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STUDIES ON SOME BIOACTIVE COMPOUNDS FROM COLORED WHEAT

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Abstract

The varieties of colored wheat registered are very limited, and those available have a low agronomic value. The anthocyanin compounds present in colored wheat vary depending on the wheat variety as well as the growing conditions. Due to their antioxidant activity, anthocyanins are of increasing interest to nutritionists, food researchers and plant breeders. The objective of this work was to determine some bioactive compounds from wheat genotypes H171-I with purple grains and H171-III - with red-purple grains. The samples were evaluated by UV-VIS spectrophotometry for anthocyanins, phenols, enzymes and total antioxidant capacity. The determinations showed that the highest level of anthocyanins was found in the genotype H171-I, followed by Pitar and H171-III; phenolic compounds were present in all wheat genotypes, but unlike the other samples, they were synthesized at higher levels in the genotype H171-I. This genotype also had the highest total antioxidant capacity. Data on the activity of polyphenol oxidase and peroxidase enzymes showed a high level for both enzymes in the red grains Pitar genotype. The analyzed compounds varied according to the investigated wheat variety.

Key words: *anthocyanins; antioxidant capacity; colored wheat; enzymes; phenols*

INTRODUCTION

Wheat (*Triticum aestivum*) is the second most consumed cereal type worldwide, after rice (Arendt and Zannini, 2013). White and red wheat varieties are the most commonly consumed. Colored wheat varieties, which contain anthocyanins, are either not widely grown or are grown as special crops. To date, colored wheat species have been studied to obtain information on the exact composition of anthocyanins in these grains, but this is still largely unknown (Abdel-Aal et al., 2006). This would mainly be due to differences in the extraction methods applied. Wheat species containing high amounts of anthocyanins in the outer layers of the pericarp are those with blue, purple or black grains. Anthocyanins are phenolic compounds classified as flavonoids, along with flavones, flavanols, flavanones and iso-flavonoids (Liu, 2004). Grain seeds are not typical sources of anthocyanins, but blue, red and purple seeds are attractive to consumers, food producers and plant breeders (Guo et al., 2012), therefore relevant knowledge has also been accumulated about maize, wheat, barley, oats and rice, which are the most important food sources in the world. There is still a need for wheat varieties with good genetic stability, excellent stress resistance and high yield (Guo et al., 2012). Anthocyanins are generally defined as bioactive, non-nutritional compounds responsible for antioxidant and UV/photoprotective functions (Ryan et al., 2001) and also play a role in plant reproduction (Kong et al., 2003). Anthocyanins participate in the formation of non-specific disease resistance in plants (Treutter, 2006), such as pre-harvest germination, where the red pigment of red-bean wheat is synthesized via the flavonoid biosynthetic pathway, in which the dihydroflavonol-4-reductase (DFR) gene is one of the genes involved in anthocyanin synthesis (Bi et al., 2014).

In purple-grained wheat, anthocyanins were found to be located in the pericarp, whereas in blue wheat they are found in the aleurone layer. In black wheat (referred to as "deep purple"), anthocyanins were found in both the pericarp and the aleurone layer (Abdel-Aal et al., 2012).

In the case of heat-treated grains, the distribution of anthocyanins in the outer layers of the grain may affect the stability of these compounds in the extraction process. Purple wheat has, on average, a lower total anthocyanin content than blue and black wheat varieties. Colored wheat seeds are a natural source of pigments as phytochemicals and can impart desirable color and stability to commercial food products.
The objective of this work was to determine biochemical compounds with antioxidant properties in four wheat genotypes with different colored pericarp, including purple.

MATERIALS AND METHODS

The biological material was constituted of the wheat genotypes "Pitar", H171-I and H171-III from the National Agricultural Research and Development Institute, Fundulea (Figure 1).

Figure 1. Wheat genotypes investigated

"Pitar" is a Romanian winter wheat variety, registered in 2015, with a high quality and red grains. The genotypes H171-I and H171-III are experimental lines with purple (H171-I) and purple-red (H171-III) pericarp respectively, but the grains still have irregular color, obtained by crossing the wheat variety "Favorit" with the couch grass species *Thinopyrum bessarabicum.*

Extracts preparation
For each sample 4 g of material was weighed to which 40 ml of methanol was added. The mixtures were incubated in a shaking incubator at 120 rpm for 48 hours. For experiments, the mixtures were centrifuged at 10000 rpm for 5 minutes. The obtained filtrate was stored at 40^0C until use (24 hours). Extracts were then analyzed for biochemical compounds.

