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Phenolic acids accumulation and their antioxidant potential in wheat cultivars under the reduced and optimal nitrogen levels

Marija Kovačević Babić[1], Daniela Horvat[1]*, Dario Novoselović[1], Filip Horvat[2], Krešimir Dvojković[1]

[1]Agricultural Institute Osijek, Južno predgrađe 17, 31000, Osijek, Croatia
[2]Croatian Agency for Agriculture and Food, Centre for Seed and Seedlings, Usorska 19, Brijest, 31000 Osijek, Croatia

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*CORRESPONDENCE
Daniela Horvat
daniela.horvat@poljinos.hr

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ABSTRACT

Three common wheat cultivars (*Triticum aestivum* L.) were grown under the reduced and optimal nitrogen (N) levels during 2018/2019 vegetation season. In this study, phenolic compounds were analysed as soluble-free and insoluble-bound fractions, because the majority of them are found in cereals in these forms. Total phenolic content in both extracts (TPC) was determined using the Folin-Ciocalteu reagent, while the antioxidant activity (AOA) of phenolics was evaluated using the 2,2–diphenyl–1–picrylhydrazyl radical scavenging test. The composition and content of phenolic acids (PAs) was analysed by HPLC. On average, TPC and AOA values were several times higher in the insoluble–bound extract compared to the soluble–free one (163.2 mg GAE/100 g DM *vs*. 60.7 mg GAE/100 g DM, and 48.5% *vs*. 17.6%, respectively). Soluble–free PAs ranged from 4.0 mg/100 g DM (p–coumaric acid) to 4.6 mg/100 g DM (p–hydroxybenzoic acid), while insoluble–bound PAs content was much higher, ranging from 2.2 mg/100 g DM (caffeic acid) to 56.5 mg/100 g DM (ferulic acid). For phenolic extracts, TPC, AOA and almost all PAs content was higher under the reduced nitrogen levels. In general, insoluble–bound PAs were more susceptible to higher nitrogen supply than soluble-free ones. Although cultivars showed a similar response to increased nitrogen rate, considerable variability in their phenolic properties was observed.

KEYWORDS

wholegrain wheat; nitrogen level; total phenolics; antioxidant activity; phenolic acids

KEY CONTRIBUTION

In the wholegrain samples, much higher content and antioxidant potential was found in the insoluble–bound phenolics compared to the soluble–free ones. The properties of dominant insoluble–bound phenolics were more affected by higher nitrogen. The most abundant phenolic acid was ferulic. Wheat grains are rich source of phenolic compounds with potential health benefits.

