J. Farm Sci., 30(2): (195-199) 2017

Studies on genotypic differences in seed phenol, anthocaynin and antioxidant activity in soybean

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(Received: April, 2017 ; Accepted: June, 2017)

Abstract: Soybean genotypes of different color seeds (5 black, 3 yellow, 1 brown and 1 green)were sown during *kharif* 2015. The seeds of all the genotypes were collected at different reproductive stages (R5 to R8) of soybean. The protein,total phenol and anthocyanin contents in whole seeds and seed coats were measured and the antioxidant activity(peroxidase and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity) were measured. The black colored soybeans were found to have higher phenol, anthocyanin contents as well as protein content on par with yellow soybean. The highest antioxidant activity was observed in the extracts of black and brown varieties. The highest total phenol content was found in Dharwad Black Local and Dharwad Soybean Mutant, with the highest total antioxidant activity. The study indicated that, varieties of black and brown seeds could be of special significance not only for their higher content of total polyphenols, ranging from 4.52 to 6.86 mg of gallic acid equivalents/g of dry material, but also for their high content of natural antioxidants such as anthocyanins. Hence, black soybean is not only a good source of phenol but also anthocyanin with biological activity in addition to a source of protein and oil contents. Therefore, development of multipurpose soybean with industrial and nutritional value is emphasized.

Key words: Anthocyanin, Antioxidant activity, Free radicals, Polyphenols

Introduction

Soybean [*Glycine max* (L.) Merrill] seeds are one of the most important sources of protein and oil in the world. It is an important crop worldwide, because of its wide range of geographical adaptation, unique chemical composition, good nutritional value, functional health benefits, and variety of end-uses (food, feed and non-edible). It contains about 40 per cent good quality protein, 20 per cent oil having about 85 per cent unsaturated fatty acids including 55 per cent poly unsaturated fatty acids (PUFA), 25-30 per cent carbohydrates and almost no starch, 4-5 per cent minerals, anti-oxidants, hence named as "Wonder Crop", "Miracle Crop", and "Golden Bean".

Commercially grown soybean varieties have yellow seed (common soybean). Some varieties are self-colored and build up considerable amount of brown-black pigments within the epidermal layer of the seed coat during seed development and become more prominent at complete maturity. Black soybeans are distinct from common soybean having high contents of tocopherol, isoflavones, flavonoids and anthocyanins which possess biological activity. They are low in net carbohydrates and high in phytonutrients, antioxidants, fibre, protein, vitamin K, iron, magnesium, copper, manganese, molybdenum and riboflavin. Anthocyanin from black soybean seed coat couldeffectively scavenge oxygenic free radical, and inhibitlipid peroxidation and DNAdamage (Azevedo *et al.,* 2003). Soybean has long been consumed as an important source of natural antioxidants in China, Brazil and European countries because of the rich anthocyanin content in its seed coat.The average intake of anthocyaninby USA citizens has been estimated at up to 180–215 mg/day which is higher than that of other flavonoids such as flavonols (Clifford, 2000). But in India the consumer acceptability of black soybean is less and demand in the market is not much high. But they are supposed to be significant in numerous ways for crop and human health and nutrition. In this perspective this study has been conducted with an objective to know the genotypic difference for phenol and anthocyanin contents and antioxidant properties of these two biochemical traits.

Material and methods

Seeds of ten genotypes of both vegetable (AGS 447, AGS 459, AGS 460 and DSb-15) and grain type (Kalitur, DSb-21, DSM, KHSb-2, DB-local and JS-335) soybean differing in seed coat color were collected at different growth stages from R5 (Beginning seed) to R8 (physiological maturity) stage sown during *kharif* 2015 at Main Agricultural Research Station, University of Agricultural Sciences, Dharwad. The biochemical parameters *viz.,* protein, phenol, anthocyanin contents were measured at all the reproductive stages starting from beginning seed. Whereas, the antioxidant actvity was measured at the physiological maturity stage. The data was analysed by Standard Fisher's method of analysis of variance (ANOVA) and results were interpreted as suggested by Gomez and Gomez (1984).

