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Research Note

Diversity of proanthocyanidin content in soybean landraces

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Abstract:
Proanthocyanidins (PAs) are natural plant antioxidants, whose radical scavenging activities (RSA) degrade harmful free radicals. In the current study, PA contents were analyzed in 52 soybean [*Glycine max* (L) Merril] landraces having different seed coat colors. PA was extracted by HCl-Butanol method; RSA was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and quantified using spectrophotometer. Soybean cultivar KaiFengKuoZhuangqingDou had the highest PA content, 946 ABS.ml g^{-1}, followed by Peking, 860 ABS.ml g^{-1}. Both the cultivars had black seed coats. Chasengoku 81Go had the highest PA content (485 ABS.ml g^{-1}) among the landraces with brown seed coat color. In landraces with green seed coats, PA content ranged from 1 to 6 ABS.ml g^{-1}. In landraces with yellow seed coats PA content ranged from 0 to 10 ABS.ml g^{-1}. The RSA was directly proportional to PA content with a high correlation coefficient (r^2 = 0.9724), so PA content contributed highly to RSA. Reasons for these wide variations in PA content and RSA within the same colored landraces are discussed.

Key words: *Glycine max*, antioxidants, genetic variation, proanthocyanidin, radical scavenging activities.

In soybean [*Glycine max* (L) Merril], proanthocyanidins (PAs) are secondary plant metabolites, which play crucial role in interacting with diverse environmental conditions they are exposed to (Harborne and Williams, 2000), in protecting the plants from various types of abiotic and biotic stresses (Dixon *et al.*, 2005) and during early developmental stages (Taylor and Grotewold, 2005).

As the demand of soybean for food, feed and commercial product is increasing continuously, its benefit as a natural source of antioxidants is looked into with a great interest. Experimental results have shown that PA possesses health-conferring benefits (Ross and Kasum, 2002; Scalbert *et al.*, 2005; Schijlen *et al.*, 2004), which have increased the focus and research on PA. Soybean rich in PA provide healthy food for humans and feed for livestock. PAs present in the fodder leaves bind to the leaf protein in the rumen of herbivores and retard rapid protein digestion by rumen organisms, thus PA prevents pasture blots in animals and increases absorption of amino acids (Tanner *et al.*, 1994). The increasing awareness of PA as a health promoter adds to the increasing demand for soybean. Thus PA rich soybean varieties can be important source of antioxidants to people and livestocks.

Free radicals are highly reactive even at low temperatures and moisture levels. The most common source of free radicals is molecular oxygen which may be converted to reactive oxygen species (ROS). ROS are the consequences of several processes including metabolism, for example, due to incomplete oxidation of water during photosynthesis or reduction of oxygen during respiration (Bailly, 2004). Diverse molecules that serve as a defense system against oxidative damage by conferring and neutralizing ROS, other free radicals, or catalysts to oxidation reactions are called antioxidants. Antioxidants inhibit free radical mediated reactions by a variety of mechanisms. Radical scavenging activity (RSA) is a process by which antioxidants scavenge ROS and lower the toxicity (Helliwell and Gutteridge, 1999). RSA found in *Cucumis melo***,** *Momordica charantia*, date extracts, sweet potato, mushrooms, etc. are reported to have anti-carcinogenic benefits (Gill *et al.*, 2011; Asiamah *et al.*, 2011; Hasan *et al.*, 2010; Islam *et al.*, 2009; Ramkumar *et al.*, 2010). Therefore, it is necessary to determine the level of RSA in soybean, which has been a common crop for thousands of years (Guo *et al.*, 2010).

Soybean landraces have various colored seed coats. Differently colored seeds possess different type of antioxidants and other beneficial components. For example: yellow seed coat contains isoflavones, tocopherols, rutein, and saponins (Monma *et al.*, 1994); red brown seed coats contain proanthocyanidins (Takahata *et al.*, 2001; Todd and Vodkin, 1993); and black seed coats are rich in anthocyanins (Yoshida *et al.*, 1996). However, it is

not known if the same colored genotypes have same amount and type of antioxidants. Therefore, it is crucial to investigate the status of PA content in different soybean landraces. The objective of this study was to determine presence and quantify PA content and RSA in various soybean landraces. To investigate the presence and amount of PA content and RSA, 52 soybean landraces were assayed. This is the first report on the diversity of PA content and RSA in so many soybean landraces.

