Effects of nitrogen nutrition, fungicide treatment and wheat genotype on free asparagine and reducing sugars content as precursors of acrylamide formation in bread

P. Martinek[1], K. Klem[2], M. Váňová[1], V. Bartáčková[3], L. Večerková[1], P. Bucher[4], J. Hajšlová[3]

[1]*Agrotest Fyto, Ltd., Kroměříž, Czech Republic*
[2]*Institute of Systems Biology and Ecology, Brno, Czech Republic*
[3]*Institute of Chemical Technology Prague, Czech Republic*
[4]*Potato Research Institute Havlíčkův Brod, Ltd., Czech Republic*

ABSTRACT

Acrylamide, a monomer with neurotoxic and potential carcinogenic effect, is formed via the Maillard reaction in heat-treated carbohydrate-rich foods. The major acrylamide precursors are reducing sugars and the amino acid asparagine. The aim of this study was to analyse effects of nitrogen nutrition, leaf disease control, wheat genotype and their interactions on acrylamide precursors content in wheat flour. Asparagine content was generally increasing at higher nitrogen doses, and nitrogen dose increase from 0 to 180 kg/ha increased the asparagine content to about 250%. The highest asparagine levels were determined at early spring nitrogen application. In the year 2006 with high leaf disease infestation, fungicide treatment decreased asparagine content particularly at higher nitrogen doses. In 2007, the effect of leaf disease control did not express in respect of very low infestation level. Close relationship between protein content and free asparagine in wheat flour was determined when leaf disease stress (fungicide treatment) and drought stress (year) were constant. Asparagine content was strongly influenced by wheat genotype and the differences between genotypes exceeded 200%. Effect of higher intensity was lower as compared to nitrogen nutrition, with regard to compensatory effect of fungicide treatments. Glucose content in wheat flour decreased both with fungicide treatment and total intensity level. Nitrogen dose increased glucose content up to 120 kg N/ha. Higher nitrogen doses decreased glucose content to initial level.

Keywords: acrylamide precursors; nitrogen nutrition; leaf disease control; wheat genotype

The presence of acrylamide in foods has attracted interest after Swedish scientists (Tareke et al. 2002) discovered in 2002 that heat treatment of starch-rich foods induced relatively high levels of acrylamide. Exposure to acrylamide causes damage to the nervous system of both humans and animals (Lopachin and Lehning 1994). Acrylamide is also considered as a significant reproductive toxin with mutagenic and carcinogenic effects in mammals (Dearfield et al. 1995), and was classified as carcinogenic to humans by the International Agency on Research on Cancer (IARC 1994). Acrylamide formation in foods was documented by numerous studies (see Stadler and Scholz 2004). First detected factors essential for acrylamide formation in heat-treated food were high starch content and insufficient water amount during the thermal process. A number of research teams simultaneously discovered that acrylamide is formed via so-called Maillard reaction and major reactants of its formation are reducing sugars and the amino acid asparagine (reviewed by Becalski et al. 2002). Much attention was paid to methods of food preparation (temperature, processing time, pH, effects of additives, and others), which resulted in lots of interesting findings that enable to reduce risks of acrylamide formation (reviewed by Taeymans et al. 2004). However, there is still lack of knowledge about effects of genotype and cropping factors on the content of acrylamide formation precursors.

