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Variability of Polyphenols in Seeds of Different Varieties and Recombinant Lines of Soybean

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Abstract

Functional foods, which are characterized by therapeutic and prophylactic properties, are becoming increasingly popular in most countries around the world, as they provide the human body not only with essential nutrients, but also with necessary biologically active supplements. Among them, flavonoids play an important role. They belong to the group of polyphenols and are characterized by antioxidant, anticancer, hepatoprotective, and antisclerotic activity. Analysis of a large number of soybean collection samples showed that the flavonoid content in seeds significantly depends on the color of their seed coat. In yellow-seeded genotypes, their content varies between 70-100 μg/g, and in black-seeded genotypes, between 250-300 μg/g. Significant variability in flavonoids has also been observed over the years.

Keywords: Polyphenols; Flavonoids; Antioxidant activity; Nutritional qualities; Black soybeans

Introduction

Legumes play a crucial role in the nutritional balance of our planet. They provide millions of people with a balanced diet, especially in developing countries. Consuming dishes made from the seeds of this group of crops helps improve immunity, regulates the normal functioning of the gastrointestinal tract, and has a beneficial effect on maintaining body weight, which allows you to keep a beautiful figure and prevent premature aging. Their main value lies in their high protein content, which is characterized by a balanced composition of essential aminoacids, ensuring healthy nutrition for humans and feeding farm animals and poultry. The significant increase in the population of our planet requires constant growth in food production. While in 1950 the population of the planet Earth was 2.5 billion, in 1970 it reached 3.7 billion, and in 2010 - 6.9 billion. According to forecasts, in 2050 there will be 9.15 billion people living on Earth. Global warming, a decrease in the amount of quality water for irrigation, and a reduction in agricultural land as a result of urbanization will lead to a significant food shortage, especially in developing countries. The only way to overcome this is to reduce the production of meat and dairy products and switch to plant-based foods that are not inferior in nutritional value and, in some cases, are even better.

In addition to high-quality protein, legume seeds contain many vitamins, minerals, and other biologically active compounds. A particularly valuable component of their seeds is flavonoids-compounds of a polyphenolic nature that have a preventive effect on the cardiovascular system and oncological diseases, lower blood pressure, inhibit platelet aggregation, prevent premature aging, and help the body better adapt to environmental factors. Today, 537 million people on our planet suffer from diabetes, and by 2040, their number will grow to 783 million [1]. An increase in such patients is particularly noticeable in developing countries. Numerous studies have clearly demonstrated the preventive effect of a plant-based diet with antidiabetic activity, which reduces blood sugar levels, increases insulin secretion in the body, regenerates pancreatic β-cells, normalizes glycolysis in the liver, and intensifies the flow of glucose into muscle tissue. Among the 100 plants recommended for the prevention and treatment of this disease are chickpea, soybean, bean, pea, and vetch [1]. We believe that this group should be supplemented with such an extremely important legume as lentils [2]. Diets that include the above-mentioned crops not only help control diabetes but also promote optimal body weight.

Polyphenols are a class of natural chemical compounds that contain one or more phenolic groups (ring-shaped structures). They are synthesized only by plant organisms and are characterized by strong antioxidant activity [3]. These ingredients are quite common in fruits, vegetables, and whole grains, especially in legumes. In recent years, this group of substances has been of great interest to physiologists, medical professionals, and nutritionists due to the accumulation of new scientific information about their positive effects on human health. Due to the presence of a significant number of double bonds, they are carriers of active oxygen and are intensively involved in redox processes as antioxidants. In plants, they are most often found in the form of glycosides.

Depending on the number of phenolic units in the polyphenol molecule, hydroxyl groups, and the level of oxidation, they are divided into the following groups: phenolic acids, flavonoids, stilbenes, and lignins [4,5]. Phenolic acids are formed as a result of microbial breakdown in the large intestine from more condensed molecules [6]. The most studied are gallic, ellagic, caffeic, chlorogenic, and rosmarinic acids. They serve as important signaling molecules in the interaction of plants with microbial organisms, improving nutrition and performing protective functions [7].