Soybean landraces native to Japan, China and America (Table 1) were grown, in row spaced 80 cm apart with 24 cm spacing between the plants in the field condition of Kyoto, Japan. Twenty seven landraces were the natives of China, 23 of Japan and 2 were from USA. The matured pods were harvested and seeds were air dried to 9 % moisture content. The dried seeds were stored at 10 °C, with seed moisture content maintained at 9 ± 0.5 % until used.

Seed coats from 4 seeds of each cultivar were removed manually, weighed and used for the extraction of PAs. Total PA was extracted using 70 % aqueous acetone as an extraction solvent according to the method described by Ariga *et al.*, (1981) and Takahata *et al.*, (2001). The seed coats of each cultivar were crushed into fine powder, and treated with 5 mL of 70 % aqueous acetone at 25 °C overnight in dark. The supernatant was saved; the acetone was removed by evaporation and adjusted to a final volume of 5 mL using 80% EtOH. This 80 % EtOH-based crude solution was used for all the downstream experiments.

Measurement of PA: The PA content was measured using the HCl−butanol method (Takahata *et al.*, 2001). The crude solution was treated with a reaction mixture, which consisted of 200 µL crude solution and 800 µL of HCl−butanol (concentrated HCl/*n*-butanol, 1:5). The first absorbance reading (R_1) was taken at 548 nm using a spectrophotometer. The reaction mixture was heated at 95 °C for 30 min, then the second absorbance reading (R_2) was taken at 548 nm. The increase in absorbance at 548 nm (R_2-R_1) was used for the measurement of PA content. In case of the black soybean, cyanidin-3-glucoside was reported to be the major anthocyanin (Takahata *et al.*, 2001), absorbance at 538 nm, which is the maximum in the spectrum of cyanidin-3-glucoside, was also measured for evaluation of anthocyanin content.

Measurement of RSA: The RSA was measured according to the 2, 2-diphenyl- 1- picrylhydrazyl (DPPH) method as described by Suda *et al.*, (1999). DPPH was dissolved in 80 % EtOH to give a 200 µM of a stable, free radical. The reaction mixture consisted of 0.5 mL of 200 µM of a DPPH solution and 0.5 mL of the test sample diluted in 80 % EtOH (or 80 % EtOH for the control). A series of diluted sample solutions were prepared before starting the reaction; for example, the crude solution was diluted as 10: 490 (ratio of crude extract to 80 % EtOH), 20: 480, 30: 470, 40: 460, 50: 450, and 100: 400. The DPPH solution was added to the diluted test sample to start the reaction. The solution was mixed well by shaking, kept at 25 °C for 20 min and the absorbance of the reaction mixture was measured at 520 nm. The volume of crude extract required to decrease absorbance by 50 %, (IC50) at 520 nm, of the control (100 %, the crude solution without DPPH) was calculated as (% DPPH-RSA= (1-($A_{sample}/A_{controlt=0}$)) x100. When the range of the dilution was unsuitable for determining a 50 % decrease, the ratio of dilution was optimized, and the reaction was repeated until a proper value was obtained. The DPPH radical-scavenging activity was expressed as the seed coat weight, calculated from the added volume of the crude solution.

All the experiments were carried out in triplicate; the mean and standard deviation were calculated using Microsoft Excel. The significance of the observed correlation coefficient was tested using Student's t test in Microsoft Excel. All parameters were considered at 95 % level of significance ($p < 0.05$).

The landraces used in this study had black, brown, brown-yellow, green and yellow seed coat colors (Fig. 1). There were 7, 6, 4, 4 and 31 landraces of each category, respectively. Landraces with yellow seed coats had either yellow or black hilum (Fig. 1E, F). The maximum and minimum PA content in black, brown, brown- yellow, green and yellow colored seed coats were found in the landraces KaiFengKuoZhuangqingDou and Kurosengoku; Chasengoku 81Go and BaiHuaMoShiDou; QiYuanShanZhuangShuiDou and Iyo Daizu (B); Iyo Daizu (A) and Matsufuku; and WeiYuanQiYueHuang and Tamahomare, respectively. The maximum and minimum RSA were in the landraces Peking and Kurosengoku; Chasengoku 81Go and TongPiDou; QiYuanShanZhuangShuiDou and HongHuLiuYueBao, Matsufuku and Iyo Daizu(A); and Geden Shirazu, respectively (Table 1).