Procedures adopted for analysis of biochemical parametrs

Determination of total seed protein content (%)

Seed nitrogen in all the genotypeswas first estimated by micro-Kjeldahl method of nitrogen estimation. Later seed crude protein content was computed by multiplying the seed N by a constant 6.25 as 16 percent of the total protein is nitrogen.

$$\% \text{ N content} = \frac{\text{Titre value x Normality of acid x } 0.014 \text{ x } 100}{\text{Weight of the sample}}$$

Determination of total phenol contents (mg g^{-1})

Total phenols were determined by the Folin–Ciocalteu method. Required amount of seeds collected at R5, R6, R7 and

R8 stages were extracted with 10 ml hot distilled ethanol (80%).The amount of total polyphenols in whole seed and seed coat alone was calculated from the calibration curve of phenol standard solutions (covering the concentration range between 0.2 and 1.0 mg/ml) and content expressed as milligrams of total phenol per gram of dry seed material(DM).

Determination of anthocyanins

For the determination of anthocyanins80% aqueous methanol was used. The extracts were centrifuged at 17,000 rpm for 20 min and kept in refrigerator before assay.

Total anthocyanins content of seeds and seed coat were determined according to the pH differential spectroscopic method [Cheng and Breen 1991]. Anthocyanins were extracted by grinding the sample with 80% (vol/vol) methanol solution at a 1:10 ratio (wt/wt) in a pestle and mortar. Samples of the extracts were centrifuged at 17,000 rpm for 20 min. The supernatant (1ml aliquots) were diluted either with 0.2 M KCl/0.2 M HCl (25:67, vol/vol) buffer to10 ml and adjusted to pH 1.0 or with 1.0M$NaCH_3COO$/1.0MHCl/water (10:6:9 by volume) to 10ml and adjusted to pH4.5.After 30 minutes of incubation at room temperature, absorption (A) was measured at a wavelength of 510nm and 700 nm. Absorbance readings were converted to total amount of anthocyanins as cyanidin-3-glucoside equivalent using a molar extinction coefficient of 2.96 $x10^4$, a molecular weight of 484, and absorbance at pH 1.0 and 4.5 and expressed as milligrams ofcyanidin-3-glucoside per gram of DM.

Peroxidase activity (µ moles of gauaicol min^{-1})

Peroxidase activity was estimated following the method of Mahadevan and Sridhar (1986). One gram of seed tissue (entire seed with seed coat) and seed coat separately of R8 stage was extracted with 3ml of 0.1M phosphate buffer (pH 7.0) by grinding with a pre-cooled mortar and pestle. The homogenate was centrifuged at 17,000 rpm at 5°C for 15 minutes and the supernatant was used as enzyme source. The reaction mixture contained 3ml buffer solution, 0.05ml guiacol solution 0.1ml enzyme extract and 0.03 ml hydrogen peroxide solution in a cuvette (bring the buffer solution to 25°C before assay). Absorbance was measured in the spectrophotometer at 436nm by waiting until the absorbance increased by 0.05 start a stop-watch and note the time required in minutes to increase the absorbance by 0.1unit.

DPPH radical scavenging activity (%)

The percentage of antioxidant activity (AA %) of seed and seed coat extract was assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay. The measurement of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was performed according to methodology described by Brand-Williams *et al.* (1995). The samples were reacted with the stable DPPH radical in an ethanol solution. The reaction mixture consisted 0.5 ml of sample, 3 ml of absolute ethanol and 0.3 ml of DPPH radical solution 0.5 mM in ethanol. The changes in color (from deep violet to light yellow) were read [Absorbance (Abs)] at 517 nm after 100 min of reaction using a UV-VIS spectrophotometer. The mixture of ethanol (3.3 ml) and sample (0.5 ml) served as blank. The control solution was prepared by mixing ethanol (3.5 ml) and DPPH radical solution (0.3 ml). The scavenging activity percentage (AA %) was determined according to Mensor *et al.* (2001).

$$AA\% = 100 - \frac{(A\ sample - A\ bllank)}{A\ control}$$

Results and discussion

Seed protein content

Seed protein content at R5, R6, R7 and R8 stages of soybean, differed significantly among the genotypes. Higher protein content was reported at R5 stage in all the genotypes but showed decrease at R6 and R7 stages and then again increased at R8 stage (Table 1). In similar report given by Hill and Briedenbach (1974) mentioned that protein accumulated rapidly between 12 and 28 DAF, but declinedat the onset of seed desiccation. Levels of protein decreased 2–6 per cent during the first3–5 weeks after flowering and gradually increased thereafter until maturity, which was attributed to the rapid synthesis of oil andstarch in early seed development (Yazdi-Samadi *et al.,* 1977). Lowry *et al.* (1951) observed that less than 5 per cent of the total protein of the mature seed had been accumulated by 12 DAF.

