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Wall bound phenolics and total antioxidants in stored seeds of soybean (*Glycine max*) genotypes

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ABSTRACT

Quality seed is the most important input for sustainable agricultural productivity. Recent studies highlight the role of antioxidants in scavenging the reactive oxygen species, which is an indicator for quality seed. In natural ageing conditions, reactive oxygen species accumulates in the seeds that lead to seed deterioration. Wall bound phenolics in seed coat are potential antioxidants that encounter the reactive oxygen species. Four soybean genotypes with different seed coat wall bound phenolics were studied in 2018 at ICAR-Indian Institute of Seed Science and found that the black coat seed such as kalitur and JS76-205 showed highest phenolic content (117.00 and 128.50 µg/mL) and antioxidant activity of 51.38 and 46.07 µg eq. ascorbic acid, respectively. On contrary, the harasoya (green coated seed) resulted lowest phenolic content 14.30 µg/mL, while in RAUS-05 (white coated seed) the antioxidant activity is 2.58 µg eq. ascorbic acid, which is significantly lower than the other genotypes. Further, the genotypes were evaluated for the seed quality parameters and observed that kalitur and JS76-205 showed higher germination percentage (80%) and vigor indices I (900.33) and II (35.01) than the other genotypes. These results substantiate the hypothesis that the seed coat wall bound phenolics with antioxidant properties could alleviate the reactive oxygen species, protects from mechanical and chemical damage and helps in enhancement of seed quality parameters.

Key words: Antioxidants, Ferulic acid, Phenolics, Protocatechuic acid, Soybean

Recent observations on climatic changes and their influence on quality seed production have become a major challenge for sustainable agricultural productivity (Ali *et al*. 2017). Studies on seed deterioration substantiate that the fluctuations in weather conditions, i.e. biotic and abiotic factors contribute reactive oxygen species (ROS) production (Yadav *et al.* 2006, Sano *et al*. 2016). These radicals have high reactivity and damage lipids, DNA, RNA and proteins that ultimately lead to cell death (Kumar *et al*. 2015, 2016, Chandusingh *et al*. 2017). Phenolics that have the antioxidant capability because of redox properties can neutralize or quench the highly reactive hydroxyl radical ($OH^{.}$), singlet and triplet oxygen (Dona *et al*. 2013). Hence, mere possessing of quality phenolics is essential for antioxidant activity (Mhamdi *et al*. 2010). Talai and Sen-Mandi (2010) have proposed that the total antioxidant potential is mainly governed under genetic control in fresh harvested seeds; whereas in aged seeds, environmental factors determine the quality of the seed, which indicates that the aged seeds are prone to oxidative damage and need to be protected with antioxidants like seed coat phenolics. However, most of the hypothesis developed is based on the control deterioration or accelerated ageing experiments that need to be verified in naturally aged seeds. The problem of seed deterioration and field weathering are prominent in soybean genotypes. To determine the role of phenolics in seed longevity, 4 soybean genotypes such as kalitur (landrace having higher longevity) and JS76-205 (used in seed chain) with black seed coat genotypes, harasoya and RAUS-5 (defoliator) with green and white coat seeds (Agarwal *et al*. 2013) that differ in seed coat phenolics were chosen in the study. Further, evaluation of seed coat phenolics and their relation with quality seed parameters have been studied.

MATERIALS AND METHODS

Soybean genotypes: Soybean genotypes such as kalitur, JS75-206 (black coat seed), harasoya (green coat seed) and RAUS-05 (white coat) were supplied by ICAR-Indian Institute of Soybean Research, Indore, India. The seeds were naturally stored at 25°C for 1 year and used for experimental studies performed in 2018.

Phenolics extraction from seed coat: Seed coat

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phenolic compounds were extracted using various solvents as AWAa-1 (70:28:02,I), AWAa-2 (70:29.5:0.5,II), AWAa-3 (70:29.8:0.2,III), MW-1 (50:50,IV), MWAa-2 (50:49.5:0.5,V), MAa-3 (99.5:0.5, VI) and EW (70:30, VII), respectively. The nomenclature of solvent systems were expanded as A (acetone), W (water) Aa (acetic acid), M (methanol) and E (ethanol). Initially, the soybean seeds were soaked in water for 24 h. After drying, the wet seed coat is removed manually and dried in an oven for 4 h at 60 °C. To the dried seed coat, 5 mL of solvent mixture is added and homogenized with mortar and pestle. The extracts were filtered and the filtrate is used for total phenolics and antioxidant activity estimation.