Introduction

Common wheat (*Triticum aestivum* L.) is the second most-produced cereal after maize in Croatia. Over the past five seasons (2019–2023), the Croatian wheat acreage has ranged from 141,602 to 158,000 ha, with grain yields between 5.6 and 6.7 tonnes (Croatian Bureau of Statistics, 2024). The common wheat kernel is typically fractionated into bran, endosperm, and embryo, and then milled into flour, which is used in a wide range of foods, including bread, cereals, cakes, pasta, etc. Consumer awareness of health-promoting foods has increased as many human diseases are related to oxidative stress (Chaudhary et al., 2023). Preclinical and clinical evidence strongly suggests that the daily consumption of foods rich in polyphenols offers protection against various chronic diseases, including neurodegenerative, cardiovascular diseases, cancer, and diabetes (Rudrapal et al., 2022). Wheat is one of the primary grains in the human diet, and the nutritional and health benefits of wheat and grain products are attributed to unique phytochemicals that complement those in fruits and vegetables (Liu, 2007). Phenolic acids (PAs), along with flavonoids, represent the most common phenolic compounds found in wheat grains. They are among the major and most complex groups of phytochemicals (Žilić et al., 2016). Andersson et al. (2014) reported that approximately 75–80% of PAs in wheat are bound to cell wall polymers, while 20–25% are esterified to sugars and other low molecular mass compounds, and only 0.5–2% are in the free form. It is well known that whole wheat flour exhibits higher antioxidant potential than white flour, primarily because phenolic compounds are mainly located in the bran (Călinoiu and Vodnar, 2018). Concerning the antioxidant potential of major cereal phenolic acids, their capacity to scavenge free radicals decreases as follows: gallic, caffeic, benzoic, sinapic, syringic, ferulic, p–coumaric, vanillic, and 4–hydroxybenzoic acid (Shamanin et al., 2023). Similar to agronomic and quality traits, wheat cultivar is an important factor influencing the distribution of PAs (Fernandez–Orozco et al., 2010; Zhang et al., 2012; Horvat et al., 2020; Tian et al., 2022). Li et al. (2008) determined the amounts and compositions of free, conjugated, bound, and total PAs in 175 wheat samples, reporting a wide range (3.5-fold) in total PAs concentration. Despite a relatively high genetic impact, some authors report that environmental factors, such as climate conditions and nitrogen management, strongly influence wheat phenolic components (Fernandez–Orozco et al., 2010; Wang et al., 2018; Tian et al., 2021; Barański et al., 2020; Laddomada et al., 2021). Recent studies indicate that the effect of nitrogen (N) fertilization on wheat PAs is inconsistent, mainly because nitrogen (N) rates often interact with environmental factors, such as agronomic practices, weather conditions, and wheat type. Some authors report increased TPC, AOA, and dominant ferulic acid under higher nitrogen (N) rates (Ma et al., 2015), while others found that higher nitrogen (N) levels did not affect ferulic acid, but significantly reduced p–coumaric and increased sinapic acid (Tian et al., 2022). In order to improve the health aspects of wheat, this study was conducted to investigate TPC, AOA, and PAs distribution in three commercial wheat cultivars grown under the reduced and optimal nitrogen (N) management.

Materials and methods

Plant materials and field experiments

A small-plot field experiment with three winter wheat cultivars characterized by high baking potential (Kraljica, Olimpija and Srpanjka) was conducted at the research fields of the Agricultural Institute Osijek, Croatia, during the 2018/2019 vegetation season. Overall, the 2018/2019 growing year was extremely warm with a multi-annual average rainfall (DHMZ, 2024). The field experiments were arranged in a randomized split-plot design with three replications. The harvested plot size was 7.56 m^2, and the seeding rate was 350 kernels/m². The nitrogen (N) treatment consisted of two fertilization levels: 74 kg N/ha as the reduced N treatment (only basal fertilization before sowing) and an additional 100 kg N/ha as the optimal N treatment, applied in two top-dressings of 50 kg N/ha at the tillering and stem extension growth stages during winter-spring period of 2019. In commercial wheat production in eastern Croatia, the total N fertilizer application typically ranges from 120–140 kg to 180–200 kg N/ha, depending on the residual soil N, cultivar, planned yield, and quality. All other management practices, including herbicide, insecticide, and fungicide applications, were standard for commercial wheat production in Croatia. The cultivars were harvested in late June and early July 2019.

Extraction of soluble-free and insoluble-bound phenolics

Soluble-free and insoluble-bound phenolics were extracted following the procedure of Zavala–López and García–Lara (2017), with some modifications. Soluble-free phenolics were extracted using 80% methanol from 0.25 g of wholegrain samples at 25 °C for 15 min. After centrifugation (Universal 320R; Hettich, Germany) for 10 min at 5000 rpm, the supernatant was stored at −20 °C until analysis. The remaining pellet residue, after removal of soluble-free phenolics, was treated with 2 M NaOH at 90 °C for 2 h (Shaking Water Bath GFL 1092, Germany), then acidified to pH 2. Insoluble-bound phenolics were extracted three times with ethyl acetate. The organic layer supernatant was evaporated to dryness (BÜCHI B–720 Vacuum Controller, Germany), reconstituted with 80% methanol, and stored at −20 °C until analysis.