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Asparagine is the main substance for transport of reduced nitrogen from roots into leaves by xylem and from leaves into newly formed seeds by phloem (Pate 1980). Free asparagine is accumulated mostly under conditions of limited protein synthesis and excessive offer of reduced nitrogen in plants (Lea et al. 2007). Asparagine is also one of main metabolic products during leaf senescence. Therefore, the increased free asparagine accumulation can be induced by stress conditions, such as drought, nutrient deficiency or effects of pathogens, which speed up the senescence. A considerable role is obviously played also by genetic regulation of enzymes participating in the synthesis and degradation of asparagine in plant. According to Weber et al. (2008), free asparagine content in wheat flour increases at increased nitrogen doses (220 kg/ha) up to 270% relative to non-fertilized control depending on cultivar and year. The increased potential of acrylamide formation is particularly caused by nitrogen fertilization resulting in protein content higher than 13%. The content of reducing sugars (glucose, fructose), as the second of important acrylamide formation precursors, is affected in winter wheat mainly by year and genotype. A limiting factor of acrylamide formation in cereal products is mostly the content of free asparagine. In rye, free asparagine is converted to acrylamide on a level of around 0.80%; in wheat it is 0.98% and in potatoes only 0.29% (Elmore et al. 2005). More detailed studies on the effect of cropping treatments, such as nitrogen application timing and dose, fungicide protection against diseases, effect of genotypes, and their interactions on the content of acrylamide formation precursors in cereals are not available. The aim of the presented work was thus to complete missing data related to the effect of cropping factors on potential acrylamide formation during heat treatment, to find relationships between individual factors and to find possibilities for reduction of risks of acrylamide formation in bakery products.

MATERIAL AND METHODS

Evaluation of the effect of nitrogen nutrition in interaction with leaf disease control on acrylamide precursors content in flour

In 2006–2007, field experiments were set up after winter wheat as preceding crop aiming at an analysis of the impact of total nitrogen dose, application timing and form in interaction with the effect of fungicide treatment on acrylamide precursors content in flour. The experiments were conducted on winter wheat cv. Ebi. Nitrogen doses were increased stepwise by 60 kg N/ha from 0 to 180 kg N/ha. Nitrogen was applied at the three basic timings: T1 – regeneration topdressing (the first half of tillering – BBCH 23-25), T2 – production topdressing (the beginning of stem elongation – BBCH 31-32), T3 – late topdressing (the end of stem elongation to the beginning of heading – BBCH 39-51).

Nitrogen was applied in the form of limestone ammonium nitrate 27.5% N (LAN) or urea (UR). The fertilization applications are surveyed in Table 1. Each fertilization variant was divided into the two sub-variants with different levels of fungicide

Table 1. Nitrogen applications in individual fertilization variants

Variant No.	T1 – regeneration BBCH 23-25	T2 – production BBCH 31-32	T3 – late BBCH 39-51
1	0 kg N		
2	60 kg N/ha LAN		
3		60 kg N/ha LAN	
4			60 kg N/ha LAN
5	60 kg N/ha LAN	60 kg N/ha LAN	
6	60 kg N/ha LAN	60 kg N/ha LAN	60 kg N/ha LAN
7	120 kg N/ha LAN		
8		120 kg N/ha LAN	
9	180 kg N/ha UR		
10	120 kg N/ha UR		60 kg N/ha LAN

LAN – limestone ammonium nitrate; UR – urea

Table 2. Fungicide applications in the treated variant (F)

T1	BBCH 31-32	Alert 0.8 + Capitan 0.4 l
T2	BBCH 39	Sfera 0.4 l
T3	BBCH 59-61	Amistar 0.4 l + Caramba 0.8 l

treatment: (a) untreated control and (b) intensive fungicide treatment. The fungicide applications in the treated variant are shown in Table 2. Each combination of nitrogen nutrition and fungicide treatment was replicated three times in 10 m^2 plots. The plots were laid out in a randomized split block design. After harvest, a representative bulk sample was obtained from the three replications, used for flour milling and analyses of asparagine and reducing sugars contents. Based on the results from 2006 and 2007, only four contrast nitrogen dose treatments were taken for analysis of acrylamide precursors (each in fungicide treated and untreated sub-variants).

Evaluation of the effect of crop management intensity in interaction with winter wheat genotype on acrylamide precursors content in flour

In 2006–2007, experiments were set up after oilseed rape as preceding crop aiming at the evaluation of effects of crop management intensity and winter wheat genotype on the content of asparagine and reducing sugars in flour. A collection of winter wheat cultivars with different breadmaking quality classifications (Ebi, Sulamit, Ludwig, Samanta, Batis, Bill, Complete, Drifter, Contra, Estica) was used to establish variants at three crop management intensities: low (L), medium (M) and high (H). These variants differed in the level of nitrogen nutrition, frequency and dose of fungicide applications, and dose of growth regulators used against lodging (Table 3).