Determination of total anthocyanins. The total anthocyanin content of diluted plant material extracts was estimated by the pH differential method (Giusti and Wrolstad, 2001). Extracts were diluted twice: once with potassium chloride buffer (pH 1.0) and then with sodium acetate buffer (pH 4.5). The dilutions were allowed to equilibrate for 15 min before measuring their absorbance in the spectrophotometer at two wavelengths, at 520 nm and 700 nm respectively. The dilution factor (10) was obtained by dividing the final sample volume by the initial volume. The dilutions were allowed to equilibrate for 15 minutes before measuring their absorbance in the spectrophotometer. Absorbance (A) was measured at 520 nm and 700 nm in both buffers respectively:

$$A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$$

The following equation were applied to estimate the total anthocyanin content:

$$\text{TAC (mg/L)} = \frac{A\ x\ MW\ x\ DF\ x\ 10^3}{\varepsilon\ x\ l}$$

Where: TAC=Total Anthocyanins Content in mg/L; DF is the dilution factor (10); MW is the molecular weight of cyanidin-3-O-glucoside (Cy3G) (MW = 449.2 g/mol), and ε its molar absorptivity (29,900 L / mol $^{-1}$ & cm-1 for cyd-3-glu); 10^3 = the conversion factor from g to mg.

Determination of total phenolic content. The spectrophotometric determination of total phenol content was carried out according to the Folin-Ciocalteu method (Singleton, 1999). The method consists of chemically reducing the Folin-Ciocalteu reagent (a mixture of tungsten and molybdenum oxides) and measuring the absorbance at 765 nm. Total phenol values were expressed as mg gallic acid equivalent (GAE)/L sample. Measurements were obtained using an Eppendorf UV/Vis spectrophotometer. The concentration of polyphenols (mg GAE/L) in the samples was determined from the standard calibration curve (y = 0.0012x + 0.0102; R2 = 0.9996) obtained for different gallic acid concentrations (50 - 500 mg/L) (Figure 2).

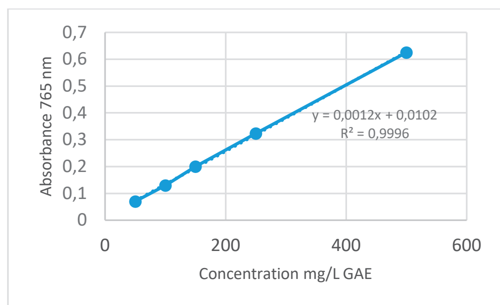

Figure 2. Standard curve of Gallic acid

Determination of polyphenol oxidase (PPO) and peroxidase (POD)

PPO activity was determined by the method of Liu et al. (Liu et al., 2005). The standard reaction mixture contained: 1.5 ml of 40 mmol/l catechol and 2.3 ml of 0.1 mol/l phosphate buffer (pH 6.5). The reaction mixture was incubated at 25°C for 5 min. Then 0.2 ml of sample was added to the test tube over the mixture with shaking. Immediately, the absorbance increase was measured at 420 nm with a UV-VIS spectrophotometer (Eppendorf).

Peroxidase activity was determined spectrophotometrically at 470 nm using the method described by Bergmeyer (1974) with minor modifications. The reaction mixture contained: 2.8 ml 0.1 M potassium phosphate buffer pH 7.0; 0.05 ml 30 mM guaiacol solution; 0.05 ml 3% H2O2 and 0.1 ml protein extract. The samples thus prepared are read in the spectrophotometer at OD 470 nm (for each sample - 1 reading/minute for 2 minutes). In both cases, the optical density variation was studied for two minutes. Enzyme activities were calculated according to the formula:

$$U/ml = \frac{\Delta OD \times Vt \times D}{\Delta t \times \varepsilon \times Vs}$$

Where: ΔOD = optical density; Vt = total volume of reaction mixture; Vs = sample volume; ε = extinction coefficient of catechol (3400 mM/cm) and guaiacol (26.6 mM/cm) respectively; Δt = reaction time.