Estimation of seed coat phenolics: Determination of total phenolics from soybean seed coats has been studied using Folin–Ciocalteau Reagent (FCR). To the samples (0.5 mL) an amount of water 0.5 mL H_2O and 2 mL of FCR (1:5 H_2O) was added and incubated for 3 min. Thereafter, 10 mL of 10 % (w/v) sodium carbonate was added and the reaction mixture was incubated for 30 min at room temperature. Absorbance was measured at 725 nm in a UV–Vis spectrophotometer and the amount of total phenolics was calculated as ascorbic acid equivalent (AAE) in mg/g of fresh weight (Złotek *et al*. 2016).

Determination of antioxidant potential from soybean genotypes: The antioxidant potential of different soybean genotypes was carried out according to Złotek *et al*. (2016) using DPPH (1,1-diphenyl-2-picrylhydrazyl) as the source of free radicals. In the assay, 100 µL of seed coat extracts was mixed with 2 mL of DPPH (1 mM) and incubated for 5 min at room temperature. Absorbance at 515 nm was measured immediately. The affinity of the seed coat extract to quench DPPH free radicals was measured by following equation.

$$Activity\ (\%) = \left[\frac{A-B}{A}\right] \times 100$$

where A, Absorbance of control at 0 min; B, Absorbance of sample after 5 min.

The scavenging activity of seed coat extract was expressed in µg/100 mg of ascorbic acid per gram of fresh weight.

Characterization of phenolics using HPLC: The phenolics particularly compounds incorporated in the cell wall were extracted using alkaline hydrolysis according to Campbell and Ellis (1992). The sample preparation for HPLC analysis and the experimental conditions maintained as per the method developed according to de Ascenosao and Dubery (2003).

Seed quality parameters: Seed germination tests of soybean genotypes were done using 'top of paper' method as per the International Seed Testing Association (ISTA) protocol (ISTA 2008). Seed germination is considered when the radicle and plumule attained 0.5 cm length. Further, mean seedling length and dry weight were also recorded to understand the seed quality parameters (ISTA 2008). Seed vigour index indexes were determined by equation 1 and 2.

Seedling vigour index I = Germination (%) × Mean seedling length (cm) (i)

Seedling vigour index II = Germination (%) × Mean seedling dry weight (mg) (ii)

Electrical conductivity test: Electrical conductivity (EC) test was carried out with 50 seeds in 8 replicates were soaked in 75 mL of deionized water for 24 h at 25ºC. EC was determined in a conductivity meter (Analyzer 600) with an electrode with constant 1 (Panobianco and Viera 1996).

Statistical analysis: The experiments were performed in triplicate and the standard error was calculated at 5% level.

RESULTS AND DISCUSSION

Colour of different soybean genotypes: Soybean cultivars with different colour are chosen to study the influence of seed coat phenolics in seed longevity. Cultivars such as kalitur and JS76-205 are having black seed coat, harasoya with green seed coat and RAUS-05 possess white seed coat. Seed coat colour of soybean is having a significant importance on the longevity and found the correlation between colour and germination. In the present study, kalitur and JS76-205 showed higher germination (80%); whereas harasoya and RAUS-05 resulted 60% each, respectively. The colour formation at seed coat is mainly attributed to the presence of phenolic compounds such as flavonoids. It is observed that in early seed development of *Arabidopsis*, the flavonoids accumulate as colorless compounds in the vacuoles (Gomes and Garcia 2013).

Wall bound phenolics extraction from seed coat: Seed coats have different phenolic compounds and to have optimum extraction, solvent systems that dissolve the desired products are essential. In the study, 07 solvent systems have been studied, of which acetone : water : acetic acid (70:29.8:0.2) solvent system resulted 129.4 µg/mL and 121.0 µg/mL of total phenolics from JS76-205 and kalitur (black seed coat) genotypes, respectively. Similarly, for harasoya (green coat) genotype, acetone: water: acetic acid (70:28:02) solvent system extracted 21.0 µg/mL of total phenolics; while for RAUS-05 (white seed coat) genotype 23.6 µg/mL of total phenolics has been obtained with ethanol:water in ratio of 7:3. Phenolic compounds have specific solubility in different solvent systems, as a result each genotype phenolics have been obtained in different solvent systems (Kumar *et al*. 2017, Singh *et al*. 2014).

Estimation of wall bound seed coat phenolics from different soybean genotypes: Total phenolic from seed coat have been standardized with different solvent systems. The black seed coated genotypes such as JS76-205 has showed highest phenolic content with the 128.50 µg/mL, while kalitur genotype resulted with 117.00 µg/mL phenolic contents. On the other hand, the white seed coated RAUS-05 genotype showed 19.40 µg/mL phenolic content; whereas, the green seed coat genotype harasoya has phenolic content of 14.30 µg/mL, which is lowest among all other genotypes (Fig 1).