After harvest, a representative bulk sample was obtained from the four replications of the variant, which was used for further analyses. First, basic

Table 3. Applications in individual variants of crop management intensity

Growth stage	Intensity		
	L	M	H
In spring after growth regeneration	30 kg N/ha (LAN 27.5)	30 kg N/ha (LAN 27.5)	30 kg N/ha (LAN 27.5)
Tillering			66 kg N/ha (UR)
End of tillering		Retacel Extra R 68 0.75 l/ha	Retacel Extra R 68 1 l/ha
		Topsin M 70 WP 0.5 kg/ha	Campofort Forte 5 kg/ha
End of tillering to the beginning of stem elongation	30 kg N/ha (LAN 27.5)	30 kg N/ha (LAN 27.5)	40 kg N/ha (LAN 27.5)
First half of stem elongation	30 kg N/ha (UAN 390)	30 kg N/ha (UAN 390)	30 kg N/ha (UAN 390)
	Retacel Extra R 68 1 l/ha	Retacel Extra R 68 1.5 l/ha	Retacel Extra R 68 1.5 l/ha
			Moddus 0.15 l/ha
			Alert 0.8 l/ha
			Capitan 0.4 l/ha
End of stem-elongation	30 kg N/ha (LAN 27.5)	30 kg N/ha (LAN 27.5)	40 kg N/ha (LAN 27.5)
			Sfera 267.5 EC 0.4 l/ha
			Sunagreen 1.5 l/ha
			12.5 kg N/ha (UR-solution)
Flag leaf initiation	Bumper 25 EC 0.5 l/ha	Artea 330 EC 0.5 l/ha	
Heading			Amistar 0.4 l/ha
			Caramba 0.8 l/ha
			12.5 kg N/ha (UR-solution)

LAN – limestone ammonium nitrate; UAN – urea ammonium nitrate; UR – urea quality parameters for evaluation of breadmaking quality (protein content, volume weight, 1000-grain weight, grain hardness, ash content, wet gluten, falling number and Zeleny /SEDI/ test) were assessed. Based on these analyses, genotypes with contrast quality parameters were selected for determination of free asparagine and reducing sugars contents in flour. A basic criterion for the selection was the protein content in grain.

HPLC/FLD analytical method for analysis of asparagine

The method is based on asparagine extraction by shaking of sample in 20 ml of methanol/water (1:1; v/v). In the next step, the sample is 10x diluted with water. Final step is addition of 0.5 ml of 0.005M HCl containing an internal standard homoserine to 0.5 ml of diluted extract. The determinative step is HPLC/FLD technique with pre-column derivatization by *o*-phtaldialdehyde. For the quantification homoserine, as an internal standard, is used.

HPLC/RID analytical method for analysis of sugars

The method is based on methanol/water (1:1; v/v) sample extraction by shaking and supported by sonication. In the next step, sample is centrifuged at 6000 rpm. The solid sediment is washed by ITL and is combined with another portion of extraction mixture. Combined extracts are analysed using Ca^{2+} ionex column with water as a mobile phase. The identification and quantification of target analytes (glucose, fructose) were carried out by simple HPLC/RID technique.

RESULTS AND DISCUSSION

Evaluation of the effect of nitrogen nutrition in interaction with leaf diseases control on acrylamide precursors content in flour

The effect of nitrogen nutrition and fungicide treatment on basic quality parameters of grain and content of acrylamide precursors in flour is

Table 4. Comparison of basic grain quality data and acrylamide precursors in flour in both experimental years (2006 and 2007)