The coefficients of correlation (r^2) between PA content and RSA for black, brown and brown-yellow landraces were positive and high i.e. 0.87, 0.96 and 0.93 respectively. For green it was -0.09 and for yellow it was 0.18 (Table 2). For long time, soybean was considered a poor source of PA. According to Gu *et al.* (2003) soybean contained no detectable amount of PA. It could be because they must have used commercial yellow landraces

for the test. In this study, we found that some soybean landraces had very high amount of PA (Table 1). Although soybean species are believed to have a narrow genetic diversity (Zhou *et al.*, 2002), a wide variation in PA content and RSA was found within the landraces and a distinct variation was observed even within the same colored groups (Table 2). The variation in PA content and RSA among the extant landraces is sufficiently wide for genetic studies and breeding purposes.

Based on the amount of PA content, the landraces were divided into five clusters: cluster 1 to cluster 5. Cluster 1 had landraces with no PA content or those with less than 1 ABS.ml g^{-1}. Cluster 2 had landraces with PA content between 1 to 10 ABS.ml g^{-1}. Cluster 3 had landraces with PA content between 10 and 100 ABS.ml g^{-1}. Cluster 4 had landraces with PA content between 100 and 500 ABS.ml g^{-1}, and the landraces in Cluster 5 had PA content more than 500 ABS.ml g^{-1} (Table 1). To use the vast collection of germplasm, it is necessary to understand the genetic basis underlying the desired trait. The landraces classified into clusters according to PA content will help the selection of landraces for breeding purposes. The first cluster contains landraces with no or negligible amount of PA content while the fifth cluster contains the landraces with maximum amount of PA content. The landraces from these two clusters i.e. 1 and 5 are ideal for crossing to create recombinant inbred lines for the genetic study of the PA content and RSA.

Inter cultivar variation of PA content and RSA:The maximum PA content and RSA were found in landraces with black seed coats followed by landraces with brown, brown-yellow, and yellow seed coats. In landraces with black seed coats, PA content ranged from 260 ABS.ml g^{-1} in Kurosengoku to 946 ABS.ml g^{-1} in KaiFengKuoZhuangShuDou (Table 2). A brown cultivar Chasengoku 81Go had the highest PA content and the lowest PA content among the brown landraces was found in BaiHuaMoshiDou. Among the colored landraces, brown (both types) seed coats had the highest variation (variance, σ^2=28658) in the PA content. The σ^2 was 85240 and 6.2 in landraces with black and yellow seed coats, respectively. This shows that there was a wide variation of PA within the landraces having brown seed coats. In landraces with green colored seed coats, PA content and RSA were low but detectable. There was a low variance (σ^2= 4.78) within the green colored landraces and the correlation between PA content and RSA was negative. In yellow landraces the PA content ranged from 0 ABS.ml g^{-1} in Tamahomare to 10 ABS.ml g^{-1} in WeiYuanQiYueHuang (Table 2).

Methods to assay PA content by HCl Butanol and RSA by DPPH are efficient and reliable (Suda *et al.*, 1999; Furuta *et al.*, 2003) and we also used the same methods. Our finding is consistent with the previous reports on PA content and RSA in other plants by different researchers (Sahloul *et al.*, 2009; Sofidia *et al.*, 2008; Muchuweti *et al.*, 2007).

Spectrophotometric readings of crude solution in HCL Butanol before heating were much lower for brown landraces than black landraces but after heating the readings for black and brown landraces were almost similar (Fig. 3 and Fig. 4). The brown seed coats showed an increase in the reading after heating. In the reading at 548 nm, the reading before heating is due to free monomeric PA present in the crude solution. During boiling, the complex PAs are broken down into the monomeric forms thus producing more monomers of PA. Thus, the increase in the reading after heating was due to the addition of monomeric PA, as a result of breakdown of complex polymeric PA present in the seed coats, to the free monomeric PA. This indicates that the brown seed coats contain more complex PAs than the black seed coats.

The high correlation between PA content and RSA (r^2= 0.9724) signify that RSA is the function of PA content (Table 3). The RSA was high in all the landraces with colored seed coats except the green landraces in which it was negative. The green color could be due to factors other than polyphenols. Borrmann *et al.* (2009) reported that the green color in the seed coats is due to chlorophyll. Taken these together, it is sensible to have no RSA in green seed coat landraces.

The correlation between whole seed weight and the weight of seed coat was 50 %, which means that only 50 % of the seed coat weight showed the influence of seed weight. Seed coat weight had very low correlation with both PA content and RSA. This low correlation means that the variations in PA contents among different seed sized landraces was not due to the variations in their seed coat weights.