Total antioxidant activity

Total antioxidant activity of the samples was assessed by the phosphomolybdate method (Prieto et al., 1999), using ascorbic acid as a standard (Garrat, 1964). Results were expressed in ascorbic acid equivalents based on the standard calibration curve (y = 0.0059x + 0.0409; R=0.993) obtained for different ascorbic acid concentrations (10 - 200 µg/ml) (Figure 3).

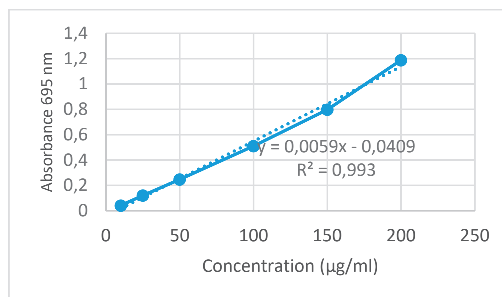

Figure 3. Standard curve of Ascorbic Acid

The method is based on the reduction of Mo (VI)-Mo(V) by extracts and subsequent formation of a green phosphate/Mo(V) complex at acidic pH. The reaction mixture consisted of: 0.3 ml of extract combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Tubes containing the reaction solution were incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the solution was measured at 695 nm relative to the control using a spectrophotometer. Methanol (0.3 ml) was used as a control. The higher the absorbance values, the higher the total antioxidant capacity. Ascorbic acid equivalents were calculated using a standard graph. Results were expressed in ascorbic acid equivalents based on the standard calibration curve (see Figure 6) obtained for different ascorbic acid concentrations (10 - 200 µg/ml).

Statistical analysis

All experiments were performed in 3 replicates. Results were expressed as mean values and standard deviation (±SD). Statistical analyses were performed using Microsoft Office Excel 2019.

RESULTS AND DISCUSSIONS

Wheat genotypes "Pitar", H171-I and H171-III were evaluated by UV-VIS spectrophotometry for their total anthocyanins, phenols and enzymes composition (polyphenol-oxidase - PPO and peroxidase - POD).

Determination of total anthocyanins content (TAC) showed that the highest level of anthocyanins was recorded in genotype H171-I (24.58±0.208 mg/L), followed by "Pitar" (16.96±0.095 mg/L) and H171-III (12.19±0.089 mg/L) (Figure 4).

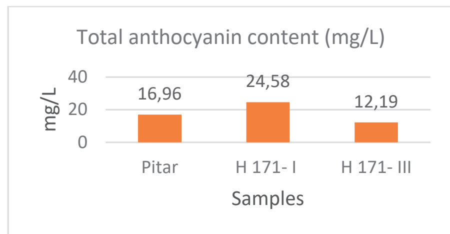

Figure 4. Anthocyanin content in grains

The anthocyanin compounds present in colored wheat depend on the wheat variety, growing conditions and maturity of the grain. These factors can sometimes make it difficult to identify and quantify anthocyanins (Rao et al., 2018). Due to their potential health-enhancing attributes through their antioxidant activity, anthocyanins are of interest to the food industry. At present, in the world, there is some cultivars with purple pericarp as Laval, Laval 19, Indigo with the TAC range between 72 and 96 mg/kg (Francavilla and Joye, 2020).
Selection of the wheat lines with intense purple pericarp, from H171-I, could increase the total anthocyanin content.
Total phenol content was determined from the standard calibration curve (see Figure 2).
The data obtained showed that the highest level of total phenols was recorded in genotype H 171-I (69.59±0.810 mg GAE/L), followed by wheat genotype 'Pitar' (50.4 ±0.483 mg GAE/L). The lowest concentrations of polyphenols were recorded in H-171-III (37.4 ± 0.813 mg GAE/L) (Figure 5).