Dona *et al.* (2013) have reported that the phenolics availability is one of the quality seed parameter. Seed coat phenolics have several functions in seed physiology. For

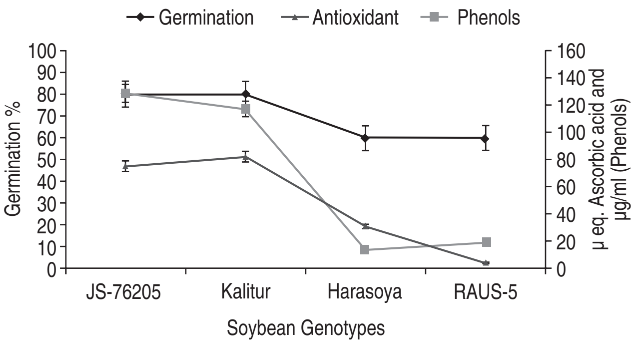

Fig 1 Correlation between seed coat phenolics, antioxidant and germination percentage of soybean genotypes.

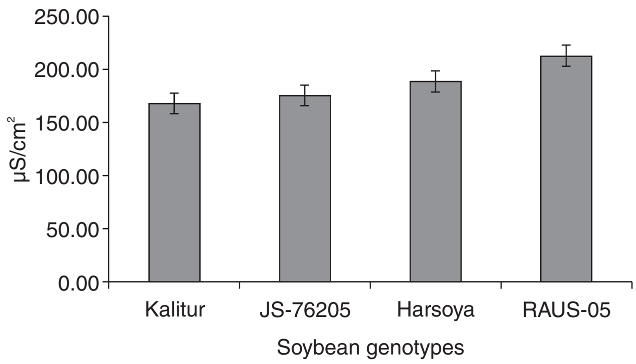

Fig 2 Electrical conductivity test from different soybean genotypes.

example, damaged seed coats are prone to chilling injury due to hydration stress in imbibition stage. Interestingly, Tully *et al*. (1981) have observed that black seed coat soybean imbibe water slowly and are less prone to chilling injury. Another function of phenolics in seed coats possess chemical defense against microorganisms. Unlike in polyphenolic form, phenolics as monomers may serve as inhibitors to fungal growth that enhance these germination under humid storage conditions (Vinutha *et al*. 2014).

Determination of antioxidant potential of soybean genotypes: Phenolic compounds having antioxidant activity is highly desirable in the seed germination process. During seed germination, free radical synthesis occurs that can be neutralized by antioxidants. Seed coat of different soybean genotypes showed different antioxidant potential. The highest antioxidant potential was determined in kalitur 51.38 µg eq. ascorbic acid followed by JS76-205 with 46.07 µg eq. ascorbic acid; whereas, in harasoya the antioxidant potential is 19.31µg eq. ascorbic acid and RAUS-05 resulted lower antioxidant potential with 2.58 µg eq. ascorbic acid (Fig 2). Phenolics with antioxidant potential are desirable because in germination process increase of ROS is widely reported (Tiwari *et al*. 2018, Sarangi *et al*. 2019).

For instance, flax seed coats are rich in polyphenolics (lignans), which act as antioxidants and may scavenge the ROS. This effect can protect the embryo, because the position of latter and its relation to seed coats and components could influence the seed storability (Chandusingh *et al*. 2018). Similar observations of viability lose in peanut and onion seeds due to radicle tip damage indicate the potential of phenolic antioxidant in the seed coats (Sinha *et al*. 2016, Singh *et al*. 2017b).

Characterization of phenolics derived from different soybean genotypes: Seed coat phenolics of soybean genotypes revealed presence of antioxidants such as protocatechuic acid *p*-coumaric acid, *t*-ferulic acid, vanillic acid, vanillin, 4-hydroxy benzoic acids and 4-hydroxy benzaldehyde. These compounds quench the free radicals and help to maintain the homeostasis of ROS. Kalitur possessed highest amount of protocatechuic acid, 4-hydroxy benzoic acid, 4-hydroxy benzaldehyde and vanillin with concentration of 3.41, 0.11, 1.39 and 0.06 µg/100 mg dry weight tissue. Similarly, in JS76-205 protocatechuic acid is 1.26 µg/100 mg dry weight tissue. On the contrary, RAUS-05 has showed 0.18 and 1.32 µg/100mg of *p*-coumaric acid and *t*-ferulic acid, respectively. However, harasoya resulted with lower levels of phenolic content and antioxidant activity than the other genotypes (Table 1).