Total phenolic content

Total phenolic content (TPC) in both phenolic extracts was determined using a modified Folin−Ciocalteu method (Singleton and Rossi, 1965), in accordance with our previous research (Horvat et al., 2020). TPC was expressed as mg of gallic acid equivalents (GAE) per g of dry matter (DM) based on a gallic acid calibration curve.

Antioxidant activity

The antioxidant activity (AOA) of soluble-free and insoluble-bound phenolics was analysed using the 2,2−diphenyl−1−picrylhydrazyl (DPPH) assay (Brand-Williams et al., 1995). Further details are available in our previous research (Horvat et al., 2020). The percentage of DPPH radical scavenging activity was calculated using the formula: $(A_c - A_t)/A_c \times 100$, where A_c is the absorbance of the control, and A_t is the absorbance of the extract.

Determination and quantitation of phenolic acids by HPLC

Individual phenolic acids (PAs) in both extracts were analysed using a Series 200 HPLC system (Perkin Elmer, USA) with a Kinetex Core-Shell RP-C18 column (150 x 4.6 mm, 100 Å, 5 µm) and a diode array detector (DAD). Prior to HPLC analysis, samples were filtered through a 0.2 µm nylon filter (Ahlstrom GmbH, Germany). The mobile phase consisted of the solvent A (Millipore water with 1% trifluoroacetic acid (v/v)) and the solvent B (acetonitrile with 1% trifluoroacetic acid (v/v)). The elution gradient was 5−40% of B over 40 min. The column temperature was maintained at 30 °C with a flow rate of 1.0 mL/min. Phenolic compounds were identified by comparing UV absorption spectra at 275 nm and retention times with standards, and quantification was performed using five−point external calibration curves.

Statistical analysis

Differences among treatments and cultivars were analysed using one-way or two-way ANOVA with a significance level of $P \leq 0.05$, followed by Tukey's significant range test. Statistica 13.1 (TIBCO Software Inc., Palo Alto, CA, USA) was used for data analysis. All determinations were made in triplicate.

Results and discussion

The variations in TPC and AOA of both soluble-free and insoluble-bound phenolic extracts from wheat cultivars grown under the reduced and optimal N conditions are presented in Table 1. On average, the TPC was 2.7 times higher in insoluble−bound extracts (163.2 mg GAE/100 g DM) than in soluble free extracts (60.7 mg GAE/100 g DM), indicating that insoluble−bound phenolics contribute significantly more to the total phenolic content than soluble-free phenolics (Liyana−Pathirana and Shahidi, 2006).

Table 1. Mean[1] values of TPC and their AOA under the reduced and optimal N levels

GENOTYPE	N (kg/ha)	SOLUBLE–FREE PHENOLICS TPC (mg GAE/100 g DM)	SOLUBLE–FREE PHENOLICS AOA %	INSOLUBLE–BOUND PHENOLICS TPC (mg GAE/100 g DM)	INSOLUBLE–BOUND PHENOLICS AOA (%)
KRALJICA	NR	63.6^{ns} ±1.3	18.7^{b} ±3.1	176.6^{b}±11.2	50.3^{ns} ±3.4
	NO	63.9 ±4.7	17.9^{a} ±5.9	160.8^{a} ±7.3	48.3 ±3.6
OLIMPIJA	NR	65.1^{b} ±2.4	16.5^{a} ±4.1	176.7^{b} ±6.1	54.3^{b}±4.2
	NO	61.2^{a} ±1.6	17.5^{b}±5.9	157.6^{a} ±2.3	47.0^{a} ±3.5
SRPANJKA	NR	56.0^{ns} ±1.8	19.0^{b} ±4.7	157.4^{b} ±14.2	47.5^{b} ±2.9
	NO	54.6 ±6.1	16.3^{a}±3.4	150.3^{a} ±14.0	43.9^{a} ±3.0
MEAN	NR	61.6^{b} ±4.5	18.1^{b} ±4.2	170.3^{b} ±13.7	49.5^{b} ±5.5
	NO	59.9^{a} ±5.8	17.2^{a} ±5.2	156.2^{a} ±9.5	47.6^{a} ±3.1
OVERALL MEAN		**60.7 ±5.1**	**17.6 ±4.6**	**163.2 ±13.6**	**48.54 ±4.5**

