Research Article

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Phenolic compounds and biological activities of rye (*Secale cereale* L.) grains

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Abstract: The rye flour is, together with the wheat flour, the basic ingredient used in traditional bread baking. The rye grain contains many compounds with significant impacts on the consumer. Considering that, various biologically active phytochemicals were determined in extracts from mature grains of 19 rye genotypes (*Secale cereale* L.). The content of total phenols, flavonoids, phenolic acids and thiols, as well as antioxidant activities and inhibitory activities against trypsin, thrombin, and urokinase were analyzed by spectrophotometric methods. The vanillic acid, vanillin, *p*-coumaric acid, and *t*-ferulic acid were analyzed in particular by high performance liquid chromatography (HPLC). The observed differences in the amounts and activities between rye genotypes reflected variations in their genetic background. Rye grain is a remarkable source of specific phytochemicals. Genetic diversity in rye makes it possible to identify individual genotypes that have a unique content and biological activity of compounds deposited in mature grains. One sub-group of rye genotypes had higher values of antioxidant properties and concentrations of polyphenols. Other sub-group had higher proteinase inhibitory activities and contents of polyphenols. The third sub-group contained as though the universal genotypes, i.e. genotypes with average values in nearly all the measured parameters.

Keywords: rye; *Secale cereale* L.; phenolic compounds; antioxidant activities; proteinase inhibition.

1 Introduction

Hundreds of phenolic compounds have been identified in extracts from mature grains of cereals [1, 2]. The high content of these compounds in the diet can effectively scavenge free radicals and reduce the risk of cancer, heart, and other diseases [3, 4]. Their beneficial effects were demonstrated in clinical trials in rats [5], hamsters [6], as well as in human [7]. Also, a nutritional survey suggested that consumption of cereal grains may reduce the risk of various diseases [8]. The high antioxidant activity had phenolic compounds in wheat grain, mainly those located in the bran [9]. Oat grains are also important sources of many compounds exhibiting antioxidant activity and diet containing oat enhanced the antioxidant status in organism of consumers [10]. Extracts from grain bran of other cereals such as corn and rye were also able to effectively inhibit oxidation of low-density lipoproteins due to the presence of phenolic antioxidants [11, 1]. Undoubtedly, increasing whole cereal grains consumption should be desirable. Recommended daily intake of cereal grains provides the organism a variety of beneficial health effects and can prevent some diseases [12]. Nevertheless, sufficient daily intake of cereal grains is rarely achieved in the Western type of diet.

The rye (*Secale cereale* L.) is a traditional crop cultivated worldwide and belongs to the main cereals feeding the world. Despite the ever-decreasing growing area over the world, rye is the second among the grains, most commonly used in bread production [13]. Rye grains have a high content of compounds with antioxidant capabilities and their consumption was proven to have beneficial effects on the human body [3]. The oat bran contains high content of dietary fibre, mainly β -glucans [14] while the rye bran consists mainly of insoluble arabinoxylans and cellulose [15]. Rye grains are an important source of many

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compounds such as dietary fiber, alkylresorcinols, folate, tocopherols, phenolic acids, and sterols. However, differences in contents of these compounds were considerable between individual rye cultivars [16]. There were found also free phenolic acids (*p*-coumaric, ferulic, sinapic) and phenolic acids liberated from soluble esters and glycosides (vanillic, caffeic, *p*-coumaric, ferulic, sinapic) [17], respectively. The ferulic acid was the most abundant phenolic acid in rye grains as well as in products made from rye [3]. Numerous studies have been focused on the antioxidant potential of rye extracts determined mainly using the DPPH (1,1-diphenyl-2-picrylhydrazyl radical) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assays, respectively. The high antioxidant activity (up to 98%) based on the ABTS free radical scavenging was observed in aqueous extracts from grains of all tested rye cultivars [18]. Moreover, enzymatic treatment of rye flour by tannase from *Paecilomyces variotii* considerably affected the antioxidant capacity of rye grain extracts and the DPPH and FRAP (ferric ion reducing antioxidant power) values were increased by 1150% and 61%, respectively [19]. Comparison of antioxidant activity determined by DPPH and FRAP in flour milling fractions of rye and other cereals ranked rye among their average [20]. The Trolox equivalent antioxidant capacity (TEAC) of the rye breads evaluated by the free radical scavenging activity against ABTS· and DPPH· radicals, respectively, showed better antioxidative properties and higher content of antioxidants in rye breads in comparison with wheat ones [21]. High antioxidant capacity of rye grains can be due to the higher content of alkylresorcinols in bran fraction that is greater than in wheat, oat, and triticale grains [22, 23].