		Thousand grain weight (g)		Volume weight (kg/hl)		Protein content (%)		Asparagine (mg/kg)		Glucose (mg/kg)		Fructose (mg/kg)	
a) Experiment with nitrogen nutrition and fungicide treatment													
Fungicide treatment	(kg N/ha)	2006	2007	2006	2007	2006	2007	2006	2007	2006	2007	2006	2007
Fungicide	0	41.3	39.2	80.1	75.4	13.3	11.7	100	114	116.0	140.1	132.5	100.2
	60 (T1)	40.4	39.5	81.1	76.1	13.6	14.4	126	149	389.8	116.3	241.0	76.4
	120 (T1, T2)	43.2	38.8	80.5	75.1	14.4	14.5	184	146	114.0	110.4	123.2	70.4
	180 (T1)	41.3	36.9	81.0	74.9	14.0	13.3	148	215	212.4	134.7	143.9	94.6
Untreated	0	43.4	38.5	79.9	74.9	12.1	12.4	102	112	270.8	147.7	160.4	107.8
	60 (T1)	35.7	38.9	79.3	74.6	12.0	12.7	116	142	535.8	111.3	150.7	71.4
	120 (T1, T2)	34.7	38.5	79.3	76.0	14.0	15.8	176	168	352.4	124.9	154.1	84.8
	180 (T1)	36.5	36.4	78.5	74.2	14.2	14.2	254	181	465.7	134.9	189.1	94.8
b) Experiment with wheat genotypes and intensity of crop management													
Variety	Intensity	2006	2007	2006	2007	2006	2007	2006	2007	2006	2007	2006	2007
Samanta	L	42.7	44.9	82.9	77.3	12.6	13.1	46	93	270.7	105.3	197.3	95.2
	M	44.0	46.2	83.4	77.7	13.2	12.9	62	90	212.3	127.9	185.4	87.8
	H	40.0	44.8	83.4	77.7	14.2	13.8	70	123	69.6	77.7	104.6	64.0
Sulamit	L	41.3	41.7	82.8	77.0	12.7	12.3	90	102	302.2	112.3	312.7	92.4
	M	39.3	42.6	83.5	77.7	13.7	12.5	124	109	262.3	75.0	231.8	55.0
	H	40.7	41.2	82.6	77.9	14.5	13.9	176	142	210.7	114.6	264.4	74.6
Drifter	L	45.3	43.3	81.3	74.0	13.3	12.2	76	67	125.0	83.4	88.8	63.4
	M	44.0	44.5	81.8	74.7	14.3	11.9	112	68	145.6	83.2	153.2	53.2
	H	42.7	43.3	80.8	75.8	15.2	13.3	138	107	101.0	84.5	171.6	44.4

compared for both experimental years in Table 4a. The highest difference between years was found for both reducing sugars (glucose and fructose). This is probably caused by very wet conditions prior to harvest which resulted in increasing sprouting and starch decomposition to monosaccharides in 2006. The content of free asparagine was influenced mainly by nitrogen dose whereas the effect of year is not apparent. The effect of the total nitrogen dose applied at T1 (regeneration dose) on asparagine content in flour is illustrated in Figure 1. There is an apparent uniform increase in the asparagine content with a rising nitrogen dose up to 180 kg N/ha in variants without fungicide treatments. Likewise, in variants with fungicide treatments the content of free asparagine increases, however, this increase is very small. In the untreated control, free asparagine content increases due to higher nitrogen offer up to a level of 250% in comparison with the non-fertilized variant, whereas this increase is only around 150% in fungicide treated variants. The increase of free asparagine content in relation to nitrogen dose in wheat confirms the findings reported by Weber et al. (2008). Enhanced offer of nitrogen in plants elevated free asparagine content in flour and above all in combination with stress factors that reduce the utilization of amino acids in protein synthesis. Biotic stress due to infection by leaf diseases decreased the assimilation area and shortened the period of photosynthetic activity. Similar results have been obtained, for instance, in infection of tomato leaves by *Pseudomonas syringae* bacterium that caused several times higher content of free asparagine (Perez-Garcia et al. 1998). Though lower nitrogen doses applied at T1 can support tillers formation and biomass production increase, in the case that no other nitrogen doses are supplied, excessive reduction in the number of tillers and early senescence of the photosynthetic apparatus take place at later stages. Consequently, in fungicide treated variants the highest free asparagine content was determined in the variant with the nitrogen dose of 60 kg N/ha applied at T1. In the untreated control, this stress effect of late nitrogen deficiency was masked by effects of the stress caused by leaf disease infection.