The protein content of vegetable soybean (AGS 460, AGS 459 and AGS 447) also followed a similar trend, but accumulated 2-5 per cent less protein when compared to grain soybean. Among the grain soybean genotypes, black soybeans (Kalitur, DSM, and DB-Local) found to accumulate more of protein at all the stages compared to yellow soybeans (DSb-21, KHSb-2, JS-335) in all the stages (Table 1). Xu *et al.,* 2007; Xu and Chang, 2008 reported a similar result that black soybeans are similar to regular yellow soybeans. But they are high in protein, phyto-nutrients, antioxidants and minerals.

Phenol and anthocyanin contents in the seed and seed coat

Plants consumed by humans may contain thousands of different phenolic compounds. The effects of dietary phenolics are of great current interest due to their antioxidative activities. According to their color and their total polyphenol content, soybean varieties could be divided into three groups: (1) black and brown seeds, with the highest contents of total polyphenols and anthocyanins (2) green seeds, with moderate amounts of total polyphenols, flavonoids, and anthocyanins and (3) yellow and reddish seeds, lower content of total polyphenols and seed anthocyanins (Takahashi *et al.,* 2005). In the present study there was a gradual increase in the amount of total phenols from R5 to R8 stage (Table 2). However, the amount of total phenols was found higher in the black and brown soybean genotypes (Kalitur, DSM, AGS 460, AGS 459, AGS 447 andDB-Local) when compared to yellow (DSb-21, KHSb-2 and JS-335) and green soybean (DSb-15). The black soybean genotype, DB-Local recorded high amount of total phenols (0.92, 1.15, 4.74 and 6.86 mg g^{-1}dry seed) at R5, R6, R7

Table 1. Genotypic differences in the total protein and phenol content at R5, R6, R7 and R8 stage of soybean.