The correlation between PA content and RSA was 97 % (Table 3), therefore, PA possesses radical scavenging ability, hence directly contributes to RSA. Varieties with high PA have high RSA. The soybean landraces with colored seed coats, especially the black and brown seed coats which contain high amount of PAs and hence have high RSA, are healthier to consume than the commercial landraces with yellow seed coats. Most commercial landraces have yellow seed coats owing to the consumers' preference for clean looking soybean products. Since these commercial landraces have fewer amounts of PAs and low RSA, it is necessary

to transform the genes for high PA content from the landraces with high PA and RSA to create high yielding landraces with high PA content which in turn will confer high RSA.

It is known that in the PA synthesis pathway, the color conferring loci, namely *i*, *R* and *T* lie in the upstream (Zabala and Vodkin, 2003). In general, positive correlation of PA content to colored landraces supports this. It was reported that immature black and brown seed coat contained significant amount of procyanidin, a 3'4' hydroxylated proanthocyanidin (Todd and Vodkin, 1993) and showed a high degree of polymerization (Takahata *et al.*, 2001). The synthesis of procyanidin is controlled by *T* gene. Thus in brown and black landraces, PA content is higher than the landraces with *t* genes. The difference in the PA content and RSA in brown (*i, r, T*) and black (*i, R,T*) landraces could be due to *R* gene. But these conditions do not explain the wide variation within the same colored groups as they will have the same genes for seed coat color. The analysis of PA content in soybean seed coats from different landraces showed a wide range of variation among the population and within the same colored landraces. Therefore, these wide variations in PA content within the same colored landraces show that the genes at *i, R* and *T* only cannot create such a wide variation. This could be due to some genes other than *i, R* and *T*, which have either additive or epistatic effect on PA content, or there are several isoforms of these genes.

PA contents were studied in red grape (Makris *et al.*, 2008); *Leonotis leonurus* (Oyedemi and Afolayan, 2011); *Malva parviflora* (Afolayan *et al.*, 2008); *Lecaniodiscus cupanioides* (Sofidiya *et al.*, 2008); and in many other plant species. In soybean, PA content was reported in only a few colored cultivars (Todd and Vodkin, 1993). Similarly, RSA were studied in black (Furuta *et al.*, 2003) and brown (Takahata *et al.*, 2001) lines only. This is the first comprehensive report on the variation of PA content and RSA in a large collection of soybean landraces. The high PA and RSA content lines need to be evaluated for anti nutritional factors *viz.*, the Kunitz trypsin inhibitor (KTI), Bowman-Birk inhibitor, lectins, phytic acid and oligosaccharides, raffinose and stachyose and used as donors in the breeding program for development of nutritionally rich soybeans varieties.

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Table 1. Performance of landraces used in the study