Interesting results were also found in a comparison of nitrogen application timing at the total dose of 120 kg/ha (Figure 2). The highest free asparagine contents were assessed at T1. At a later application or dividing it into two timings, the content of free asparagine decreased in both fungicide treated variants and untreated control. These results are partly in contrast with our expectation because increased levels of free asparagine were supposed rather at later application timings when nitrogen use efficiency declines. It is probably, similarly to previous results, a negative effect of non-uniform distribution of nitrogen during the growing season, which first causes excessive biomass increase at early application; however, later stress conditions due to nitrogen deficiency prevail and lead to the reduction in a number of productive stems and early senescence of the photosynthetic apparatus. Nevertheless, it is apparent, similarly to previous results, that the application of fungicides facilitated a longer functioning of the photosynthetic apparatus and decreasing in free asparagine content. Another reason for such a small effect of later nitrogen applications on free asparagine content can be also a very low activity of asparagine synthetase in wheat leaves at grain filling (Simpson and Dalling 1981).

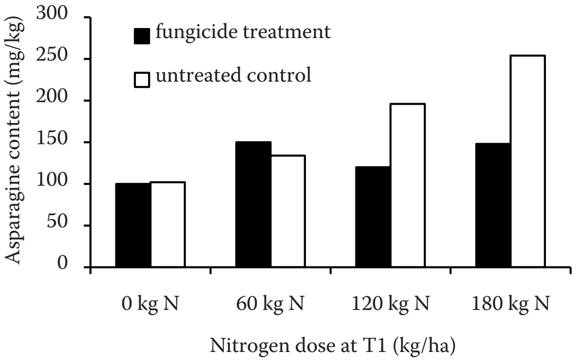

Figure 1. Effect of the total nitrogen dose at timing T1 in interaction with fungicide treatment on free asparagine content in flour in 2006

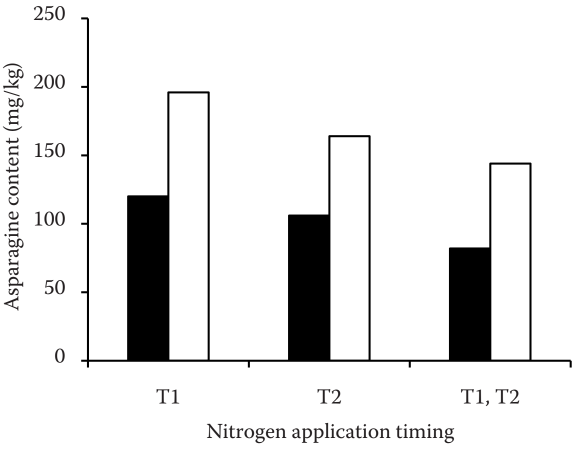

Figure 2. Effect of nitrogen application timing at the total dose of 120 kg/ha in the form of LAN on free asparagine content in flour in 2006

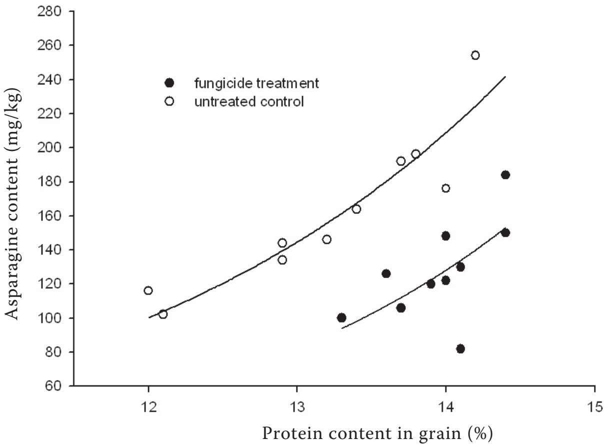

Figure 3. Relationship between N-substances content in grain and free asparagine content in a set of variants treated with fungicides and untreated controls in 2006

