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ORIGINAL ARTICLE

Anthocyanin's as marker for selection of buckwheat plants with high rutin content

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Abstract This study is focused on the selection analysis of high rutin contents in various buckwheat species and cultivars, such as *Fagopyrum esculentum* Moench. (Cultivars Lileya, Bilshovik, Rubra), *F. tataricum* G. (ssp. rotundatum (Bab) Krot. and ssp. tuberculatum Krot.), *F. cymosum* Meissn, and *Fagopyrum giganteum* Krot. Rutin contents in vegetative organs of plants showed good correlation with anthocyanins contents in vegetative organs of Rubra cultivar. The presence of anthocyanin's contents in the vegetative organs of buckwheat can be a reliable genetic marker for screening plants with high content of rutin. In the third generation of selection process with the proposed selection method by us, a genetic line of Rubra cultivar with high rutin content in the vegetative mass has been obtained. The proposed method of selection based on the color visual assessment of plant parts of buckwheat which is correlated with anthocyanin contents. The color visual assessment of vegetative organs of buckwheat plants can be marker for selection buckwheat cultivars with high anthocyanin's and rutin contents.

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Keywords Anthocyanins · *Fagopyrum* species · Rutin

Anthocyane als Marker zur Selektion von Buchweizenpflanzen mit hohem Rutingehalt

Zusammenfassung Diese Studie konzentriert sich auf die Selektionsanalyse hoher Rutinkonzentrationen in verschiedenen Buchweizenunterarten und -sorten wie Fagopyrum esculentum Moench, (Sorten Lileya, Bilshovik, Rubra), F. tataricum G. (ssp. Rotundatum (Bab) Krot. und ssp. Tuberculatum Krot.), F. cymosum Meissn und Fagopyrum giganteum Krot. Der Rutingehalt in vegetativen Organen der Pflanzen zeigte eine eindeutige Korrelation mit dem Anthocyangehalt in vegetativen Organen der Sorte Rubra. Das Vorhandensein von Anthocyanen in den vegetativen Organen von Buchweizen kann ein verlässlicher genetischer Marker zum Screenen von Pflanzen mit hohem Rutingehalt sein. In der dritten Generation des Selektionsverfahrens mit der in der Studie vorgeschlagenen Selektionsmethose wurde eine genetische Linie der Sorte Rubra mit hohem Rutingehalt in der vegetativen Masse erzielt. Die vorgeschlagene Selektionsmethode basiert auf der optischen Einschätzung der Farbe von Pflanzenteilen des Buchweizens, die mit dem Anthocyangehalt zusammenhängt. Die optische Einschätzung der Farbe vegetativer Organe von Buchweizenpflanzen kann ein Marker zur Selektion von Buchweizensorten mit hohem Gehalt an Anthocyanen und Rutin sein.

Schlüsselwörter Anthocyane · Fagopyrum · Rutin

Introduction

The use of bioactive substances in food and pharmaceutical industries is limited due to lack of information about their biological activity and limited number source of raw materials. The search for efficient and economically profitable sources of flavonoids and drug development with P-vitamin activity is one of the major concerns of the pharmaceutical industry (Hollman and Katan 1999). Buckwheat could be an important nutritional source of flavonoids, especially in countries with an average daily flavonoid intake (Kreft et al. 1999). Seeds, dehulled seeds, hulls, straws, and leaves of buckwheat have good antioxidative capabilities over oats and barley (Holasova et al. 2002). The antioxidative properties of this plant are directly connected with the phytochemicals such as phenolic components, which have potential for preventing various metabolic diseases (Holasova et al. 2002; Velioglu et al. 1998). The use of buckwheat for production of gluten-free pasta, bread, and flour from plant extract has been of great interest for food industries since the last decade (Verardo et al. 2011). The incorporation of buckwheat in wheat flour allowed enhancing the antioxidant properties and functional components of wheat bread. It has also been suggested that the bioactive compounds from these plants play an important role in *in vitro* foregut fermentation in ruminant animals (Leiber et al. 2012). Therefore to select buckwheat species and cultivars with high phenolic content or find a way to increase the production of phenolic compounds in the different cultivars of buckwheat—regarding needs of agriculture and functional food industry—is a topic issue.

Rutin (rutinoside) is one of the phenolic compounds which is presented in buckwheat plants (Park et al. 2000). Rutin content in vegetative mass of buckwheat (leaves, inflorescences and stem) ranges from 2 up to 9% (Park et al. 2000; Kreft and Fabjan 2006), which may vary according to growth location and cultivar. It has been reported that groats and hulls of buckwheat contained approximately 0.20 mg g^{-1} and 0.84–4.41 mg g^{-1} rutin contents, respectively (Oomah and Mazza, 1996). Tartary buckwheat is an excellent source of rutin because groats of Tartary buckwheat showed levels of rutin at 80.94 mg g^{-1}. Rutin content in buckwheat flowers in full bloom stage is 6.8%, in the leaves 5.5%, and in the stalk 3%.