High content of phenolic acids and other phenolic compounds correlated with inhibition of proteases such as thrombin, trypsin, and urokinase [24]. Another endoproteolytic, exoproteolytic, carboxypeptidase, aminopeptidase, and N- α -benzoyl-arginine-*p*-nitroanilide hydrolyzing activities were also detected in extracts of whole meal of the rye [25]. The occurrence of protease inhibitors in cereals, including the rye, their properties, physiological role in the plant, and nutritional influence were reviewed [26]. Many reports presented enzyme inhibitors in rye (*Secale cereale* L.), particularly against trypsin [25], subtilisin [27], α -amylase [28], and bi-functional proteinase/ α -amylase inhibitors [29].

The aim of this study was to determine profiles of selected polyphenols, antioxidant activity and proteinase inhibitory properties of extracts from mature grains of different rye (*Secale cereale* L.) genotypes.

2 Experimental Procedure

2.1 Plant material, chemicals and extraction procedure

Rye (*Secale cereale* L.) genotypes were obtained from the rye collection of genetic resources maintained in the Genebank of the Slovak Republic (Research Institute of Plant Production, Piešťany, Slovakia). The set of 19 genotypes included: SVKPOL2007-40 (Slovakia), SVNDOL2007-34 (Slovenia), HGP 4 (Germany), KR-54 (Czech Republic), Pluto (Germany), České (Czech Republic), Universalne (Poland), Pancerne (Poland), Voschod 1 (Russia), Tetra Leningradska (Russia), Bosmo (Poland), Amando (Germany), Marlo (Germany), Albedo (Czechoslovakia), Kisvárdai legelő (Hungary), Bernburger Futterrogen (Germany), Dankowskie nowe (Poland), Luco (Poland), Niawo (Poland). Rye genotypes were different in morphological, agronomical, phytopathological, and other characteristics. All were grown in the field conditions and cultivated by the same growing technology, in the same year, and in the same location (Víglaš-Pstruša, Slovakia, 48°32'N, 19°10'E, altitude 375 m, average annual temperature 8.0°C, average annual rainfall 666 mm).

All chemicals used in laboratory experiments were products of the Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Extracts were prepared from mature milled rye grains (flour size particles were 5 – 10 μ m). Flour (2.5 g) was extracted in 75 mL of 80% (v/v) methanol, at 32°C, with stirring (250 rpm), 48 h in the dark. The solid yield was filtrated off, liquid part was centrifuged and the supernatant evaporated by rotary vacuum vaporizer at 35°C. Dried yield was re-dissolved in 1 mL of acidified water (0.1% water solution of formic acid, pH~2) for HPLC analysis. Supernatants were kept at -20°C and immediately and repeatedly centrifuged before chromatographic analysis. The same extraction protocol was used for samples to be analysed by microplate assays, but dry yield was dissolved in 4 mL of methanol.

2.2 HPLC analysis

The HPLC separation, identification, and quantification were performed using the Waters HPLC system (Waters Corp., Milford, MA, USA) equipped by Waters 1525 binary pump, Waters 2998 photodiode array (PDA) detector, Waters 2707 Autosampler, Thermostat Waters Model 5 CH, Waters Symmetry C18 column (75 $\times$ 4.6 mm i.d., 3.5 μ m) with an adequate security guard column and software

Empower 2. Two phases were prepared for gradient elution, the phase A was 0.1% water solution of formic acid and the phase B was 0.1% methanol solution of formic acid. Gradient was non-linear; 0-8 min of 15% to 25% solvent B, 8-15 min 25% solvent B, 15-18 min of 25% to 40% solvent B, 18-25 min of 40% to 65% solvent B and 25-30 min 100% solvent B over 30 min. The non-linear gradient of the identified phenolic acids was used [30]. Flow rate of 1.0 mL/min was used, the column temperature was set at 30°C, and injection volume was 20 µL. The detector operated at wavelength of 260 nm and 320 nm and UV scan at 200 – 400 nm.

2.3 Total polyphenols, flavonoids and thiols

The content of total phenolics (TPC) in extracts was determined by the method modified for the microplate screening system [31] based on the reaction of phenol hydroxyl groups with the Folin-Ciocalteu reagent. The reaction mixture contained 20 µL of sample (extract/standard/ethanol) and 20 µL of Folin-Ciocalteu reagent. After 5 min was added 200 µL of 10% sodium carbonate (w/v) and incubated 30 min in the dark. Absorbance at 760 nm was measured and the content of total phenolics was expressed as milligram of gallic acid equivalent (GA eqv.) per kilogram of grains.