[1] Mean ± SD across three replicates (n = 3). TPC = total phenolics content, AOA = antioxidant activity, GAE = gallic acid equivalent, DM = dry matter, NR = reduce nitrogen level, NO = optimal nitrogen level. Means with different superscript letters (a–b) in a column are significantly different (p ≤ 0.05), ns = non−significant.

TPC for both extracts was significantly reduced by increased N application (Table 1), which is consistent with findings by Cartelat et al. (2005), Chen et al. (2011), and Kumar et al. (2023), who noted that under biotic and abiotic stress, plants induce phenolic biosynthesis to increase tolerance. A reduction in phenolics due to N fertilization aligns with the carbon–nitrogen balance theory (Bryant et al., 1983), which suggests that high N availability limits the production of carbon-rich secondary metabolites. Conversely, Stumpf et al. (2015) found that while TPC of free phenolics increased with more N, TPC of conjugated soluble phenolics decreased, while insoluble phenolics were less affected. Ma et al. (2015) reported that TPC decreased under N180 compared to NO, but further N rate increases (N240−N300) led to higher TPC in both phenolic extracts. On average, AOA followed the same pattern as TPC, with a considerably higher levels in insoluble−bound phenolic extracts (48.5%) than in soluble-free (17.6%) (Table 1). Both extracts showed higher AOA under the reduced N rate (Table 1), in line with Mao et al. (2015). However, Stumpf et al. (2015) observed no significant effects of N treatments on AOA in both phenolic fractions. The response to N regime was cultivar–specific. The influence of N on TPC and AOA for individual cultivars was more pronounced in insoluble−bound phenolic extracts. For example, the cultivar Olimpija exhibited the highest TPC and AOA, measured as DPPH scavenging ability, under reduced N (176.7 mg GAE/100 g DM and 54.3%, respectively), whereas Srpanjka showed the lowest values under optimal N (150.3 mg GAE/100 g DM and 43.9%, respectively) (Table 1).

Table 2. Mean[1] values of soluble−free phenolic acids under the reduced and optimal N levels

GENOTYPE	N kg/ha	*p*-HB	SYR	*p*-COU	TOTAL
		(mg/100 g DM)			
KRALJICA	NR	4.4^{a} ±0.19	5.5^{ns} ±0.65	3.9^{ns} ±0.08	13.8^{ns} ±0.53
	NO	4.6^{b} ±0.25	5.5 ±0.37	4.0 ±0.17	14.1 ±0.37
OLIMPIJA	NR	4.6^{ns} ±0.30	3.4^{ns} ±0.17	4.2^{ns} ±0.05	12.2^{ns} ±0.42
	NO	4.5 ±0.25	3.6 ±0.33	4.1 ± 0.17	12.2^{ns} ±0.34
SRPANJKA	NR	4.5^{a} ±0.15	3.5^{ns} ±0.10	3.8^{ns} ±0.08	11.8^{a} ±0.08
	NO	4.9^{b} ±0.23	3.6 ±0.13	3.9 ±0.02	12.5^{b} ±0.16
MEAN	NR	4.5^{a} ±0.92	4.2^{ns} ±1.08	4.0^{ns} ±0.20	12.6^{a} ±0.97
	NO	4.7^{b} ±0.25	4.2 ±0.97	4.0 ±0.16	12.9^{b} ±0.93
OVERALL MEAN		**4.6 ±0.24**	**4.2 ±1.00**	**4.0 ±0.18**	**12.8 ±0.94**

[1] Mean ± SD across three replicates (n = 3). p−HB = p−hydroxybenzoic acid, SYR = syringic acid, p−COU = p−coumaric acid, FER = ferulic acid, DM = dry matter, NR = reduce nitrogen level, NO = optimal nitrogen level. Means with different superscript letters (a−b) in a column are significantly different ($p \leq 0.05$), ns = non−significant.