Name of the Landraces	Seed coat color	Origin	Mean Seed coat wt	Mean wt. of 100 seeds (± SD)	Mean PA content (±SD)	Mean RSA (±SD)	Cluster no.
Akatsuka	Brown	Japan	0.060	20.16± 3.41	108.33± 47.14	0.74 ± 0.32	4
Amakusa	Yellow	Japan	0.051	16.315± 0.26	1.23± 1.73	0.00 ± 0.00	2
Aobatake	Green	Japan	0.074	28.60± 0.28	3.39± 0.00	0.16 ± 0.01	2
Aogozen	Green	Japan	0.073	26.01± 3.35	1.7± 0.00	0.15 ± 0.02	2
Arakushi Shirazu	Yellow	Japan	0.053	18.25± 0.47	1.17± 1.65	0.19 ± 0.05	2
Asahi 60 go	Yellow	Japan	0.084	26.09± 4.48	5.19± 1.04	0.06 ± 0.07	2
BaDaJia	Yellow	China	0.065	22.25± 1.08	5.8± 2.73	0.31 ± 0.04	2
BaiHuaMoShiDou	Brown	China	0.090	7.35± 1.41	4.15± 0.00	0.25 ± 0.25	2
BaoAnDaDou	Yellow	China	0.062	18.56± 2.77	7.11± 4.31	0.03 ± 0.01	2
BaoShan	Yellow	China	0.067	24.25± 1.65	7.45± 0.00	0.18 ± 0.02	2
Chamame Shoryu	Brown	Japan	0.050	14.02± 1.79	8.73± 1.76	0.89 ± 0.38	2
Chasengoku 81 go	Brown	Japan	0.051	14.20± 1.42	485.56± 5.18	15.66 ± 3.30	4
DaYeHuang	Yellow	China	0.056	21.50± 2.11	3.37± 4.76	0.14 ± 0.01	2
DuLuDou	Black	China	0.049	12.241± 1.86	260.53± 109.75	7.17 ± 3.00	4
Enrei	Yellow	Japan	0.082	31.65± 22.37	4.32± 2.03	0.22 ± 0.31	2
Fukutaka	Yellow	Japan	0.077	34.47± 24.37	3.26± 0.00	0.36 ± 0.12	2
Geden Shirazu	Yellow	Japan	0.071	24.54± 4.58	0.87± 1.23	0.44 ± 0.63	1
Goyoumame	Black	Japan	0.078	27.52± 0.51	451.34± 178.81	9.99 ± 1.66	4
GuangShanWenShuTianEDan	Yellow	China	0.065	23.01± 2.22	5.78± 2.72	0.08 ± 0.11	2
HeiYaoHuangDou	Yellow	China	0.122	30.75± 22.89	8.19± 0.00	0.35 ± 0.18	2
HeiYaoHuangDou	Yellow	China	0.057	7.60± 10.75	5.47± 4.64	0.37 ± 0.45	2
HongHuLiuYueBao	Brown-Y.	China	0.042	14.78± 1.19	8.97± 4.22	0.14 ± 0.14	2
Iyo Daizu	Green	Japan	0.042	11.89± 1.65	5.89± 0.00	0.11 ± 0.16	2
Iyo Daizu	Brown-Y.	Japan	0.063	19.07± 0.60	8.00± 0.00	0.15 ± 0.03	2
Jack	Yellow	USA	0.053	16.19± 11.44	5.86± 1.65	0.19 ± 0.27	2
Kaburekara	Yellow	Japan	0.069	22.65± 3.48	9.01± 2.55	0.38 ± 0.31	2
KaiFengKuoZhuangqingDou	Black	China	0.052	15.47± 1.20	945.94± 457.29	18.81 ± 2.59	5
Kairyo Shirome	Yellow	Japan	0.056	14.92± 3.45	3.36± 4.75	0.06 ± 0.09	2
Kara Shirazu	Brown	Japan	0.042	17.29± 4.56	361.21± 222.56	10.97 ± 4.22	4
Kurosengoku	Black	China	0.045	12.24± 1.86	260.53± 109.75	7.17 ± 3.00	4
Matsufuku	Green	Japan	0.067	22.34± 0.20	0.92± 1.31	0.24 ± 0.29	1
Misuzu Daizu	Yellow	Japan	0.087	31.65± 22.37	4.32± 2.03	0.22 ± 0.31	2
NanTongXiaoYuanDou	Yellow	China	0.063	22.98± 2.63	2.98± 4.21	0.27 ± 0.21	2
PaiSaPoa A	Yellow	China	0.049	17.47± 2.58	6.37± 5.41	0.12 ± 0.15	2
Peking	Black	China	0.057	13.93± 1.41	860.33± 46.43	23.19 ± 1.52	5
QiYuanShangZhuangShuiDou	Brown-Y.	China	0.059	18.72± 0.33	128.39± 73.53	2.28 ± 0.79	4
ShangRaoAiZiWu	Yellow	China	0.075	29.28± 0.36	5.03± 2.37	0.10 ± 0.01	2
ShangTiePai	Brown-Y.	China	0.054	17.5± 0.82	63.3± 17.9	1.88 ± 0.52	3
Shirome Teppo	Yellow	Japan	0.077	24.69± 5.05	7.33± 1.15	0.16 ± 0.04	2
Shiroyama Dadachya	Yellow	Japan	0.063	22.98± 2.63	2.98± 4.21	0.27 ± 0.21	2

Table 1. Contd..

