principal component described the variations in AME and AOH content and trade quality parameters, such as: VW, TKW and CP.

The first principal component is affected by rheological properties, WG, FN and GI. Ostro variety is characterized by the highest extensibility, as well as the highest WG value, regardless of the AME and AOH content, while Nirvana cultivar is noticed for the highest resistance, ration extension and C2 properties, as well as the higher FN and GI parameters.

The second principal component is influenced by the AOH and AME content, by the trade quality parameters VW, TKW and CP, and also by the spelt wheat treatment and variety. The samples treated by *Alternaria* spp. are located at the bottom of the PCA graphic, where the highest AME and AOH content and the highest CP in samples were found. Austria is the most prone variety for AOH and AME infection, while Ostro is the most resistant.

4. Conclusion

The negative impact of *Alternaria* spp. infection and *Alternaria* toxins of the quality of spelt wheat was confirmed by standard methods for trade quality detection followed by the complex rheological evaluation. The significantly reduced quality, both trade and technological, was markedly influenced by *Alternaria* spp. compared to fungicide treatments, with negative impact of mycotoxins as well. *Alternaria* spp. additionally destroyed spelt weaker gluten structure, subsequently significantly affected the quality of dough properties.

In terms of food safety, continuous monitoring of *Alternaria* contamination must be of high priority, in both filed and storage facilities of spelt wheat, particularly taking in consideration that spelt wheat is commonly grown in low input systems with limited pesticides use. In the case of a strong infection, herewith stimulated by artificial inoculation, *Alternaria* toxins are strongly correlated with reduction of trade quality, potentially providing risks for human and animal consumption.

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