Buckwheat is one of the traditional crops of Ukraine and other European countries, and the selection of buckwheat with high flavonoids content such as rutin and anthocyanins can provide the pharmaceutical and food industry with nutrient premixes or nutrient systems. The selection of better plant type in buckwheat can be achieved through days to maturity, leaf length, leaf width, seed weight, number of internodes, and seeds/cyme (Rana and Sharma 2000). Thus, we have aimed to develop a method for selection of better varieties among selected buckwheat species and cultivars on the basis of rutin estimation to observe the relationship between quantity of anthocyanins, rutin, and pink color in the vegetative organs of the selected plant.

Materials and methods

Plant description

Four different buckwheat species and cultivars, such as *Fagopyrum esculentum* Moench (cultivars Lileya, Bilshovik, Rubra), *F. tataricum* G. (ssp. rotundatum and ssp. tuberculatum Krot.), *F. cymosum* Meissn, and *F. giganteum* Krot, were selected for the present study. Cultivar Rubra with high anthocyanins content (3.87−4.41 mg/100 g DW) in the vegetative organ has been received by family selection method from chemo mutants from Taras Shevchenko National University of Kyiv. Cultivar Lileya is received from the Institute of Agriculture at the Ukrainian Academy of Agrarian Science (UAAS). The tetraploid cultivar Bilshovik has been selected by academic Sakharov V. with colleagues. The cultivar Bilshovik is characterized by the high mass of 1000 grains (test weight). *F. tataricum* G. is a one-year plant which, among the species researched, has a better pollination of flowers and a higher grain production. *F. giganteum* Krot. is amphidiploid and received after crossing *F. tataricum* G. with the perennial plant *F cymosum* Meissn. (Krotov and Dranenko 1973). *F. cymosum* Meissn is polyploid with 32 chromosomes. The collection of buckwheat germplasm which is maintained at the Scientific Research Institute of Groat Crops in Ukraine comprises nearly 1000 samples which are readily available for breeding research.

Rutin estimation

In all selected plants, rutin content was analyzed by using Folin-Ciocalteu reagent with slight modifications (Singleton and Rossi 1965). Plant materials (inflorescences, leaves) of 50 and 100 mg were homogenized with 0.2 g glass powder and 2 ml methanol. The homogenate was centrifuged at 3,000 × g for 5 min, and the supernatants were collected in separate tubes and used for rutin analysis.

0.5 µl of rutin extracts (concentrations 0.5, 1, 2, 4 mg ml−1) and series of standard solutions were estimated on the plate with silica gel (Sorbfil). The chromatogram is placed in the S-chamber. The solvent system for the separation of flavonoid compounds is ethyl acetate-acetonitrile (13:5:2, v/v/v). After drying the plates with a hot air stream chromatograms were measured in the wavelength dimen-

sion of 450 nm. The rutin content is determined by the following formula:

$$X = C \times V/a$$

Where,

X – rutin (flavonoids) content in the samples, mg g^{-1}

C – rutin (flavonoids) concentration according to calibration, mg ml^{-1}

a – weight of plant material, g

Anthocyanin estimation

Anthocyanins were extracted from 0.5 g weight of fresh leaves with a 10 ml mixture of n-propanol: HCl: H_2O (18:1:81, v/v/v). The samples were heated in boiling water bath for 30 min followed by incubation for 24 h in the dark at 4 °C. Extracts were filtered, and absorbance was taken at 510 and 700 nm. The quantitative content anthocyanin pigment has been defined by using a differential spectrophotometer with pH factor (Giusti and Wrolstad 2001).

Statistical analysis

The means and standard deviations were calculated by the Microsoft Office Excel 2003. All results were expressed as mean ± standard deviations from three and four replications. The one-way analysis of variance by Fisher and a correlation coefficient was used for the statistical analysis.

Results and discussion

The anthocyanin's, flavonoids, and rutin are biochemically closely related (Ehlting et al. 2001; Taylor and Grotewold 2005). Therefore, the correlation index between quantitative levels of these compounds can be used as a selection criterion for analysis. The anthocyanins of buckwheat (cyanidin-3-glucoside and other glycoside forms of cyanidin) which have a pink color are very convenient for visual estimation as part of the selection method (Klykov and Moiseenko 2003). So, the aim of the first experiment was to choose buckwheat plants with high rutin content which were suitable for the next steps of the selection process and to observe the connection between the pink color of vegetative organs and rutin content.