Name of the Landraces	Seed coat color	Origin	Mean Seed coat wt	Mean wt. of 100 seeds (± SD)	Mean PA content (±SD)	Mean RSA (±SD)	Cluster no.
Tamahomare	Yellow	Japan	0.072	36.31± 0.70	0.00± 0.00	0.00 ± 0.00	1
Tamanishiki	Yellow	Japan	0.088	33.58± 4.24	0.71± 1.00	0.00 ± 0.01	1
Thorne	Yellow	USA	0.063	22.80± 16.12	4.01± 0.00	0.21 ± 0.05	2
TieJiaQing	Yellow	China	0.082	31.48± 4.27	3.82± 1.08	0.10 ± 0.14	2
TongPiDou	Brown	China	0.055	14.47± 2.05	10.32± 8.1	0.23 ± 0.26	2
WeiYuanQiYueHuang	Yellow	China	0.035	10.20± 0.18	10.68± 10.07	0.32 ± 0.34	2
WuChangLiuYueBao	Yellow	China	0.056	17.77± 1.66	4.45± 3.15	0.30 ± 0.34	2
YiChangHeihuangDou	Black	China	0.051	13.39± 3.66	828.43± 225.3	20.10 ± 1.34	5
YlXianHeiDou	Black	China	0.078	27.52± 0.51	451.34± 178.81	9.99 ± 1.66	4
YuHuiZhen	Yellow	China	0.056	17.77± 1.66	4.45± 3.15	0.30 ± 0.34	2
YuShanBaYueBao	Yellow	China	0.088	33.11± 4.51	4.26± 4.02	0.02 ± 0.04	2
ZaoHuangDou	Yellow	China	0.051	16.97± 0.72	4.87± 3.44	0.015 ± 0.02	2

Table 2. The mean and standard weight of 100 seeds, seed coat weight of 4 seeds, proanthocyanidin content and radical scavenging activities according to the color of the seed coat.

Seed Coat Color (No. of Landraces)	Seed Coat Weight	100 Seed Weight	PA	RSA
Black (n=7) Mean	0.06	17.47	579.78	13.77
SD	0.01	6.95	291.96	6.71
Brown (n=6) Mean	0.06	14.58	163.05	4.79
SD	0.02	4.27	209.12	6.77
Brown (Y.) (n=4) Mean	0.05	17.52	52.17	1.11
SD	0.01	1.95	57.01	1.13
Green (n=4) Mean	0.06	22.21	2.98	0.17
SD	0.01	7.34	2.19	0.05
Yellow (n=31) Mean	0.07	23.29	4.64	0.19
SD	0.02	7.26	2.48	0.13

The digits in the parenthesis show the number of landraces in each group.

Table 3. The coefficient of correlation between seed weight, seed coat weight, proanthocyanidin content and radical scavenging activities

	Seed Coat wt.	PA	RSA
Seed Wt.	0.5051	0.0856	0.0972
Seed Coat Wt.	-	0.0572	0.0643
PA	-	-	0.9724

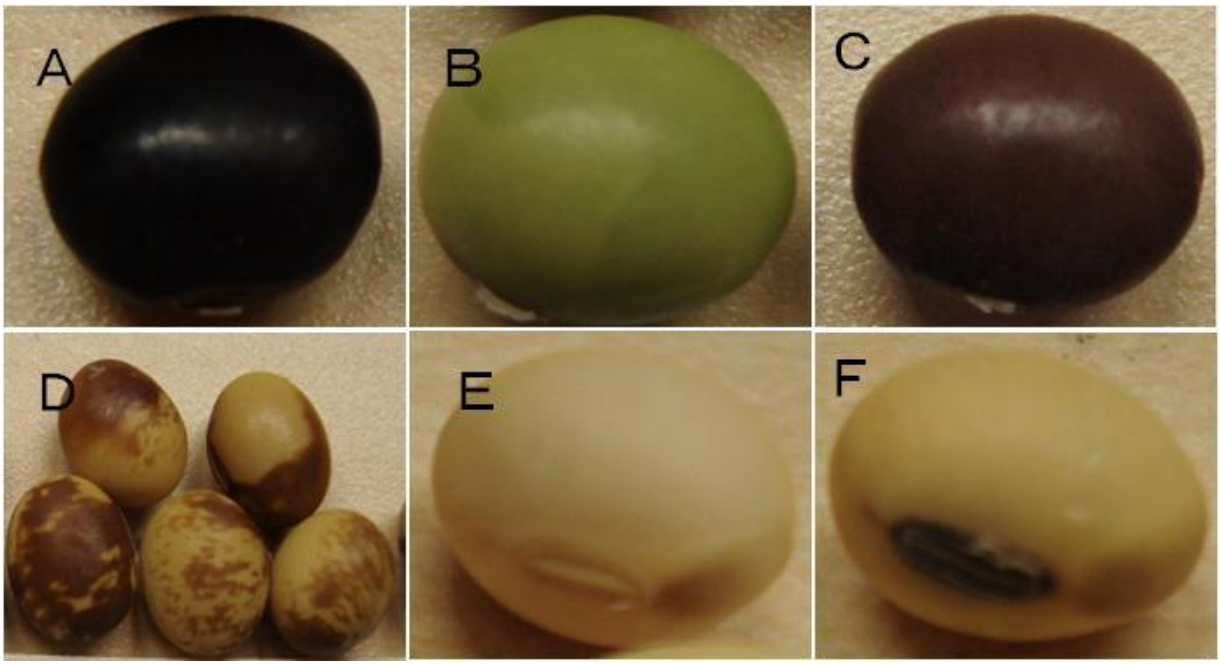

Fig. 1 Representative pictures of soybean landraces, with different seed coat colors, used in this study. A: black, B: green, C: brown, D: brown with yellow patches designated as 'brown-yellow', E: yellow with yellow hilum, and F: yellow with black hilum.