Flavonoids are one of the most common and important groups of polyphenols. Their biological role was discovered in 1936 by Hungarian-born American biochemist and Nobel laureate Albert de Sautard, who isolated them from red pepper and proved their positive effect on the resistance of blood vessel walls to damage. Depending on the level of heterocyclic oxidation, this group of biologically active compounds includes the following classes of substances: flavones, flavanols, isoflavones, flavanones, catechins, anthocyanidins, leucoanthocyanidins, and chalcones [8]. Studies show that flavonoids have a wide range of effects: they regulate oxidative-reductive processes and the activity of enzymes and receptors, have a positive effect on the capillary system, and protect against stress, inflammation, viral, fungal, bacterial, and carcinogenic diseases [9-11].

It should be noted that they play an active role in plant metabolic functions, especially those related to the effects of environmental stress factors and protection against harmful organisms and diseases [12,13]. Flavonoids are regulators of auxin metabolism, a hormone that regulates plant growth processes. In small concentrations, growth is accelerated, and in large concentrations, it is inhibited. Therefore, this physiologically active substance is usually concentrated in growth points, buds, and young leaves [11,13]. Anthocyanin glycosides, which belong to this class of compounds, determine the diverse color of flowers, as their molecules are capable of selectively absorbing a certain spectrum of sunlight. A similar mechanism is also involved in determining the color of fruits. Ultraviolet radiation induces intense biosynthesis of flavonoids, especially quercetin and isorhamnetin, which are characterized by an increased number of OH groups. But they are most beneficial as preventive substances for maintaining a healthy vascular system by improving blood circulation, increasing elasticity, and strengthening capillary walls, which helps prevent atherosclerosis, normalizes blood pressure, and lowers low-density lipoprotein levels. Isoflavones are the most accessible to the human body, while anthocyanins are much less easily absorbed. In addition, all of these types of polyphenols have antioxidant, anti-inflammatory, and anti-diabetic effects and have a positive effect on reducing excess body weight in humans [14].

It is important to note that, on average, each Japanese person consumes 27.8 mg of isoflavones daily [15]. It is believed that significant amounts of these biologically active substances have a preventive effect on breast cancer in women and prostate cancer in men [16-18]. Studies have shown that isoflavones neutralize estrogens, which contribute to the development of cancerous tumors. In the human body, bioactive compounds are converted into phytoestrogens, which significantly reduce the activity of the hormone estrogen, which is an activator of cancer cell division. Thus, in the body, the hormone on the receptors of cancer cells is replaced by a plant biocomponent similar in structure to it, but with a significantly weaker effect. Under such circumstances, cell division is significantly reduced. In addition, the formation of capillaries and small vessels is also blocked, resulting in the death of the tumor cell. Therefore, in countries where foods rich in isoflavones, mainly soy, are consumed, cancer mortality is significantly lower. For example, in China it is 5 times lower, in Korea 10 times lower, and in Japan 4 times lower than in the United States. In many Asian countries, about 80 mg of isoflavones are consumed daily with food, while in the US, it is up to 5 mg.

It has been clearly proven that there are significantly more isoflavones in hypocotyls than in cotyledons [19]. That is why it is so important for health to consume young soybean sprouts.

The aim of our research was to determine the total amount of flavonoids in the seeds of collection varieties and recombinant soybean lines created by hybridizing parental forms that are distinguished by their increased drought resistance. Our breeding program also pays considerable attention to increasing protein levels.

Flavonoids are the main phenolic components of legume seeds, giving them their color and aroma. They are characterized by low molecular weight and have two aromatic rings connected by a third ring containing oxygen. This group includes anthocyanins (colored components) and anthoxanthins (non-colored compounds). The development of soybean varieties with improved nutritional qualities is one of the most important areas of breeding. The value of seeds increases significantly with an increase in protein and phenolic components.