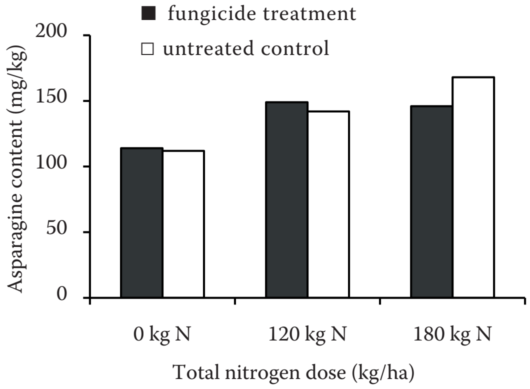

Figure 4. Effect of the total nitrogen dose in interaction with fungicide treatment on free asparagine content in flour in 2007

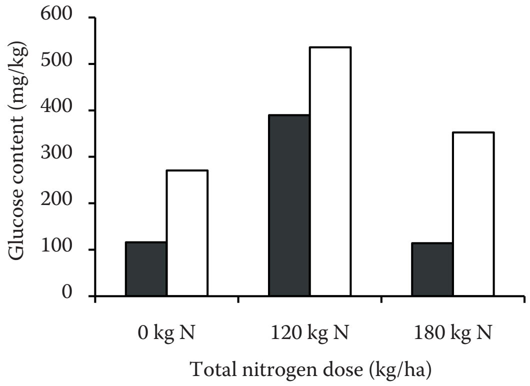

Figure 5. Effect of the total nitrogen dose in interaction with fungicide treatment on glucose content in flour in 2006

When dataset of asparagine content in flour was compared with individual quality parameters, it was found that the asparagine content was correlated very closely with protein content in grain (Figure 3). The whole dataset is divided into two groups depending on fungicide application. Parameters of fitted exponential equation and regression statistics for individual treatment groups and years are shown in Table 5. As the effect of fungicide treatment was very low in 2007 and the relationship between protein content and free asparagine was significant, only the data for 2006 are displayed in figures. The direction and course of relationships are very similar; however, the values for free asparagine content are approximately 50% higher in untreated controls than in fungicide treatments. These results reveal that the protein content can be a good indicator of elevated free asparagine content in a comparable set of samples at similar impact of stress conditions (drought, leaf diseases infection).

The year 2007 was considerably drier than 2006 and with very low infection by leaf diseases. Therefore, effects of individual factors were smaller than those in 2006. Figure 4 shows the effect of a total nitrogen dose on free asparagine content in flour in 2007. It is apparent that the higher nitrogen dose increased free asparagine content, and namely to the maximum level of 150% relatively to non-fertilized variant. Practically no effect was found for fungicide treatment. It is in full correspondence with unfavourable conditions for the development of leaf diseases.

Even though the effect of nitrogen nutrition on the content of reducing sugars was not expected,

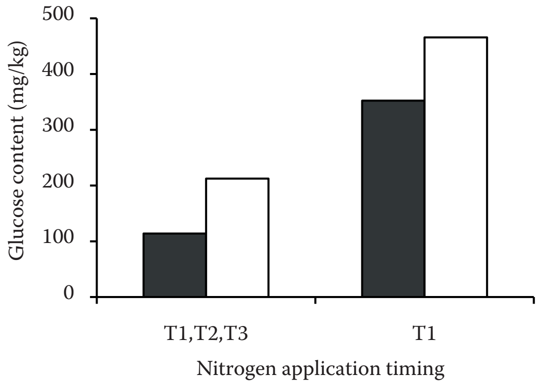

Figure 6. Effect of nitrogen application timing (a comparison of a single application with split application) at the total dose of 180 kg N/ha in interaction with fungicide treatment on glucose content in flour in 2006

Table 5. Statistics of nonlinear relationship between protein and asparagine content in flour for experiment with nitrogen nutrition and fungicide treatments

Year	Fungicide treatment	Regression coefficients		Correlation coefficient	ANOVA for regression	
		a	*b*	*r*	*F*	*P*
2006	untreated	1.231	0.367	0.931**	52.029	< 0.001
	fungicide	0.259	0.443	0.627*	5.192	0.052
2007	untreated	42.575	0.091	0.748	2.532	0.253
	fungicide	76.010	0.053	0.268	0.155	0.732

The relationship was fitted using exponential growth equation: As = a × exp (b × P); where As – free asparagine content (g/kg); P – protein content (%); significance: $^{**}P = 0.01$; $^{*}P = 0.05$