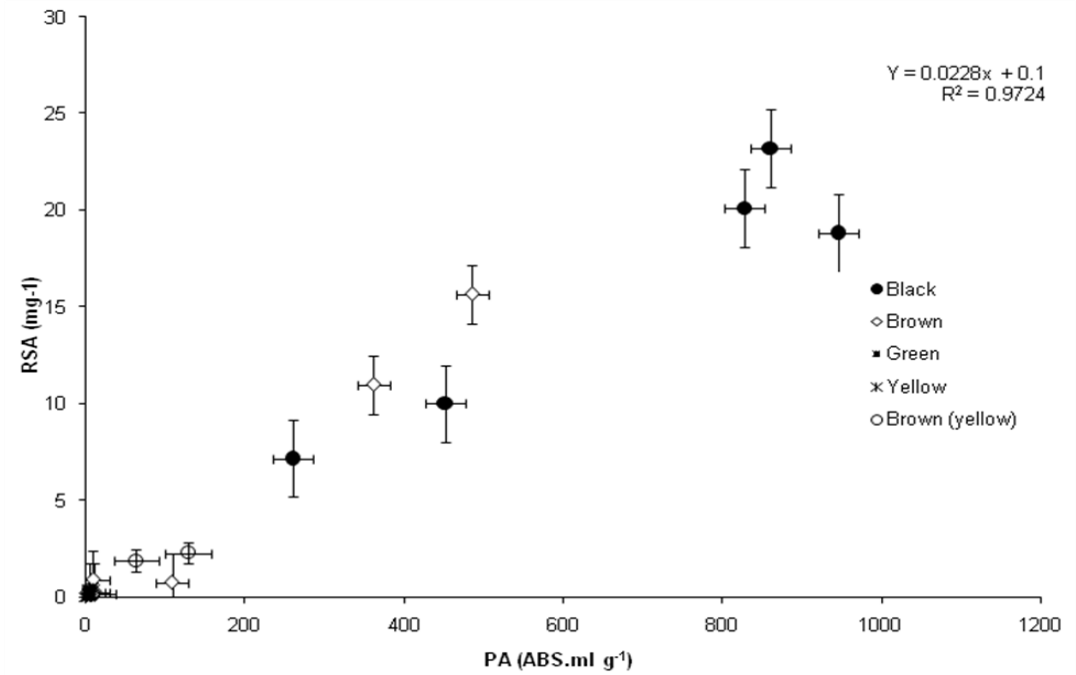

Fig. 2 The correlation between proanthocyanidin content measured in ABS.ml g^{-1} and radical scavenging activities measured in mg^{-1}. The PA and RSA of the soybean landraces are shown according to the color of the seed coats, (•) black, (◊) brown, (▪) green, (*) yellow, (ɷ) brown yellow. The vertical and horizontal bars show the error bars with standard error.