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Materials and Methods

The land of the Plant Breeding and Genetics Institute, where the research was conducted, is located in the southern part of the Black Sea lowlands, in the steppe zone of the Odessa region. The relief is almost perfectly flat. The soil cover consists of southern medium-humus high-loam chernozems on loess deposits. The thickness of the humus layer is 40-50 cm, with a humus content of 3.5-4.5%. The amount of available nutrients (in mg per 100 g of soil) is 3-4 nitrogen, 10-15 P2O5, and 20-30 K2O. The reaction of the soil solution is neutral or slightly alkaline (pH of the salt extract is 6.0-7.2).

The climatic conditions in the research area are moderately warm, formed mainly under the influence of Atlantic and Mediterranean air masses. The average annual air temperature is +9.6°C, the sum of effective temperatures is 3300°C, and the average annual precipitation is 430 mm. Winters are mild and short. The coldest month is January, with an average long-term air temperature of -2°C. Spring comes early, with temperatures rising above +5°C in the second or third decade of March. Summers are hot and long. During the summer, the soil loses moisture due to high temperatures and low relative humidity, sometimes down to 35-40%, which leads to frequent dry winds. The temperature regime of the region does not limit the development of leguminous crops, but arid conditions are usually accompanied by high temperatures, which inhibit plant growth. The arid nature of the climate is due not only to the lack of total precipitation (380-450 mm), but also to its uneven distribution throughout the growing season.

The studies used collection varieties and recombinant stable soybean breeding lines, which form the basis of our breeding program. The area of the plots in the ecological test was 10 m^2 with three replicates. The competitive test was also sown on the same plots with five replicates.

The flavonoid content, extracted with 70% ethanol, was determined based on their reaction with aluminum chloride [20,21]. The amount of protein was determined by mineralizing organic matter with concentrated sulfuric acid in the presence of a catalyst [20]. Ammonia distillation was performed using a Kjeltec Auto 1030 device.

Results and Discussion

Our soybean breeding program is aimed not only at increasing yields in arid conditions, but also at improving the nutritional quality of the seeds. This is because this crop is becoming increasingly important as a raw material for the production of functional foods with therapeutic and preventive properties. Therefore, we constantly monitor the protein content in seeds at all stages of the breeding process when creating new varieties, and also analyze this component of seeds in a large volume of the global gene pool in order to select high-protein genotypes for hybridization. In addition, in recent years, a number of biologically active substances, so-called minor food components, have been found to play a significant role in human health, exerting a positive therapeutic and prophylactic effect on the human body. These compounds include a group of polyphenols, which are abundant in legume seeds [22-25]. Reactive oxygen and nitrogen species, which damage proteins, fats, and DNA in the body, are bound and inactivated by natural antioxidants that come from food. Numerous studies have shown that legumes are an important source of these compounds.

Determining their content in soybeans, as well as other biochemical analyses of seed quality, is very important when selecting promising breeding lines. Knowledge of biochemical indicators helps to identify varieties that will not only produce high yields, but also high-quality seeds suitable for use both for food and industrial production, including raw materials for medical preparations.

We began assessing the flavonoid content of soybeans in ecological and competitive trials in 2015. The analysis of seeds of hybrid origin with different seed coat colors (yellow, black, and brown) revealed significant variability in this indicator depending on the year and genotype (Table 1).