An amount of total phenolic acids (TPAC) in extracts was determined by the reaction with the Arnov's reagent modified for the microplate screening system [32]. 30 µL of sample (extract/standard/ethanol as blank) was mixed with 150 µL of distilled water, 30 µL of 0.5 M HCl, 30 µL of Arnov's reagent (10% sodium nitrite and 10% sodium molybdate in distilled water), 30 µL of 1 M sodium hydroxide, and 30 µL of distilled water. The absorbance was measured at 490 nm and total phenolic acid content was expressed as milligram of caffeic acid equivalent (CA eqv.) per kilogram of grains.

The total content of flavonoids (TFC) was determined [33] in the reaction mixture and it consisted of 100 µL of sample (extract/standard solution/ethanol as blank) and 20 µL of 5% aluminium chloride in methanol. After 30 min of incubation, the absorbance at 405 nm was measured (Microplate Reader Opsys MRTM, Dynex, Chantilly, USA) and the total flavonoid content was expressed as milligram of quercetin equivalent (Q eqv.) per kilogram of grains.

For determination of the total content of thiols (TTC), the Ellman's reagent [(5,5-dithio-bis-(2-nitrobenzoic acid), DTNB] was used [34] in reaction of sulfhydryl groups with DTNB yielding a yellow coloured product. Reaction mixture contained 150 µL of DTNB in 1% phosphate

buffer, pH 7.0 and 20 µL of the sample (extract/standard solution/ethanol as blank). The absorbance at 405 nm was measured after 15 min of incubation and total thiols were expressed as milligram of cysteine equivalent (CYS eqv.) per kilogram of grains.

2.4 Radical scavenging activity

The DPPH radical scavenging activity of extracts from rye grains was measured by 2,2-diphenyl-1-picrylhydrazil radical (DPPH·) method [35] modified for the microplate screening assays. Decreasing of absorbance indicated increasing of free radical scavenging potential of extracts. Grain extract (25 µL) was mixed with 100 µL of 0.3 M methanolic solution of DPPH, incubated 10 min in the dark, and absorbance measured at 515 nm. The radical scavenging activity was expressed as equivalent of Trolox per kilogram of grains.

The ABTS radical scavenging activity in grain extracts was determined by the method [34] modified for microplate screening assays (ABTS). 100 µL of the ABTS reagent ((2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) diammonium salt) was mixed with 25 µL of grain extract, incubated 10 min in the dark, and absorbance was determined at 730 nm. Radical scavenging activity was expressed as equivalent of Trolox per kilogram of grains.

The ability of rye extracts to change the oxidation status of transition metals were measured by reduction of complex $\text{Fe}^{(III)}\text{-TPTZ}$ [2,4,6-Tris(2-pyridyl)-s-triazine] by the ferric ions reducing antioxidant power (FRAP) assay [36] modified for microplate screening system. Reaction mixture contained 165 µL of $\text{Fe}^{(III)}\text{-TPTZ}$ reagent and 35 µL of grain extract. After incubation for 5 min in the dark at 37°C, absorbance was determined at 630 nm and antioxidant activity was expressed as equivalent of Trolox per kilogram of grains.

The ability of rye extracts to change the oxidation status of transition metals was measured with method based on reduction of $\text{K}_3[\text{Fe}(\text{CN})_6]$ complex [37]. The microplate screening system contained 30 µL of grain extract was mixed with 100 µL of distilled water, 45 µL of 1M HCl, 45 µL of 1% (w/v) $\text{K}_3[\text{Fe}(\text{CN})_6]$, 15 µL of 1% (w/v) sodium dodecyl sulphate, and 15 µL of 0.2% (w/v) FeCl_3 solution. The reaction mixture was incubated for 20 min at 50°C, followed by measuring of absorbance at 700 nm. Antioxidant activity was determined as the equivalent of Trolox per kilogram of grains.