It was estimated that cultivar Rubra has the highest rutin content among the experimental plants of different buckwheat species and cultivars, such as cultivars Lileya, Bilshovik, Rubra, ssp. rotundatum (Bab) Krot. and ssp. tuberculatum Krot., *F. cymosum* Meissn, and *F. giganteum* Krot. (Table 1). Thus, we observed plants which contain small quantities of rutin, plants with intensive pink color of stems, leaves and inflorescences, and plants' of various intermediate forms.

Table 1 Rutin content in the vegetative organs of buckwheat plants from the full-bloom stage (Percent Dry Weight)

Species	Stem	Leaves	Inflorescences
F. esculentum, diploid (Lileya)	0.7	5.5	6.5
F. esculentum, diploid (Rubra)	1.3	8.0	13.4
F. esculentum, tetraploid (Bilshovik)	0.7	5.9	6.8
*F. tataricum*v. rotundatum	1.0	5.3	6.1
*F. tataricum*v.tuberculatum	0.7	4.6	4.8
F. cimosum	1.1	2.5	6.4
F. giganteum	1.0	2.8	6.6
$LSD_{0.05}$	0.15	0.32	0.41

The results of quantitative analysis in this experimental variation of plants showed that the content of rutin corresponded with the color of vegetative organs. In the plants with no intensive pink color of vegetative organs, the rutin content amounted to 1.0 %, in the leaves to 4.6 %, and in the inflorescences to 6.6 % of DW. At the same time, the cultivar Rubra with intensive pink color had a rutin content in the stems of 1.3 % of DW, in the leaves of 8.0 % of DW, and in the inflorescences 13.4 % of DW. So we suggest a direct correlation the between pink color of vegetative organs and the rutin content.

The next series of experiments with a high quantity of plant samples for biological replication were done for defining the correlation between the pink color and anthocyanins content; the correlation between anthocyanins and rutin contents in the vegetative organs of buckwheat. For these experiments, buckwheat seedlings of cultivar Rubra in the earliest development stages (cotyledons and 2nd true leaf) were taken. All plants were divided into five groups:

1. Color of anthocyanins was absent on the surface of leaves of seedlings.
2. Lower leaf surface partially painted.
3. Lower leaf surface with pink (anthocyanins) color.
4. Lower leaf surface with pink (anthocyanins) color and upper surface of leaves partly with pink color.
5. Lower surface and upper surface of leaves with pink (anthocyanins) color.

For the plants which were selected out of these five groups, the content of rutin and anthocyanin was estimated (Fig. 1). Another group plants was planted in an isolated small area of 1 m^2. Rutin content in the vegetative organs of this experimental buckwheat plants is presented in the Table 1. The results of the experiment with buckwheat seedlings (Fig. 1) showed a correlation between anthocyanins and rutin contents. The degree of correlation in the group 5 with lower and upper leaves surface with pink (anthocyanins) color was 0.920 ± 0.163 at the 95 % confidence level (Table 2).

The analysis of the samples of the selected five groups of buckwheat plants showed correlative dependence between

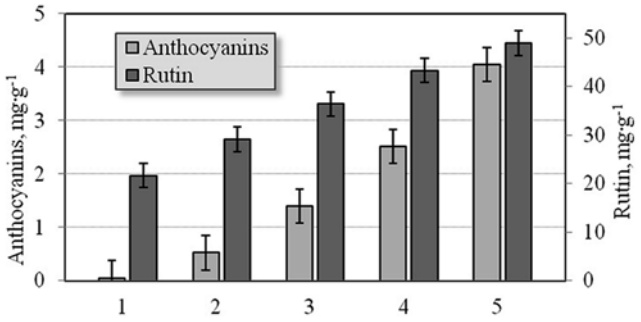

Fig. 1 Dependence of the rutin content from the content of anthocyanins in leaves of buckwheat seedlings

rutin content and visual assessment of the source material (Table 2). The quantitative analysis of anthocyanins confirmed that. In group 5 with lower and upper surface of leaves with pink (anthocyanins) color, the highest anthocyanins content was found: 1.89 mg g^{-1} FW in the stems, 2.72 mg g^{-1} FW in the leaves, and 7.39 mg g^{-1} FW in the inflorescences. The correlation coefficient for stems is 0.984, for leaves 0.913, and for inflorescences 0.938.

From buckwheat plants with lower surface and upper surface of leaves with pink color (group 5), seeds for further breeding experiments were selected. The selection was carried out during 3 years (2009−2011). The genetic line of cultivar Rubra with highest rutin content in the vegetative mass was obtained after 3 years selection process which based on the color visual assessment of vegetative organs of buckwheat (Table 3). The rutin content in the stems increased to 3.6%, in the leaves to 14.1%, and in the inflorescences to 17.8% of DW. The content of rutin in the vegetative mass of leaves and inflorescences received by the genetic line of buckwheat is closely to that of buds of Sophora japonica (Couch et al. 1952) in terms of quality and content of rutin, and can be recommended as rutin source for pharmaceutical industry.