In this study, the amount of flavonoids was estimated in 12 breeding lines with different seed colors, which were obtained by crossing the collection variety Cobra, which has black seeds, with the Ukrainian variety Yug 30 and the collection sample K-4937. The recombinant lines had different seed color intensities, ranging from yellow and light brown to completely black. This made it possible to differentiate the amount of flavonoids depending on the color of the seed coat. In black-seeded lines (5 pcs.), the total amount of these compounds varied between 257.0-291.0 μg/g, in light brown (2 pcs.) - 180.8-227.7, in red-brown (2 pcs.) - 212.3-219.7, brown (1 pc.) - 165.1 μg/g. In the yellow-seeded line of this hybrid combination, the amount of flavonoids was only 84.4 μg/g. The results clearly indicate that the presence of pigments in the seed coat contributes to an increased amount of flavonoids.

It is known that the biosynthesis of anthocyanin pigments and flavonoids in the soybean seed coat is controlled by loci I, T, W, R, and O [26]. Genotypes with dominant T and R alleles are characterized by completely black seeds. The breeding lines carrying TrO alleles are distinguished by their brown color. Different shades of brown depend on the combination of the above loci in the dominant and recessive states. The presence of the I gene in the soybean genotype inhibits the biosynthesis and accumulation of pigments, resulting in the formation of yellow-colored seeds, while its recessive allele leads to an even distribution of pigments in the seed coat. Thus, by combining certain gene loci, it is possible to regulate the biosynthesis of flavonoids in individual soybean genotypes. For specific purposes, through targeted selection of parents and repeated selections in early hybrid generations, it is possible to create recombinant lines with an increased amount of these compounds.

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Table 1: Flavonoid content in seeds of recombinant soybean lines with different seed coat colors.

Field No 2018	Breeding line	Colour			Total flavonoid content, µg/g				
		seed	pubescence	flower	2015	2016	2017	2018	average
26345	(K-4937 /Cobra) / Yug 30	light brown	gray	white	161,1	301,1	267,0	181,7	227,7
26347	(K-4937 /Cobra) / Yug 30	reddish brown	gray	white	169,6	266,9	231,0	211,5	219,7
26348	(K-4937 /Cobra) / Yug 30	black	absent	purple	268,8	303,0	230,0	362,3	291,0
26351	(K-4937 /Cobra) / Yug 30	black	absent	purple	344,2	165,7	261,0	381,3	288,0
26353	(K-4937 /Cobra) / Yug 30	black	red	purple	316,2	167,9	225,0	337,7	261,7
26354	(K-4937 /Cobra) / Yug 30	reddish brown	gray	white	205,1	208,3	187,0	248,9	212,3
26361	(K-4937 /Cobra) / Yug 30	yellow	absent	purple	115,8	79,5	59,0	83,3	84,4
26363	(K-4937 /Cobra) / Yug 30	brown	absent	white	139,0	221,1	170,0	130,4	165,1
26367	K-4937 /Cobra	black	absent	purple	245,8	268,5	254,0	259,8	257,0
26370	(ms1Tonica / Tokyo) / Noir 2	light brown	reddish gray	purple	286,3	160,4	136,0	140,7	180,8

The flavonoid content in soybean collection samples was studied in an ecological nursery (Table 2). Initially, it included a large number of genotypes from different countries, but under our arid conditions, only varieties that tolerate temperature stress and are capable of forming seeds at low soil moisture remained. These are mainly varieties of Ukrainian breeding.

Table 2: Flavonoid content in soybean varieties of ecological testing.