2.5 Proteinase inhibitory assays

Proteinase inhibitory activities of rye extracts were determined by photometric methods with the chromogenic substrate Z-Lys-SBzl.2HCl in equimolar combination with DTNB as was published previously [38]. Substrates were cleaved by trypsin, thrombin, or urokinase, respectively, and released DTNB-SBzl was detected at 405 nm. Each reaction sample contained 0.06 mM substrate, 1% DMSO (v/v), and grain extract in potassium phosphate buffer (50 mM, pH 7.0). The reaction started by addition of enzyme solution: 0.3 U/mg of trypsin, 1.714 U/mg of thrombin, and 1.072 U/mg of urokinase, respectively. Reactions were incubated at 37°C and differences in optical density were measured for each sample as: $\Delta OD = (OD_{405nm} \text{ in the } 61^{st} \text{ minute}) - (OD_{405nm} \text{ in the } 1^{st} \text{ minute})$. The inhibitory activity was calculated according to the equation: $\% IA = \{[(1 - (\Delta OD_{sample} / \Delta OD_{control})) \times 100]\}$.

2.6 Statistical analysis

Statistical methods were applied to evaluate relationships between the variables (inhibition activities and components) and objects (genotypes). Correlation analysis with the Spearman correlation coefficient was used to reveal statistically significant correlations ($\alpha < 0.05$; $\alpha < 0.01$; $\alpha < 0.001$) between variables. The principal component analysis (PCA) and cluster analysis (CA) were used to evaluate relationships among all the objects (genotypes) and/or variables. Calculations were performed using the software Microsoft® Office Excel 2010, JMP®, Version 11.0. (SAS Institute Inc., Cary, NC, USA), and IBM SPSS Statistics for Windows, Version 22.0. (IBM Corp. Released 2013, Armonk, NY, USA).

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and Discussion

3.1 Grain compounds

The compounds, including TPC, TFC, TPAC, and TTC were determined in whole grains of rye taking into consideration that many of bioactive compounds are concentrated in the bran of the rye grain and only in low amounts in the flour endosperm [39]. The contents of four composite parameters determined in the rye grain extracts are presented in Table 1. The highest content of

TPC was found in genotype SVKPOL2007-40 (3369.19 mg GA eqv./kg), followed by HGP 4 (2732.78 mg GA eqv./kg), and Universalne (2608.66 mg GA eqv./kg). The genotype SVKPOL2007-40, significantly different in this parameter, is an accession obtained during the collecting mission aimed for acquisition of new rye genetic resources. The high content of TPC just in this genotype confirmed an importance of searching for new plant accessions. Some of them may be the source of new and unique traits.

The highest TFC was found in the rye hybrid Luco (203.36 mg Q eqv./kg) followed by Kisvárdai legelő (138.58 mg Q eqv./kg), and SVNDOL2007-34 (126.29 mg Q eqv./kg). The hybrid Luco was one of the first hybrid cultivar of rye released on the market. Hybrid rye cultivars have generally much higher yielding potential in comparison to the standard cultivars [40]. It is advantageous that just such a cultivar is unique in the high content of total flavonoids in grains.

The highest value of TPAC was found in genotype Voschod 1 (550.58 mg CA eqv./kg) followed by SVNDOL2007-34 (350.88 mg CA eqv./kg) and SVKPOL2007-40 (346.83 mg CA eqv./kg). Very high content of TPAC in extract from grains of the old Russian cultivar Voschod 1 is remarkable. It was much higher than the TPAC content in native rye grains as well as in rye seeds processed by germination and fermentation. These processes are tailored to increase the bioactive potential of wholemeal rye products [41]. Other interesting genotypes the SVNDOL2007-34 and SVKPOL2007-40 are accessions again obtained during collecting missions searching for landraces and obsolete cultivars. Their origin and genetic background is untraceable.

The highest TTC was observed in hybrid cultivar Amando (723.94 mg CYS eqv./kg), followed by hybrid cultivar Luco (578.25 mg CYS eqv./kg), and cultivar Niawo (456.64 mg CYS eqv./kg). The specific trait of the winter hybrid rye cultivar Amando is its proven high resistance to *Fusarium* head blight caused by pathogenic fungi of the *Fusarium* species [42]. Generally, plant thiols are involved in response of plant to almost all stress factors, especially abiotic, and their accumulation is one of the key factors of plant stress tolerance [43].

Significant variation and differences among rye genotypes in the contents of all determined parameters (TPC, TFC, TPAC, TTC) were observed in this study. It could be explained by obvious genetic differences between genotypes influenced by effects of environment (soil parameters, geographical and climatic conditions, growing technology, nutrition, protection and others). However, all rye genotypes analyzed in this study were grown in the same year and location, and by the same

Table 1: Contents of phytochemicals, antioxidant activity, and enzyme inhibitory activity of rye grain extracts.