Several studies regarding the phenolic compounds have been performed and strong positive correlation between the phenolic compound contents and the antioxidant potential (Pang *et al*.2018, Singh *et al*. 2019) has been observed. The seed antioxidants reduces the lipid oxidation (Kumar *et al*. 2015) and delay the ageing in seed tissues (Khurana *et al*. 2013). Besides, they protect the genetic material and essential biomolecules like proteins from the oxidative stress and enhance the nutraceuticals properties (Kumar *et al*. 2019).

Quality seed parameters: Quality seed parameters compromise of germination percentage, seedling length and vigour indices I and II. Among the soybean genotypes

Table 1 Determination of seed coat phenolics in soybean genotypes using HPLC (±SD)

Sample	Protocatechuic acid	4-Hydroxy benzoic acid	Vanillic acid	4-Hydroxy benzaldehyde	Vanillin	*p*-Coumaric acid	*t* -Ferulic acid
JS-76205	1.2623 ± 0.0931	0.1076 ± 0.0105	0.0611 ± 0.0061	0.9048 ± 0.0836	0.0275 ± 0.0021	0.0335 ± 0.0029	0.0977 ± 0.0093
Harasoya	0.0059 ± 0.0004	0.0271 ± 0.0022	0.4581 ± 0.0413	0.9827 ± 0.0923	0.0229 ± 0.0019	0.0918 ± 0.0083	0.7457 ± 0.0718
Kalitur	3.4114 ± 0.3112	0.1169 ± 0.0108	0.4343 ± 0.0425	1.3953 ± 0.1014	0.0645 ± 0.0058	0.1276 ± 0.0112	0.8672 ± 0.0832
RAUS-5	0.0308 ± 0.0029	0.1059 ± 0.0098	0.5611 ± 0.0523	1.3711 ± 0.1132	0.0279 ± 0.0023	0.1810 ± 0.0168	1.3259 ± 0.1214

Table 2 Seed quality parameters of soybean genotype (±SD)

Genotype	Seed quality parameter		
	Germination (%)	Vigor Index-I	Vigor Index-II
Kalitur	80.00 ± 1.52	900.33 ± 1.76	35.01 ± 1.58
JS-76205	80.00 ± 0.90	818.67 ± 2.84	30.95 ± 1.17
Harasoya	60.00 ± 0.76	665.16 ± 2.48	28.17 ± 1.00
RAUS-05	60.00 ± 1.61	622.00 ± 2.16	25.22 ± 0.75

studied, the black coat genotypes kalitur and JS76-205 have germination percentage with 80 percent; whereas, white and green seed coat genotypes showed 60 percentage of germination. The vigour index-I of different soybean genotypes showed the descending order of kalitur (900.33 ± 1.76) >JS76-205 (818.67 ± 2.84) >harasoya (665.16 ± 2.48) > RAUS-05 (622.00 ± 2.16). Similarly, vigour index-II of soybean genotypes corresponds to the trend that the kalitur (30.95±1.17) > JS76-205 (35.01 ± 1.58) >harasoya (28.17 ± 1.00) > RAUS-05 (25.22 ± 0.75) as shown in Table 2.

Electrical conductivity test: Electrical conductivity (EC) is one of the important parameter that determined firmness of seed coat. Generally, in aged seed, the seed coat looses the firmness and allows leakage of electrolytes in the solvent. Higher the value of EC lower the quality of seed; while, lower the EC value signifies the quality of seed. In the present study, the highest EC among different soybean genotypes was in white coated seed of RAUS-05 with 213.00 $\mu S/cm^2/g$, lowest amount of EC was found in kalitur with the 168.37 $\mu S/cm^2/g$. The seed coats of harasoya and JS76-205 genotypes have 188.55 and 175.50$\mu S/cm^2/g$ EC, respectively (Fig. 2). Thus, the current study exhibit correlation with Capeleti *et al*. (2005) that in soybean the phenolics polymerize to form lignin (polyphenol), which plays important role in seed permeability and resistance to mechanical damage.

Correlation of wall bound phenolics, antioxidant activity and seed quality parameters: Evidences on correlation of antioxidant potential, phenolics and seed quality parameters substantiate that the phenolics could increase the quality seed parameters by protecting from mechanical and chemical damage in maize (Vinutha *et al*. 2014). In the present study, soybean black seeded coat showed highest germination, vigour indices –I and II. Presence of phenolics and antioxidant activity is higher in these genotypes and showed positive correlation. However, with the harasoya (green black coat) and RAUS-05 genotypes the phenolics is low concomitantly resulted with poor seed quality parameters (Fig 2). These results indicate that the presence of phenolics coupled with antioxidant potential could enhance the quality of seed parameters.

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