Variety	Total flavonoid content, µg/g							
	2015	2017	2018	2019	2020	2021	2022	average
Smolyanka	399,6	380,0	367,0	354,4	144,0	96,0	370,0	301,6
Oriana	209,3	243,0	136,0	114,1	54,0	25,0	120,0	128,8
Oksana	229,3	232,4	127,0	54,9	50,0	-	90,0	-
Farvater	150,4	180,0	175,0	111,3	53,0	-	130,0	-
Khutorianka	154,6	156,0	129,0	76,8	-	-	-	-
Artemida	128,5	124,3	128,0	91,4	50,0	43,0	100,0	95,0
Mentor	176,9	70,0	120,0	93,9	-	-	-	-
Ametyst	67,0	96,0	162,0	121,2	55,0	33,0	160,0	99,2
Melpomena	62,6	87,0	155,0	120,4	52,0	44,0	140,0	94,4
Medeya	61,0	82,3	159,0	109,1	47,0	28,0	160,0	92,3
Valuta	67,8	81,0	147,0	114,1	57,0	35,0	160,0	94,6
Znakhidka	55,9	95,0	89,0	121,2	47,0	34,0	-	-
Taverna	35,5	81,3	130,0	111,4	53,0	29,0	190,0	90,0
Izumrudna	57,7	66,0	98,0	122,9	70,0	27,0	170,0	87,4
Feniks	50,0	70,0	98,0	126,4	72,0	32,0	120,0	81,2
Vasyl'kivska	54,0	64,0	84,0	121,5	56,0	36,0	100,0	73,6
Siaivo	56,1	67,0	91,0	95,5	58,0	31,0	190,0	84,0
average	118,6	127,9	140,9	121,2	61,2	37,9	157,1	118,2

In this nursery, the Smolyanka variety clearly stood out in terms of flavonoid content, with an average of 301.6 µg/g of this phytochemical in its seeds. A distinctive feature of this variety is the black color of the seed coat. Thus, the results in Tables 1 and 2 indicate that the presence of anthocyanins in the seed coat significantly affects the total amount of flavonoids. Similar results have also been obtained by other scientists. The color of the seed coat affects the level of flavonoids, isoflavones, and fatty acids in

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soybeans [27,28]. A study by Vietnamese scientists clearly showed that the flavonoid content was strongly dependent on the color of the seed coat, while there was a weaker correlation with the total amount of phenolic compounds [29]. The color of the seeds did not affect the protein content.

It should be noted that the content of this ingredient in the seeds of yellow-seeded genotypes was also characterized by a certain level of variability. For example, in the Oriana and Oksana varieties, the amount of flavonoids exceeded 200 µg/g in some years, which is a fairly high indicator. It is also necessary to note the significant variability in flavonoid content over the years. Thus, in the new Taverna variety, the amount of these compounds was 35.5 µg/g in 2015, 81.3 in 2017, 130.0 in 2018, 111.4 in 2019 - 111.4, and an average of 90.0 µg/g over 7 years. The Smolyanka variety, which is distinguished by its high flavonoid content, showed the following variability over the years: 2017 - 380 µg/g, 2018 - 367.0, 2019 - 354.4, 2020 - 144.0, 2021 - 96.0, 2022 - 370.0 µg/g.

The variability in the amount of flavonoids in the recombinant lines of the competitive trial is shown in Table 3.

Table 3: Flavonoid content in recombinant soybean lines of the competitive trial (2017-2021).

Plot No	Origin	Total flavonoid content, µg/g					
		2017	2018	2019	2020	2021	average
	Siaivo, standard	65,0	93,0	111,4	58,0	47,0	74,9
102/18	Hybrid 905 / Senhae № 20	128,0	180,0	111,5	74,0	38,0	106,3
105/18	Hybrid 905 / Senhae № 20	89,0	174,0	131,8	50,0	31,0	95,2
120/18	Volgogradka/ Montreal	79,0	114,0	129,8	70,0	30,0	84,6
145/18	Л 29/91 / Л 892/94	50,0	109,0	153,3	-	-	-
108/18	Evans/ Л 21-22 (Serenada)	61,0	131,0	103,1	62,0	35,0	78,4
119/18	Breeding No11217/95	90,0	103,0	98,0	57,0	41,0	77,8
149/18	Hybrid 905 / Senhae № 20	64,0	98,0	115,5	68,0	40,0	77,1
118/18	Hybrid 905 / Senhae № 20	62,0	94,0	117,8	76,0	41,0	78,2
109/18	Ioshioka dairynu / Palmira	58,0	104,0	106,8	29,0	37,0	66,9
111/18	Hybrid 905 / Senhae № 20	86,0	113,0	-	-	45,0	-