Rye genotype	TPC [mg GA ekv./kg]	TFC [mg Q ekv./kg]	TPAC [mg CA ekv./kg]	TTC [mg CYS ekv./kg]	ABTS [mg Trolox ekv./kg]	DPPH [mg Trolox ekv./kg]	FRAP [mg Trolox ekv./kg]	RP [mg Trolox ekv./kg]
SVKPOL2007-40	3369.19±53.26	119.89±8.09	346.83±15.90	215.82±10.99	747.08±58.52	395.41±13.04	2888.66±145.47	12891.72±108.80
SVNDOL2007-34	1587.24±29.20	126.29±9.95	350.88±27.97	196.95±18.03	302.30±40.16	506.61±12.46	2892.72±123.60	6665.60±81.73
HGP 4	2732.78±122.94	0.00±0.15	315.80±16.05	440.98±14.83	775.27±14.18	2949.76±126.35	4478.51±110.96	9120.83±11.03
KR-54	1396.71±12.28	95.05±8.17	321.19±0.77	168.05±6.17	353.76±18.19	663.60±26.03	2797.29±122.51	14130.96±256.06
Pluto	2205.47±82.07	0.00±0.55	170.07±8.36	312.14±0.68	566.97±27.55	3041.34±17.10	5365.82±46.66	5672.21±51.16
České	984.29±42.09	89.68±3.68	284.76±5.84	111.06±2.28	417.48±29.48	926.89±19.25	3250.08±136.15	12891.72±309.16
Universalne	2608.66±149.02	43.07±3.37	323.89±27.05	442.19±5.51	655.19±40.94	882.73±46.30	3266.32±269.07	879.69±27.96
Pancerne	2257.12±68.59	79.43±4.34	218.64±0.64	203.37±6.99	358.66±33.82	781.34±51.90	2277.49±52.64	10732.18±35.98
Voschod 1	2208.63±49.24	93.77±1.31	550.58±21.57	191.33±6.70	335.38±3.19	1311.18±46.49	5012.52±222.40	19815.54±213.67
Tetra Leningradskaja	1422.80±10.13	0.00±0.06	94.50±1.13	200.97±8.74	633.13±34.71	2890.89±36.40	5120.13±47.58	9758.72±96.87
Bosmo	1588.82±61.33	109.65±1.14	345.48±21.44	266.79±5.77	392.97±17.09	568.75±26.56	3416.58±179.73	20104.58±171.42
Amando	2123.70±115.23	0.00±0.58	52.67±1.44	723.94±50.08	630.68±27.41	2701.19±22.17	3940.43±53.21	9758.72±174.33
Albedo	2282.42±50.95	42.05±0.89	143.08±10.54	402.45±24.48	530.21±40.26	1579.37±9.50	4188.15±253.11	14639.29±253.72
Kisvárdai legelő	2354.62±122.04	138.58±9.08	149.83±4.13	259.16±3.16	343.96±16.82	437.93±6.27	3225.71±169.05	3280.10±24.30
Bernburger Futterroggen	1510.55±66.18	81.23±4.57	21.64±0.99	188.93±14.03	320.68±3.17	1903.16±76.70	3778.00±134.63	13682.44±263.24
Dankowskie nowe	1841.27±115.46	0.00±0.70	12.19±0.51	298.10±19.43	655.19±39.00	2913.78±35.70	3664.29±172.15	11566.09±155.57
Luco	2470.05±74.68	203.36±15.18	194.35±4.21	578.25±39.46	375.82±6.47	120.68±1.74	4297.80±121.57	5751.95±43.01
Niawo	2534.35±163.84	119.12±0.46	175.46±2.83	456.64±19.92	1570.49±3.56	2133.74±150.05	2959.72±71.07	13263.82±1464.92
Marlo	1531.89±66.16	0.00±0.18	325.24±10.84	111.06±3.85	451.79±49.86	1975.12±14.74	4929.27±398.07	12755.50±129.43

Table 2: Parameters of the HPLC analyses of four used phenolic compounds.