Genotypes	Protein content (%)				Total phenol content (mg g^{-1} dry seed or seed coat)				Total anthocyanin (mg g^{-1} dry seed or seed coat)		
	Whole seed				Whole seed			Seed coat	Whole seed	Seed coat	
	R5	R6	R7	R8	R5	R6	R7	R8	R7	R8	
Black seeded genotypes											
AGS 459(V)	38.72^{de}	37.62^{b}	36.13^{bc}	39.29^{bc}	0.60^{c}	1.00^{bc}	3.30^{c}	5.14^{c}	27.40^{b}	0.41^{bc}	1.11^{bc}	15.93^{a}
AGS 460(V)	41.81^{c}	40.14^{a}	38.81^{ab}	40.77^{ab}	0.62^{c}	0.92^{c}	3.61^{bc}	5.62^{c}	31.77^{a}	0.55^{ab}	1.35^{b}	16.77^{a}
DB-Local (G)	46.34^{a}	41.12^{a}	38.09^{ab}	42.39^{a}	0.92^{a}	1.15^{a}	4.74^{a}	6.86^{a}	35.20^{a}	0.62^{a}	1.90^{a}	17.80^{a}
DSM (G)	44.54^{b}	40.44^{a}	40.25^{a}	41.63^{a}	0.75^{b}	1.08^{ab}	3.93^{b}	6.36^{ab}	33.57^{a}	0.28^{e}	1.67^{a}	18.03^{a}
Kalitur (G)	42.63^{c}	40.65^{a}	39.74^{a}	41.96^{a}	0.67^{bc}	1.05^{b}	3.25^{c}	6.18^{b}	32.07^{a}	0.36^{c}	1.87^{a}	16.07^{a}
Mean	42.81	39.99	38.61	41.21	0.71	1.04	3.77	6.03	32.00	0.44	1.58	16.92
Brown seeded genotypes												
AGS 447 (V)	38.67^{de}	33.89^{d}	34.48^{c}	35.93^{d}	0.58^{c}	0.83^{d}	2.61^{d}	4.52^{d}	20.27^{c}	0.34^{e}	1.07^{bc}	10.47^{b}
Green seeded genotypes												
DSb-15 (V)	38.26^{de}	34.81^{cd}	31.57^{d}	38.83^{c}	0.63^{c}	0.82^{d}	1.42^{e}	1.88^{e}	2.13^{d}	0.04^{d}	0.68^{de}	1.60^{c}
Yellow seeded genotypes												
DSb-21 (G)	39.08^{d}	37.38^{b}	38.00^{ab}	38.00^{c}	0.38^{d}	0.72^{e}	1.28^{e}	1.97^{e}	3.60^{d}	0.05^{d}	0.41^{e}	3.03^{c}
JS-335 (G)	37.03^{e}	36.36^{bc}	29.50^{d}	35.67^{d}	0.57^{c}	0.62^{f}	1.43^{e}	1.97^{e}	2.77^{d}	0.04^{d}	0.79^{cd}	1.80^{e}
KHSb-2 (G)	37.73^{de}	33.01^{d}	31.83^{d}	37.78^{c}	0.33^{d}	0.67^{ef}	1.28^{e}	2.02^{e}	3.57^{d}	0.06^{d}	0.59^{de}	2.33^{c}
Mean	37.94	35.58	33.11	37.15	0.43	0.67	1.33	1.98	3.31	0.05	0.60	2.39
Grand Mean	40.48	37.54	35.84	39.23	0.61	0.89	2.69	4.25	19.23	0.28	1.14	10.38
S.Em.±	0.530	0.773	0.853	0.572	0.03	0.03	0.15	0.17	1.07	0.05	0.11	1.14
C.D. 5%	1.576	2.297	2.536	1.699	0.10	0.09	0.45	0.51	3.17	0.15	0.32	3.40

Values in the column followed by the same letters do not differ significantly by DMRT

G: Grain soybean V: Vegetable soybean

and R8 stages of crop growth respectively. A similar result was reported in a study conducted by Malencic *et al.* (2012) where the amounts of total polyphenols ranged from 2.68 (in the yellow variety) to 6.22 (in the black 2 variety) mg of GAE/g of DM. Comparison between contents of polyphenols in yellow and colored seeds showed that varieties with black and brown seeds possessed higher levels of polyphenols with 2.5-fold higher content compared with yellow samples.

The data pertaining to the seed coat phenol content (mg g^{-1}) at R8 stage also showed significant difference among the genotypes. Among the genotypes seed coat from DB-Local had the highest amount of total phenol (35.2 mg g^{-1}) which was on par with other black soybean genotypes (Kalitur, DSM and AGS 460). Seed coat from yellow and green soybean had very small amount of total phenols. Takahashi *et al.* (2005) and Astadi *et al.* (2009) reported that the seed coat of black soybean had higher polyphenol content than that of yellow soybean (29.0 ± 0.56 and 0.45 ± 0.02 mg/g, respectively). Xu and Chang (2008b) suggested that almost three-quarters, 73.4 per cent, of the phenolic antioxidant compounds are found in the seed coat part of the soybean.

On whole seed basis, black soybean genotypes (Kalitur, DSM, AGS 460, AGS 459 and DB-Local) recorded a higher amount of anthocyanin at R8 stage. Among the genotypes, highest anthocyanin content was observed in DB-Local (1.9 mg g^{-1}) followed by Kalitur (1.87 mg g^{-1}) and DSM (1.67 mg g^{-1}). Whereas, normalyellow soybean genotypes (DSb-21, KHSb-2 and JS-335) had lower anthocyanin content in the range of 0.41-0.79 mg g^{-1}.With regards to the seed coat anthocyanin, the genotype DSM had 18.03 mg g^{-1} of seed coat anthocyanin which was significantly highest among the genotypes. The genotypes Kalitur (16.07 mg g^{-1}), AGS 460 (16.77 mg g^{-1}), AGS 459 (15.93 mg g^{-1}) and DB-Local (17.8 mg g^{-1}) were found on par with DSM. The least anthocaynin content was recorded in DSb-15 (1.6 mg g^{-1}) and same range of low seed coat anthocyanin content was observed in yellow soybean genotypes.