Since all recombinant lines in this nursery were characterized by yellow seed coat color, the content of this component varied to a lesser extent compared to Table 1. The seeds of the standard variety Syaivo contained 74.9 µg/g of flavonoids, and most lines contained approximately the same amount. Only recombinant lines No. 102/18 and No. 105/18 stood out with an increased amount. In the first of these, the flavonoid content exceeded 100 µg/g in three out of five years, and in the second, in two years. A number of studies have shown that the amount of isoflavones in soybean plants is highly variable. Thus, when analyzing 200 collection forms of different origins, the range of variability of this indicator was 520.30-7000.91 and 1999.96-6448.99 µg/g for recombinant lines [30]. Approximately the same data were also observed in other studies [31-33]. Despite this, the heritability coefficient was quite high - 0.47-0.67 for collection samples and 0.54-0.71 for recombinant lines. These indicators testify to the important role of genetic factors in increasing the level of isoflavones in soybean seeds [30]. These authors identified 37 loci (QTL) that control 4 isoflavones in recombinant lines.

It has been established that the metabolic processes occurring in seeds also significantly depend on their size [34-36]. Studies show that genetic characteristics of a variety account for 83% of the variability in protein content in seeds [37]. In this regard, it is important to constantly search for valuable genotypes among global collections, which are continuously maintained by a number of research institutions around the world. Today, this is the most effective approach in breeding work.

In recent years, interest in bioactive plant compounds has grown significantly due to their potential use as functional products in the food industry. For this purpose, grains, seeds, or individual plant organs are used in food, as well as in the form of concentrated solutions and food additives. They are extracted using a number of solvents [38-41]. This requires specific varieties, the seeds of which will serve as raw materials for the extraction of functional components. Black-seeded varieties are just right for this kind of production. In addition, it has been found that flavonoids and tannins are also easily soluble in water [42,43]. Legume seeds are usually used for food after soaking and boiling. A number of studies have shown that during cooking, high temperatures and moisture reduce the level of phenolic components [44-46]. When there is a significant amount of phenolic acids in the environment, they interact with the macromolecules that are next to them [47]. It is important to note that after boiling, the amount of phenolic compounds in the cotyledons was significantly higher than in the skin [42]. This situation can be explained by the fact that during the swelling period of the seeds, the active ingredients of the skin

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migrate mainly to the cotyledons. In addition, during extraction and processing, phenolic compounds react with other molecules, especially co-pigments, organic acids, and metals, and form more complex associations with each other [48, 49]. When exposed to high temperatures during the preparation of certain dishes, the amount of free and bound phenolic components decreases significantly, while more complex structures are broken down into simpler ones [42,47].

Thus, the studies conducted indicate that under our conditions, characterized by high temperatures during the growing season and insufficient moisture in the soil, the total flavonoid content in yellow-seeded genotypes ranges from 70 to 100 μg/g. The increased level of total flavonoids in black-seeded genotypes is mainly due to the biosynthesis of proanthocyanidins and anthocyanins in the seed coat [50].

In vitro studies have shown that a small amount of phenolic compounds is absorbed in the small intestine [42]. However, the rest of them enter the large intestine, where they perform an important prebiotic function, i.e., they serve as a substrate for beneficial microorganisms [51,52]. Thus, there is no significant loss of these extremely important food components during digestion. These studies also showed that the absorption of phenolic compounds from the skin and cotyledons of legumes in humans is the same.

Conclusion

Evaluation of a large number of collection samples and recombinant soybean lines showed that seed coat color significantly affects the amount of flavonoids in seeds. In the black-seeded Smolyanka variety and black-seeded recombinant lines, the content of this ingredient was 250-300 μg/g, while in yellow-seeded genotypes it was 70-100 μg/g. To obtain flavonoids from soybeans, black-seeded genotypes must be used.

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