Phenolic compounds	Linear range [µg/mL]	Calibration equation	R ²	LOD [ng/mL]	LOQ [ng/mL]
Vanillic acid	0.5 – 20	$y = 76194x + 12631$	0.9997	10	30
Vanillin	0.5 – 20	$y = 62346x + 7259$	0.9997	23	75
<i>p</i> -Coumaric acid	0.5 – 20	$y = 131693x - 32202$	0.9997	15	45
<i>t</i> -Ferulic acid	0.5 – 20	$y = 106291x - 40271$	0.9997	20	67

farming practices. Therefore, the observed differences between them are results of constitutive genetic determinants. The contents of all four parameters in rye extracts were much higher than in extracts from oat grains [38]. It can be stated that the rye grain is a remarkable source of these compounds and even in this small, but a genetically diverse set of rye genotypes, it is possible to identify within them genotypes with unique contents of specific compounds.

The HPLC analysis of phenolic compounds was done according to previously published approaches [15, 17, 44] and was similar to the analysis of the same compounds in oat [45]. Analytical characteristics of the HPLC determination – the equations of the calibration curves, linear ranges, coefficients of determination, limit of detection (LOD) and limit of quantification (LOQ) were determined at the signal-to-noise ratio equal to 3 and 10, respectively. The concentration of all four standards ranged from 0.5 to 20.0 µg/mL. The linearity of these functions expressed by the coefficient of determination (R^2) was higher than 0.9997 for all studied polyphenols. The precision, expressed as the relative standard deviation (RSD), was evaluated from three replicate analyses of each standard.

Four phenolic compounds were analysed in extracts from rye grains – vanillic acid (VA), vanillin (V), *p*-coumaric acid (CA), and *t*-ferulic acid (FA) (Fig. 1.). The contents of these analytes in 19 rye genotypes are in Table 3. The highest content of vanillic acid was observed in Amando, HGP 4, and Marlo, the highest content of vanillin in HGP 4, Amando, and Luco. The same genotypes, i.e., HGP 4, Luco, and Amando showed also the highest amounts of both the *p*-coumaric acid and *t*-ferulic acid. In summary, three the most interesting rye genotypes from the point of view of content of four analysed phenolics were HGP 4, Luco, and Amando.

In comparison with other cereals, extracts from oat grains contained ferulic acid and *p*-coumaric acid in a similar range (0.53 – 5.72 mg/kg) [46] and extracts from wheat grains ferulic acid, vanillic acid, syringic acid, *p*-coumaric acid (0.15 – 2.31 µg/g) [47]. Generally, the

majority of the total amount of phenolic acids (ferulic, coumaric) is in cereal grains bounded to non-extractable arabinoxylans [48, 49]. This should be beneficial for the production of “functional” foods with a higher antioxidant activity associated with different positive biological effects on the consumer organism because they are released by hydrolyzing under alkaline or acid conditions [50]. Processes used in food production such as fermentation, extrusion, different types of hydrolysis, microwave and ultrasound treatment can release phenolics bounded in cell walls [51].

3.2 Antioxidant activity

The antioxidant activity of extracts from rye grains was determined by four different methods, based on different mechanisms (scavenging of free radicals or reduction of ferric to ferrous ions, respectively). Antioxidant activities determined by various methods (ABTS, DPPH, FRAP, RP) are influenced by different chemical structure of compounds in plant extracts, especially by functional groups attached to the main backbone of molecules. Although these results are not independent, classification of samples on the basis of a single method should be avoided [52]. The genotyp Niawo expressed the highest antioxidant activity by the ABTS assay (1570.49 mg Trolox eqv./kg), whereas the genotype Pluto by the DPPH (3041.34 mg Trolox eqv./kg) and FRAP (5365.82 mg Trolox eqv./kg) methods. The genotype Bosmo expressed the highest antioxidant activity by the RP method (20104.58 mg Trolox eqv./kg). However, also several other genotypes expressed remarkable antioxidant activity (Table 2). The differences among 19 analyzed rye genotypes in their antioxidant activities determined by each of all four approaches were considerable and multiple. For example, the range of values determined by the DPPH assay was from 121 mg Trolox eqv./kg (Luco) to 3041 mg Trolox eqv./kg (Pluto). It was confirmed that the profile and amounts of antioxidants in the rye grains as well as their distribution within the grains are determined by genotype [53].

Figure 1: Overlay of UV-spectra of four phenolic compounds (black lines – standards, red line – grain sample) and two chromatograms (black line – standard mixture, red line – grain sample of genotype Tetra Leningradskaja).

Antioxidant properties stored in rye grains are exceptional because rye flour, along with the wheat one, is the basic source for baking of traditional bread. The rye flour and rye breads showed better antioxidative properties in comparison with the wheat based bakery products [21]. The same was true for rye whole-grain breakfast cereals in which the reduction of DPPH radicals

was the highest, higher than in those made from wheat and barley, respectively [54].