Soybean seed and seed coat extracts exhibited antioxidant activity because of phenols and anthocyanin contents. The antioxidant activity measured enzymatically (Peroxidase enzyme activity) and non-enzymatically (DPPH radical scavenging activity) showed significant difference among the genotypes. Antioxidants are first line of defense against free radical damage. They have the potential to avert the free radicals that induce tissue damage by preventing the formation of radicals, by stabilizing reactive oxidative species scavenging, or by

Table 2. Genotypic differences in the peroxidase and radical scavenging activity in different soybean genotypes

Genotypes	Peroxidase activity (μ moles of gauaicol min^{-1})		DPPH radical scavenging activity(%)	
	Whole seed	Seed coat	in seed	in seed coat
Black seeded genotypes				
AGS 459	3.30	24.10	63.31	67.05
AGS 460	3.53	26.85	42.77	56.21
DB-Local	3.88	27.15	51.53	62.52
DSM	3.57	29.25	63.07	68.35
Kalitur	4.01	28.06	55.35	66.63
Mean	3.66	27.08	55.21	64.15
Brown seeded genotypes				
AGS 447	3.02	21.13	31.17	39.64
Green seeded genotypes				
DSb-15	2.22	15.51	33.23	34.65
Yellow seeded genotypes				
DSb-21	1.71	11.95	24.63	26.07
JS-335	2.08	14.58	31.65	35.62
KHSb-2	2.48	19.34	32.13	34.99
Mean	2.09	15.29	29.47	32.23
Total Mean	2.98	21.79	42.88	49.17
S.Em.±	0.22	1.67	4.35	1.41
C.D. @ 5%	0.67	4.96	12.92	4.18

promoting their decomposition (Moller, 2001 and Ahmad *et al.,* 2008)**.** Normally antioxidant properties of soybean are associated with seed coat phenol and anthocyanin contents (Malencic *et al.,* 2007 and Kumar *et al.*, 2010).

Either on whole seed or seed coat basis, the black seeded soybean genotypes (Kalitur, DSM, AGS 460, AGS 459 and DB-Local) had higher amount of peroxidase enzyme activity than yellow soybean types. Among the black soybean types, highest enzyme activity was recorded in DSM followed by Kalitur. Henriksen *et al.* (2001) reported that soybean seed coat peroxidase (SBP) is a peroxidase with extraordinary stability and catalytic properties. It belongs to the family of class III plant peroxidases that can oxidize a wide variety of organic and inorganic substrates using hydrogen peroxide.

The data pertaining to the percentage antioxidant activity by DPPH radical scavenging method differed significantly in both whole seed and seed coat. All the black seeded genotypes recorded higher percentage of antioxidant activity which is attributed to higher phenol and anthocyanin contents. Significantly higher antioxidant activity was recorded in AGS 459 which was on par with the genotype DSM. DPPH radical scavenging activity of the seed coat was significantly higher in AGS 459 which was on par with Kalitur and DSM. This result is in agreement with the study of Takahata *et al.* (2001), who reported that the antioxidant activity of 70 per cent aqueous acetone extract from the seed coat of the brown soybean variety, Akita-Zairai was much higher than that of three other reddish-brown varieties, but lower than that of two black varieties, and was closely dependent on the content of phenolic compounds.

Conclusion

In the present study, significant genotypic variation were observed for total protein content, DPPH radical scavenging activity which was associated with the phenol and anthocyanin contents. Black soybean types, besides having higher protein and oil contents also have higher phenol and anthocyanin contents. These cultivars need to be brought in the main stream of domestic consumption to meet the nutritional and health security of growing population. Among the black soybean types, the mutant DSM (mutant of KHSb-2) is superior in seed yield with higher content of seed coat phenol and anthocyanin contents. Further attempts have to be made to develop agronomic strategies for cultivation of this mutant and popularize its potential use in human and animal nutrition.

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