On average, in the soluble−free extract, four PAs were identified, with the average concentrations ranging from 4.0 mg/100 g DM (p–coumaric) to 4.6 mg/100 g DM (*p*-hydroxybenzoic) (Table 2). In the insoluble−bound extract, ferulic acid was the most abundant (56.5 mg/100 g DM), followed by sinapic, *p*–hydroxybenzoic, *p*–coumaric, and caffeic acids (Table 2), supporting previous studies on the dominance of ferulic acid in wheat wholegrain (Mpofu et al., 2006; Stumpf et al., 2019; Tian et al., 2022). Cultivar Kraljica had the highest content of both total soluble-free and total insoluble−bound PAs (14.1 mg/100 g DM at NO and 90.2 mg/100 g DM at NR, respectively), while cultivars Srpanjka and Olimpija had the lowest content of total soluble−free and insoluble−bound PAs (11.8 mg/100 g DM at NR and 82.8. mg/100 g DM at NO, respectively).

Although cultivars responded similarly to increased N rate, considerable variability in their PAs was observed (Tables 2 and 3), consistent with Tian et al. (2022), who concluded that PAs concentration, especially for dominant ferulic acid, is primarily determined by cultivar and natural environmental factors. Considering N treatment, in the free–soluble extracts only *p*–hydroxybenzoic acid is affected by the higher amounts of N, while in the insoluble–bound extract N rate contributed significantly to variation in *p*–hydroxybenzoic, *p*–coumaric, ferulic and sinapic acids, which means that soluble–free phenolics were less susceptible to higher N supply (Table 2 and 3).

Recent studies on wheat grain showed that the effect of N fertilization on some single PAs appear to be inconsistent, and the causes of this may be the result of the application of different agro–technical measures, different climatic conditions, and their interaction (Ma et al 2021; Tian et al 2022).

On average, in the soluble−free extract, four PAs were identified, with the average concentrations ranging from 4.0 mg/100 g DM (p–coumaric) to 4.6 mg/100 g DM (p-hydroxybenzoic) (Table 2). In the insoluble−bound extract, ferulic acid was the most abundant (56.5 mg/100 g DM), followed by sinapic, p–hydroxybenzoic, p–coumaric, and caffeic acids (Table 2), supporting previous studies on the dominance of ferulic acid in wheat wholegrain (Mpofu et al., 2006; Stumpf et al., 2019; Tian et al., 2022). Cultivar Kraljica had the highest content of both total soluble-free and total insoluble−bound PAs (14.1 mg/100 g DM at NO and 90.2 mg/100 g DM at NR, respectively), while cultivars Srpanjka and

Olimpija had the lowest content of total soluble−free and insoluble−bound PAs (11.8 mg/100 g DM at NR and 82.8. mg/100 g DM at NO, respectively).

Table 3. Mean[1] values of insoluble−bound phenolic acids under the reduced and optimal N level