Generally, antioxidant compounds present in plant seeds play role in detoxifying mechanisms participating in seed survival undermined by oxidative stress and cellular damage, as well as during the completion of germination and storability of seeds [55].

Table 3: Contents (mg/kg) of four phenolic compounds in grain extracts from 19 rye genotypes.

	Vanillin	Vanillic acid	p-Coumaric acid	t-Ferulic acid
SVKPOL2007-40	2.259±0.041	2.120±0.275	0.832±0.083	4.451±0.165
SVNDOL2007-34	2.530±0.160	2.016±0.171	0.764±0.024	4.813±0.254
HGP 4	3.188±0.348	3.285±0.201	1.280±0.201	6.227±0.209
KR-54	2.133±0.232	2.236±0.155	0.782±0.078	3.786±0.080
Pluto	2.204±0.099	2.393±0.354	0.876±0.135	4.398±0.580
České	1.734±0.155	1.691±0.054	0.631±0.108	3.803±0.236
Universalne	1.233±0.318	1.308±0.577	0.532±0.046	2.407±0.141
Pancerne	1.086±0.052	1.407±0.127	0.533±0.113	3.346±0.266
Voschod 1	1.901±0.247	2.501±0.109	0.489±0.053	3.501±0.014
Tetra Leningradskaja	1.974±0.207	1.874±0.146	0.675±0.004	3.817±0.620
Bosmo	2.037±0.437	2.947±0.653	0.802±0.083	4.343±0.052
Amando	3.130±0.432	3.421±0.466	1.087±0.274	5.838±0.557
Albedo	1.705±0.212	2.371±0.416	1.078±0.332	5.030±0.840
Kisvárdai legelő	1.773±0.182	3.231±0.459	0.805±0.160	4.557±0.218
Bernburger Futterroggen	0.750±0.017	0.814±0.109	0.248±0.029	1.903±0.080
Dankowskie nowe	1.562±0.514	2.430±0.625	0.897±0.194	4.251±0.163
Luco	2.898±0.035	2.312±1.121	1.193±0.087	6.164±0.565
Niawo	1.348±0.103	1.122±0.123	0.343±0.041	2.262±0.016
Marlo	2.360±0.516	3.284±0.521	0.892±0.143	3.723±0.130

3.3 Proteinase inhibitory activity

Proteinase inhibitory activities for all rye genotypes are presented in Table 4. Extracts from genotypes SVKPOL2007-40, Luco, SVNDOL2007-34, Bosmo, and HGP 4 had the highest inhibition activities simultaneously against all trypsin, thrombin, and urokinase, respectively. The inhibitory activity of extracts from rye seeds against above-mentioned enzymes basically has not yet been published. An exceptional inhibition activity against thrombin (90% or higher) has been detected in extracts from eight plant species among 45 studied [56], but extracts from plant species of the family *Poaceae* had not been analysed in that study. Inhibitory properties of grain extracts against other proteinases were published. In comparison with extracts from poppy seeds [33] were rye extracts more effective in inhibition of trypsin, thrombin, and urokinase. Also in comparison with extracts from the hop cones [57], extracts from some rye genotypes had up to four times higher inhibitory activity against trypsin and three-times higher against thrombin and urokinase.

3.4 Relationships between seed extracts parameters

The principal component analysis (PCA) and cluster analysis (CA) were used for evaluation of primary data. Several relationships revealed by the PCA (Fig. 2.) were confirmed by correlation analysis. Significant correlation (in range $R=0.77 - 0.94$, $p<0.0001$) was within contents of three phenolic acids and vanillin (CA, VA, FA, V). It can be explained by their common biosynthesis in the shikimate metabolic pathway. Significant correlation was between vanillin (V) concentration and IA_TR ($R=0.630$; $p=0.003$) and between vanillin and IA_UR ($R=0.463$; $p=0.040$). Content of vanillin ($R=0.471$; $p=0.036$) and vanillic acid ($R=0.480$; $p=0.032$) significantly correlated with FRAP antioxidant activity. The total content of phenolic acids (TPCA) significantly correlated ($R=0.501$; $p=0.023$) with IA_TR and total content of flavonoids (TFC) significantly correlated ($R=0.472$; $p=0.036$) with IA_TY. This was the only correlation found in trypsin inhibitory activity. Another significant correlation ($R=0.610$; $p=0.004$) was

Table 4: Proteinase inhibitory activities of extracts from rye grains.