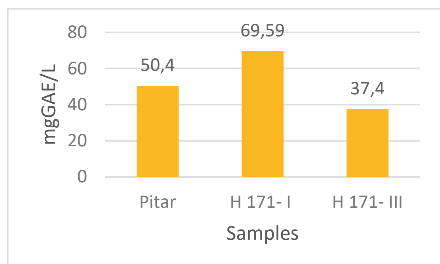

Figure 5. Total phenols content in wheat grains

Biosynthesis of phenolic compounds is not yet well understood, but has been observed to depend on genotype and growth temperature (Ma et al., 2016; Wu et al., 2016). Moisture level has also been shown to affect the concentration of total phenolics in cereal grains (Abdel-Aal et al., 2003).

Determination of enzyme activity.
Data on the enzyme activity of polyphenol-oxidase (PPO) and peroxidase (POD) showed that it was maximum for both enzymes in the red grain wheat genotype "Pitar" (0.206±0.009 U/ml PPO and 0.055 ± 0.002 POD U/ml, respectively). The results suggest that these enzymes are among those responsible for the oxidation of catechol in wheat. The wheat lines H171-I and H171-III showed similar levels of activity, but slightly higher for genotype H171-I (0.156±0.002 U/ml PPO and 0.026±0.001POD) compared with genotype H171–III (0.144 ± 0.009 PPO and 0.022 ± 0.002 POD) (Figure 6).

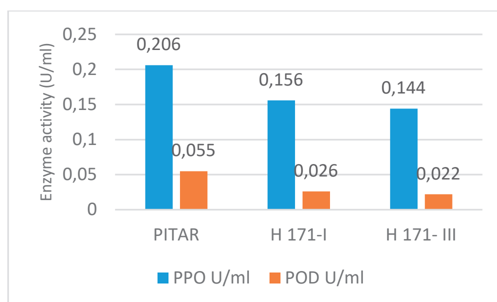

Figure 6. Polyphenol-oxidase (PPO) and peroxidase (POD) activities in wheat grains

Polyphenol oxidase (PPO) is predominantly found in the outer layers of wheat kernel (Baik et al., 1994). High levels of PPO have been associated with discoloration of wheat end-use products (Singh & Sheoran 1972; Edwards et al., 1989). In wheat, polyphenol oxidase (PPO) is responsible for the oxidation of polyphenolic compounds to quinones, which, following further reactions, leads to the production of brown melanin (Stauffer, 1987). Peroxidase (POD), the phenol-oxidizing enzyme, has been studied in many plant-pathogen interactions because of its significant role in the biosynthesis of plant cell wall components (Grisebach, 1981; Lamport, 1986). In wheat grains, peroxidase is present in the epidermis, seed coat, embryo and

endosperm. POD can oxidize ferulic acid and other major phenolic acids, producing chromophoric groups and brown substances (Fraignier et al., 2000). A high activity of POD can influence the quality of the flour and consequently can cause an undesirable color of the paste (Taha and Sagi, 1987; Kobrehel et al., 1974). In addition, peroxidase has been reported to play a key role in wheat resistance to leaf rust (Johnson and Lee, 1978).

Total antioxidant capacity. Total antioxidant activity was expressed in ascorbic acid equivalents.
Table 1 shows the results of the total antioxidant activity of the tested samples.

Table 1. Total antioxidant activity of the samples

Genotype	µg/ml
PITAR	24.86 ± 0.294
H171 I	29.13 ± 0.191
H171 III	22.23 ± 0.350

Results were expressed as mean values and standard deviation (±SD).

These results showed that total antioxidant activity was recorded in the genotype H171- I (29.13±0.19 1µg/ml), followed by “Pitar” (24.86±0.294 µg/ml) and H171-III (22.23±0.350 µg/ml).
A number of other reports have observed certain correlations between total anthocyanin content, total phenolic content and antioxidant potential (Lee et al., 2013; Harakotr et al., 2014).

CONCLUSIONS

The results obtained highlighted the presence of anthocyanins and phenolic compounds in all investigated wheat genotypes, but at higher levels in the case of the H171-I line with purple pericarp; Data on polyphenol-oxidase (PPO) and peroxidase (POD) enzyme activity showed that it was higher, for both enzymes, in the red “Pitar” grain genotype; The genotypes H171-I with purple grains and H171-III with red-purple grains recorded similar enzyme activity levels, but slightly higher in the H171-I genotype. Phenolic compounds can promote the total antioxidant capacity in the plant.

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