Understanding the complex relationships between diet and health has become a key factor for developing preventive strategies to reduce the rising incidence of chronic disease globally. The natural plant pigments that are responsible for the colorful palette of edible fruits, vegetable or

Table 3 Effect of selection process on the rutin content in the vegetative organs of buckwheat plants (cultivar Rubra) (Percent Dry Weight)

Selection	Stems	Leaves	Inflorescences
M1 (2009)	1.3	8.0	13.4
M2 (2010)	2.1	11.7	16.8
M3 (2011)	3.6	14.1	17.8
$LSD_{0.05}$	0.19	0.34	0.50

crops are recognized resources for human health maintenance; this evidence is the premise behind the 'color code' for prescriptive, proactive healthful dietary guidelines. These rich pigments protect their plant hosts from biotic and abiotic stressors, and likewise provide metabolic protection to the animals which consume the pigmented food sources, through their antioxidant, anti-inflammatory, and detoxifying mechanisms of action.

Flavonoids, especially anthocyanins and rutin, have been cited as key inhibitors of various cancers (Wang and Stoner 2008; Aherne and O'Brien 1999), CVD, stroke, cataracts, and cognitive impairment, and more recently revealed as effective natural countermeasures against the ravages of metabolic syndrome (type II diabetes and obesity) (Muraki et al. 2013).

Nowadays, the search for plant material with high content of anthocyanins, quercetin and rutin is crucial to improved plant genetics and biotechnology. The presence of high content of anthocyanins, flavonols and phenolics in the plant cells can be useful as bioreactor to produce natural components of high antioxidant activity as well as natural pigments (Hamamatskui et al. 2004). The buckwheat plants can be a resource of plant material with high rutin content. The methods of selecting buckwheat species and cultivars with high rutin content are developed nowadays by different scientific schools. Klykov and Moiseenko from the Russian Federation were granted a patent on their method of selecting buckwheat plants with high rutin content in plant tops (Klykov and Moiseenko 2003). This selection method is based on the selection of plants with an intensive pink color of stems at the stage of fruitification. It has been supposed that the pink color of stems correlates with the anthocyanins content. In our research, we found dependence between the pink color of different vegetative organs and anthocyanins content.

Table 2 Rutin and anthocyanins contents in vegetative organs of cultivar Rubra

Species	Stems		Leaves		Inflorescences	
	Anthocyanins (mg g^{-1} FW)	Rutin (mg g^{-1} DW)	Anthocyanins (mg g^{-1} FW)	Rutin (mg g^{-1} DW)	Anthocyanins (mg g^{-1} FW)	Rutin (mg g^{-1} DW)
1	0.32	11.08	0.48	55.51	1.48	65.46
2	0.64	12.35	0.59	65.95	1.97	84.78
3	0.94	19.93	1.10	94.02	3.14	116.50
4	1.45	25.22	1.46	109.43	4.93	143.36
5	1.89	29.90	2.72	121.75	7.39	153.99
$LSD_{0.01}$	0.14	1.75	0.21	5.37	0.65	11.20
$r \pm s_r$	0.984 ± 0.102		0.913 ± 0.236		0.938 ± 0.201	

For the selection of buckwheat plants with high rutin content, a selection method which is based on the pink color of seedlings was chosen. The result of our experimental selection was that F. esculentum, a cultivar Rubra, had the highest rutin content—in stems 1.3 %, in the leaves 8.0 %, and at the inflorescences 13.4 %. In the third generation, the rutin content in the stems increased to 3.6 %, in the leaves to 14.1 %, and in the inflorescences to 17.8 %. So the presence of anthocyanins in vegetative organs of buckwheat can be a reliable genetic marker for screening plants with high content of rutin. For the proposed method of selection visual assessment of vegetative organs is needed just. In addition, the plant would remain intact and could be used for further breeding work.

Conclusion

The buckwheat plant could be a resource of plant material with high rutin content. The selection method which based on pink color of seedlings has been proposed for the selection of buckwheat plants with high rutin content. A correlation between the pink color of different vegetative organs and the amount of anthocyanins content could be proved. As a result of the selection from experimental buckwheat plants, the cultivar Rubra revealed to be one with the highest content of rutin among the various buckwheat species and cultivars. In the third generation of selection process, the rutin content in the stems increased to 3.6 %, in the leaves to 14.1 %, and in the inflorescences to 17.8 %.

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