Rye genotype	IA_TY [%]	IA_TR [%]	IA_UR [%]
SVKPOL2007-40	100.00±1.30	100.00±2.70	100.00±0.10
SVNDOL2007-34	100.00±2.70	99.80±2.00	100.00±0.50
HGP 4	92.80±0.30	98.00±3.20	100.00±0.80
KR-54	100.00±1.40	70.70±0.10	81.20±0.20
Pluto	62.80±0.70	100.00±7.30	100.00±0.60
České	100.00±1.50	41.40±0.80	97.90±0.40
Universalne	100.00±2.20	0.00±5.00	100.00±0.20
Pancerne	100.00±0.80	74.50±4.10	68.90±0.10
Voschod 1	65.40±0.90	100.00±3.70	0.00±0.10
Tetra Leningradskaia	0.00±3.60	82.50±4.50	72.10±0.20
Bosmo	100.00±0.90	100.00±4.90	95.80±0.10
Amando	0.00±0.40	37.30±1.50	45.80±0.30
Albedo	100.00±2.70	13.20±0.30	57.50±0.20
Kisvárdai legelő	49.00±0.60	17.40±0.60	41.80±0.20
Bernburger Futterroggen	99.40±1.70	0.00±0.60	28.10±0.10
Dankowskie nowe	100.00±2.30	0.00±0.10	51.90±0.20
Luco	100.00±1.00	100.00±0.80	100.00±0.50
Niawo	100.00±2.10	68.50±1.30	100.00±0.20
Marlo	0.00±0.70	100.00±2.70	100.00±0.20

Figure 2: Two-dimensional PCA plot presenting 19 rye genotypes (points) and determined variables (vectors). The colour of points corresponds to colours in dendrogram (Figure 3.).

found between IA_TR and IA_UR, which could be due to the relative similarity of both enzymes. Significant correlation ($R=0.509$; $p=0.022$) was found also among antioxidant activities DPPH and ABTS what could be explained by similarities in both radical scavenging mechanisms.

Cumulative information about the differences or similarity of tested rye extracts presents also the dendrogram constructed by cluster analysis (CA) presented in Fig. 3. An interesting group of rye genotypes represents those with the blue diamonds that had higher values of antioxidant properties (FRAP, DPPH, ABTS), as well as concentrations of polyphenols determined by the HPLC (CA, FA, V, VA). Rye genotypes in group marked by green crosses had the average values in nearly all the measured parameters. The third group (red circles) represents rye genotypes with higher proteinase inhibitory activities (IA_TY, IA_TR, IA_UR) and contents of polyphenols.

Figure 3: Cluster analysis presenting relationships among 19 rye genotypes.

4 Conclusions

Composite parameters (total phenolic content, total flavonoid content, total phenolic acids content, total content of thiols), antioxidant activities determined by different methods (ABTS, DPPH, FRAP, RP), and inhibitory activities against three proteinases were determined in extracts from mature (*Secale cereale* L.) grains of 19 rye genotypes (cultivars, hybrids, genetic resources). The trypsin inhibitor is known as a pancreatic disorder promoter [58], thrombin inhibitor as promoter of diseases associated with hypercoagulation [59], and urokinase inhibitor acts as a promoter of metastatic process of onco-transformed cells [60]. Effect of environment on results has been eliminated by growing of all analyzed genotypes in the same year and in the same location. Variations and relationships between identified component parameters and biological activities within a set of analyzing rye genotypes were observed after their separation by statistical methods into three main sub-groups. One group contained less interesting genotypes (Universalne, Pancerne, Bernburger Futterrogen) which

have not been anything special trait, but they were relatively good in all parameters. The second group of genotypes (HGP 4, Amando, Pluto, Tetra Leningradskaja, Marlo, Albedo, Dankowskie nowe, Kisvárdai legelő) expressed higher values of polyphenols and antioxidant properties in grain extracts. The third sub-group contained genotypes (SVKPOL2007-40, Luco, SVKDOL2007-34, KR-54, České, Bosmo) with high proteinase inhibitory activities. Genotypes that were placed between the second and third groups could be of interest for further studies as well as use for specific purposes. The rye grain is an interesting source of natural compounds with potent biological activities. Increased consumption of foods containing rye as a whole grain or as wholegrain flour can lead to the consumption of phytochemicals beneficial to the consumer's health.

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