Table 6. Statistics of nonlinear relationship between protein and asparagine content in flour for experiment with wheat genotypes and intensity treatments

Year	Regression coefficients		Correlation coefficient	ANOVA for regression	
	a	*b*	*r*	*F*	*P*
2006	1.103	0.326	0.726**	14.502	0.022
2007	0.470	0.420	0.888**	48.698	< 0.001

The relationship was fitted using exponential growth equation: As = a × exp (b × P); where As – free asparagine content (g/kg); P – protein content (%); significance: $^{**}P = 0.01$; $^{*}P = 0.05$

it was demonstrated that the content of reducing sugars increased at a medium nitrogen dose of 120 kg N/ha, whereas it decreased at 0 kg N/ha and 180 kg N/ha. This effect was expressed most in the glucose content (Figure 5), but similar response to nitrogen dose was also apparent for fructose. In glucose the effect of fungicide treatments was also apparent when the glucose content was reduced in treated variants. This effect was not so clear in the case of fructose. The effect of application timing on glucose content can be seen only at the highest nitrogen dose of 180 kg/ha (Figure 6). Higher glucose content was found at a single nitrogen application at timing T1 in comparison with the application divided into three timings. Relatively substantial decrease in glucose content in relation to fungicide treatments is apparent again.

Evaluation of the effect of crop management intensity in interaction with winter wheat genotype on acrylamide precursors content in flour

The effect of crop management intensity and wheat genotype on acrylamide precursors in the years 2006 and 2007 is summarized in Table 4b. Beside the considerable effect of year on reducing sugars content, which was analogous to the results of the first experiment, there is apparent interaction between year and genotype in the effect on asparagine content. Whereas cv. Samanta provided higher values of asparagine content in 2007, cv. Sulamit did not give explicit response to year, and cv. Drifter showed higher values in 2006 at all intensity treatments. Such changes allow us to assume that not direct traits are responsible for asparagine content, but traits interacting with environmental conditions and influencing the protein synthesis during ripening (earliness, drought tolerance, and susceptibility to pathogens).

The results of the previous experiment reveal that a level of total intensity, including increased nitrogen doses and a higher level of fungicide treatments, can exhibit different effects on free asparagine content in dependence on the year (drought stress, pressure of leaf diseases) as well as winter wheat genotype. The dataset of free asparagine and protein content in grain from various genotypes were used to compare relationships for the years 2006 and 2007 (Figure 7). The year affected a shift of relationship and partially its

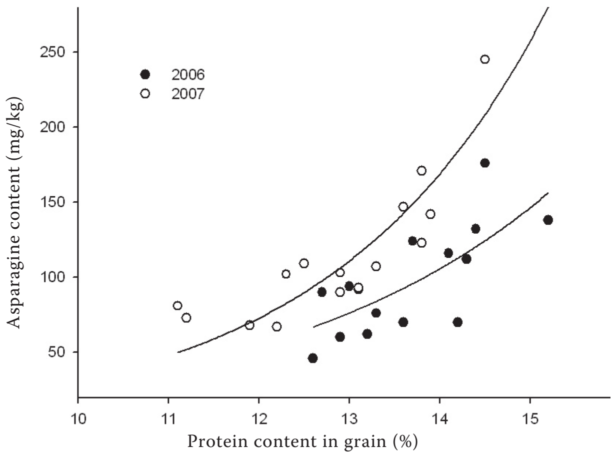

Figure 7. Relationship between the contents of N-substances and free asparagine in flour in datasets from 2006 and 2007 (experiments with crop management intensity and genotype effect)