GENOTYPE	N kg/ha	*p*−HB	CAF	*p*−COU	FER	SIN	TOTAL
		(mg/100 g DM)					
KRALJICA	NR	6.1^{ns} ±0.74	2.4^{ns} ±0.24	4.8^{ns} ±0.11	59.8^{b} ±2.49	15.5^{b} ±2.15	90.2^{b} ±5.40
	NO	5.9 ±0.96	2.2 ±0.21	4.7 ±0.10	54.9^{a} ±0.45	14.5^{a} ±1.64	83.7^{a} ±2.97
OLIMPIJA	NR	5.0^{b} ±0.15	2.1^{b} ±0.16	5.1^{b} ±0.07	56.1^{b} ±0.54	17.1^{a} ±1.76	86.8^{b} ±1.98
	NO	4.5^{a} ±0.43	1.9^{a} ±0.15	4.8^{a} ±0.03	54.4^{a} ±0.43	15.6^{b} ±0.98	82.8^{a} ± 0.74
SRPANJKA	NR	4.7^{ns} ±0.46	1.9^{ns}±0.01	4.7^{b} ±0.20	58.3^{b} ±4.92	16.7^{b} ±0.50	87.6^{b} ± 4.06
	NO	4.9 ±0.51	2.5 ±0.78	4.3^{a} ±0.10	55.2^{a} ±5.01	15.3^{a} ±0.65	83.5^{a} ±3.51
MEAN	NR	5.3^{b} ±0.77	2.1^{ns} ±0.26	4.8^{b} ±0.18	58.1^{b} ±3.29	16.5^{b} ±1.65	86.7^{b} ±3.99
	NO	5.1^{a} ±0.90	2.2 ±0.51	4.6^{a} ±0.23	54.8^{a} ±2.66	15.1^{a} ±.17	81.8^{a} ±2.47
OVERALL MEAN		**5.2 ±0.82**	**2.2 ±0.40**	**4.7 ±0.22**	**56.5 ±3.36**	**15.8 ±.55**	**84.3 ±4.08**

[1] Mean ± SD across three replicates (n = 3), p−HB = p−-hydroxybenzoic acid, CAF = caffeic acid, p−COU = p−coumaric acid, FER = ferulic acid, SIN = sinapic acid, DM = dry matter, NR = reduce nitrogen level, NO = optimal nitrogen level. Means with different superscript letters (a−b) in a column are significantly different (p ≤ 0.05), ns = non−significant.

Although cultivars responded similarly to increased N rate, considerable variability in their PAs was observed (Tables 2 and 3), consistent with Tian et al. (2022), who concluded that PAs concentration, especially for dominant ferulic acid, is primarily determined by cultivar and natural environmental factors. Considering N treatment, in the free–soluble extracts only p–hydroxybenzoic acid is affected by the higher amounts of N, while in the insoluble–bound extract N rate contributed significantly to variation in p–hydroxybenzoic, p–coumaric, ferulic and sinapic acids, which means that soluble–free phenolics were less susceptible to higher N supply (Table 2 and 3). Recent studies on wheat grain showed that the effect of N fertilization on some single PAs appear to be inconsistent, and the causes of this may be the result of the application of different agro–technical measures, different climatic conditions, and their interaction (Ma et al 2021; Tian et al 2022).

Ma et al. (2015) reported that increased N application led to increased concentrations of insoluble ferulic and vanillic acid. However, applied range of N (180–300 kg N/ha) is much higher than that of farmers' general practices. Stumpf et al. (2019) found that higher amounts of N led to the decrease of the soluble ferulic acid, while bound ferulic acids were not affected. Tian et al. (2022) observed no effects of various N rates (0, 42, 84, 126, and 168 N kg/ha) on ferulic acid content in bound phenolic extract, though they significantly affected p–coumaric and sinapic acids. The authors concluded that the intensive field management, including high N input, fungicide, and micronutrient use, might reduce wheat phytochemical levels and that soil N levels could impact wheat phytochemical accumulation. Tian et al. (2021) also reported an interaction between N and sulfur (S) affecting ferulic acid content: at 0 kg S/ha, an increase in N from 56 to 146 kg/ha raised ferulic acid, whereas at 22 kg S/ha, ferulic acid was not affected by the higher N rate.

Conclusions

There are significant differences in the phenolic properties and PAs content between soluble–free and insoluble–bound extracts of wheat. The properties of dominant insoluble–bound phenolics were more affected by higher N level. Although cultivars showed a similar response to increased N rate, considerable variability in their phenolic properties was observed. Further studies are necessary to understand the relationships among cultivars, nitrogen management, climate conditions, and their interaction with phenolic component. The results revealed that the assessed modern cultivars showed significant health benefit properties.

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