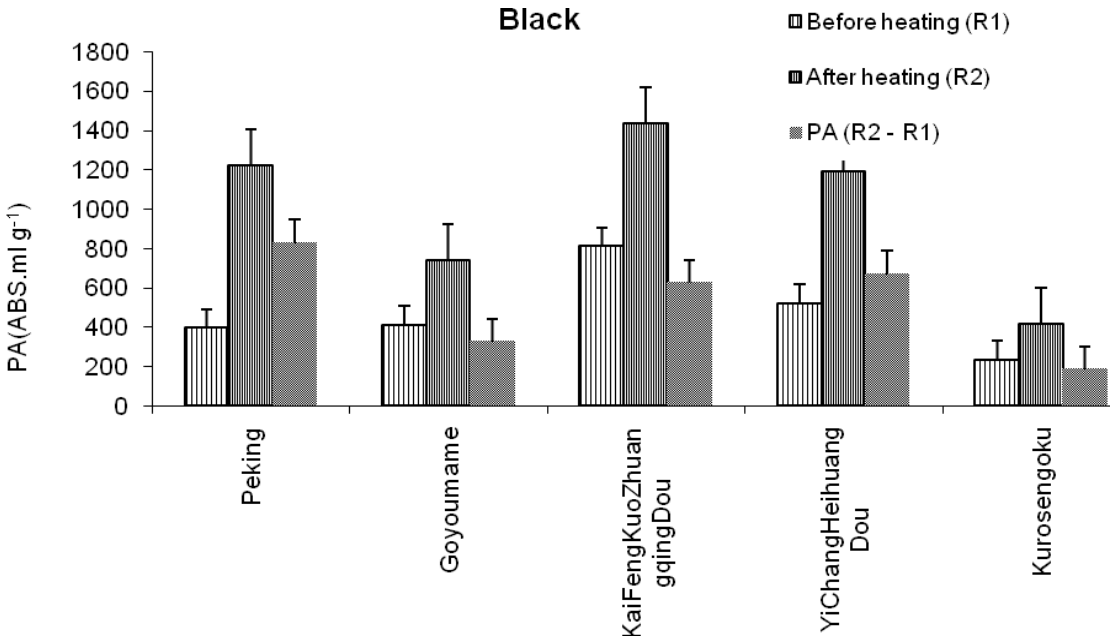

Fig. 3 Average spectrophotometric readings of the samples from landraces with black seed coats, before heating the crude solution (R_1), after heating (R_2) and the difference between the two ($R_2 - R_1$).

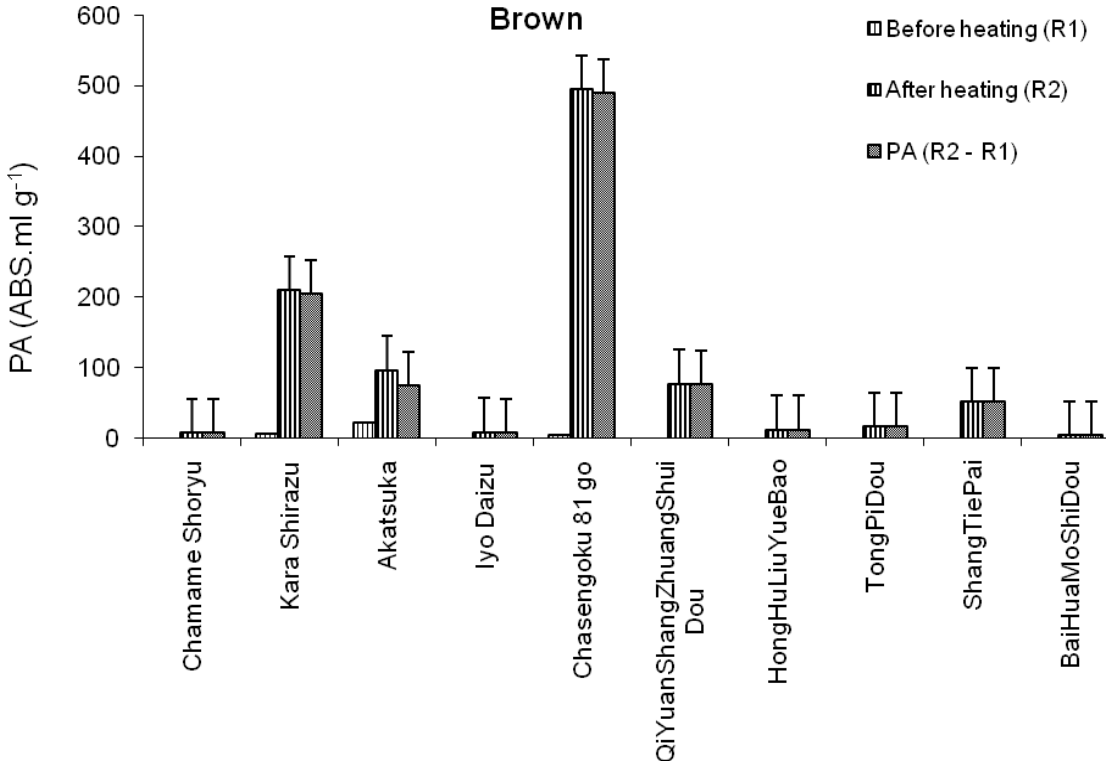

Fig. 4 Average spectrophotometric reading of samples from landraces with brown seed coats, before heating the crude solution (R_1), after heating (R_2) and the difference between the two ($R_2 - R_1$).