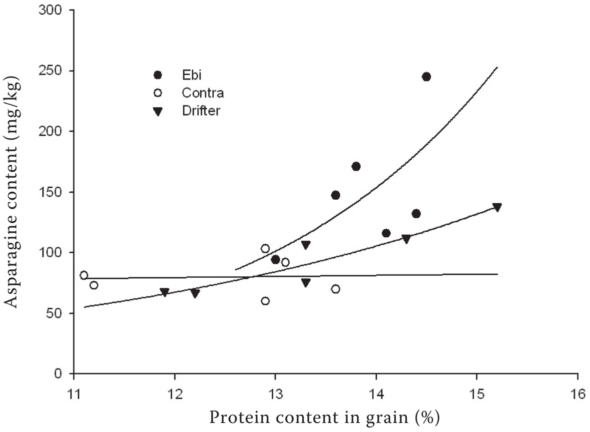

Figure 8. Effect of winter wheat genotype on the relationship between the contents of N-substances and free asparagine in flour in datasets from 2006 and 2007 (experiments with crop management intensity and genotype effect)

direction. Parameters of fitted exponential equation and regression statistics for individual years are shown in Table 6. The year 2007 was typical for high gradient of the relationship, whereas the relationship had smaller slope in 2006. In 2006, a main stress factor was the effect of leaf diseases that was at increasing nitrogen doses (leading to rising protein content in grain) inhibited by higher intensity of fungicide treatments. Therefore, the response of asparagine content to protein content was low. By contrast in 2007, disease pressure was very low and the main stress factor was drought, which was impossible to compensate by intensity. The direction of relationship had higher slope in 2007 because increased nitrogen doses were used less for conversion to proteins, and free asparagine content increased due to drought. The differences in the relationship between protein content in grain and free asparagine content (data from 2006–2007 together) were confirmed also among different genotypes of winter wheat (Figure 8). While cv. Ebi (high breadmaking quality) exhibited a rapid increase in free asparagine content with rising protein content, this relationship had lower slope in cv. Drifter, and there was no relationship between the protein and free asparagine content in cv. Contra (low breadmaking quality). It means that free asparagine content in cv. Contra stays on a stable low level independently of protein content, and thereby on year and intensity. Figures 9 and 10 enable to compare responses of selected winter wheat cultivars to crop management intensity in 2006 in free asparagine and glucose contents. The results demonstrate differences among cultivars in the content of both precursors. While cv. Sulamit is characterized by high free asparagine content

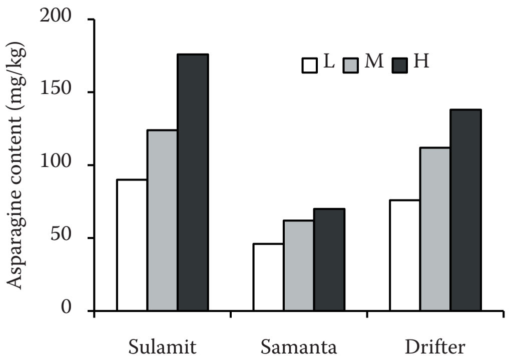

Figure 9. Free asparagine content in flour in selected winter wheat cultivars in response to crop management intensity in 2006

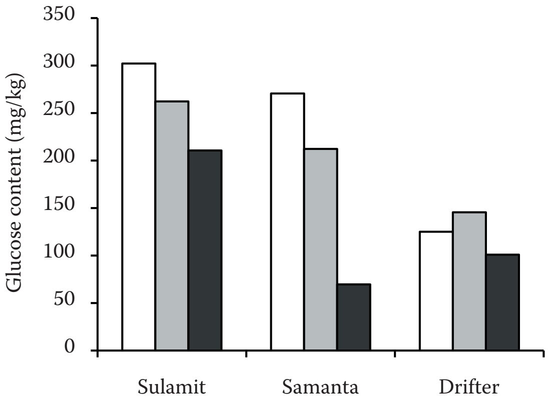

Figure 10. Glucose content in flour of selected winter wheat cultivars in response to crop management intensity in 2006

as well as glucose content, cv. Samanta has low free asparagine content and high glucose content, and finally cv. Drifter has higher free asparagine content but low glucose content. Likewise, the response to intensity was different in both precursors. While higher intensity increases free asparagine content, there is an opposite trend in glucose, whose content decreased in response to increased intensity.

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Corresponding author:

Ing. Petr Martinek, CSc., Agrotest fyto, s. r. o. Havlíčkova 2787, 767 01 Kroměříž, Česká republika
tel.: + 420 573 317 158, fax: + 420 573 339 725, e-mail: martinek.petr@vukrom.cz