

FOLIAR MICRONUTRIENTS SHAPE SEED COAT QUALITY IN VEGETABLE SOYBEAN

S. R. Nishchala¹, B. N. Radha^{1*}, Nagaraj Hullur, B. Basavaraja¹, C. S. Shantharaja¹, Geetha Govind², T. Onkarappa³

¹Department of Seed Science and Technology, University of Agricultural Sciences, Bangalore, Karnataka, India E-Mail ¹Ds: nishchala.16@gmail.com , nagarajhullur4@gmail.com, basavaraja.sst@gmail.com, shantharajacs@gmail.com

²Department of Crop Physiology, University of Agricultural Sciences, Bangalore, Karnataka, India, E-Mail ¹d: geeagri@gmail.com

³All India Coordinated Research Project (AICRP) on Soybean, Zonal Agricultural Research Station, University of Agricultural Sciences, Bangalore, Karnataka, India, E-Mail ¹d: onkarappa.t@gmail.com

*Corresponding Author: B. N. Radha, radhabn29@gmail.com, ORCID ¹d: <https://orcid.org/0000-0003-1670-1250>

ABSTRACT

The present study investigated the response of vegetable soybean seed-coat properties to foliar application of ferrous sulfate (FeSO₄) and zinc sulfate (ZnSO₄). The experiment was conducted during Kharif 2023 at the University of Agricultural Sciences, GKVK, Bengaluru, using vegetable soybean (*Glycine max* (L.) Merr.) cv. Karune (KBVS-1). Nine treatments were evaluated in a randomized block design with three replications. Among the treatments, 0.2% ZnSO₄ consistently recorded the highest values for the principal measured physical and biochemical attributes, including seed-coat thickness (0.83 mm), rupture force (69.59 N), total phenolics (0.39 mg g⁻¹), flavonoids (0.64 mg g⁻¹), proanthocyanidins (0.35 mg g⁻¹) and tannins (0.41 mg g⁻¹). Peroxidase-assay absorbance at 450 nm also reached its maximum under this treatment (2.77). Scanning electron microscopy showed comparatively greater apparent continuity among seed-coat tissue regions, while energy-dispersive X-ray spectroscopy revealed localized variation in the reported elemental composition. Collectively, the findings establish 0.2% ZnSO₄ as the most promising treatment among those tested for modifying vegetable soybean seed-coat properties.

KEYWORDS: Vegetable soybean; seed coat; zinc sulfate; ferrous sulfate; phenolics; seed-coat integrity

1. INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is a globally important legume and oilseed crop, valued for its substantial contribution to dietary protein, edible oil and industrial raw materials. The seeds typically contain approximately 40% protein and 20% oil, making soybean an important component of human nutrition and livestock feed. Soybean is broadly classified into grain-type and vegetable-type forms based on harvest maturity and intended use. Vegetable soybean, harvested at the green, full-seed stage, is gaining popularity because of its bold seeds, desirable flavour and nutritional composition (Shanmugasundaram *et al.*, 2015). Karune (KBVS-1) is a vegetable soybean variety characterized by bold seeds, a reported protein content of approximately 14%, and a relatively thin seed coat. Although nutritionally valuable, its delicate seed coat may increase susceptibility to cracking and mechanical injury, potentially affecting seed quality and storability.

The seed coat protects the embryo and cotyledons while regulating water exchange and interactions with the external environment (Souza and Marcos-Filho, 2001). Its protective efficiency depends on thickness, anatomical integrity and biochemical composition (Black and Halmer, 2006). In soybean, cracking may disrupt the palisade and hourglass cell layers, exposing underlying parenchymatous tissues and weakening the protective barrier (Wolf and Baker, 1972; Wolf *et al.*, 1981). Therefore, maintaining seed-coat integrity is important for preserving seed quality.

Seed-coat physical properties are closely associated with biochemical constituents, including phenolics, flavonoids, tannins and proanthocyanidins. These secondary metabolites contribute to plant defence and influence pigmentation, permeability and other seed-coat characteristics (Debeaujon *et al.*, 2001, 2003). Associations between proanthocyanidin accumulation, sclereid composition and seed-coat hardness have also been reported in *Rubus* species (Sugae *et al.*, 2011). Furthermore, peroxidase activity provides insights into biochemical processes associated with plant defence and oxidative metabolism.

Iron and zinc are essential micronutrients involved in enzymatic reactions, metabolic pathways and cellular protection (Cakmak, 2000; Broadley *et al.*, 2007). Previous studies have associated foliar zinc application with changes in phenolic and flavonoid accumulation, while iron application has been linked to alterations in flavonoid and procyanidin biosynthesis (Song *et al.*, 2015; Shi *et al.*, 2018; Ying *et al.*, 2021). These findings suggest that micronutrient nutrition may influence seed-coat development and biochemical composition.

However, the integrated effects of foliar iron and zinc application on vegetable soybean seed-coat characteristics remain insufficiently understood. Accordingly, this study evaluated different concentrations of ferrous sulfate and zinc sulfate for their effects on seed-coat thickness, hardness, phenolics, flavonoids, proanthocyanidins, tannins and peroxidase activity in vegetable soybean cv. Karune. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) were

employed to characterize seed-coat ultrastructure and elemental composition, respectively, to identify an effective micronutrient treatment for improving seed-coat quality.

2. MATERIALS AND METHODS

2.1 Experimental site, soil properties and plant material

The experiment was conducted during Kharif 2023 at the AICRP on Soybean, Zonal Agricultural Research Station, University of Agricultural Sciences, GKVK, Bengaluru, Karnataka, India (13°08' N, 77°57' E). The reported elevation was 954 m above mean sea level. The soil contained 30.02% coarse sand, 26.23% fine sand, 7.35% silt and 36.59% clay. Soil pH, electrical conductivity and organic carbon content were 6.95, 0.17 dS m⁻¹ and 0.54%, respectively. Available N, P₂O₅ and K₂O were 280.15, 16.67 and 200.12 kg ha⁻¹, respectively, while exchangeable Ca and Mg were reported as 31.50 and 13.50 meq 100 g⁻¹. Soil analyses were conducted following Piper (1966) and Jackson (1973). Seeds of vegetable soybean (*Glycine max* (L.) Merr.) cv. Karune (KBVS-1) were obtained from the same research station.

2.2 Experimental design and foliar treatments

Nine treatments, comprising an unsprayed control and four concentrations each of ferrous sulfate and zinc sulfate, were evaluated using a randomized block design with three replications. The gross plot measured 1.43 × 4.50 m, whereas the net plot measured 0.83 × 4.30 m. All plots received a basal fertilizer application of 30:80:37.5 kg ha⁻¹ of NP₂O₅K₂O. Sowing was performed on 10 July 2023 at 30 × 10 cm spacing and approximately 3 cm depth.

The experiment comprised nine treatments: C₁, untreated control; C₂, 0.1% ferrous sulfate; C₃, 0.2% ferrous sulfate; C₄, 0.3% ferrous sulfate; C₅, 0.4% ferrous sulfate; C₆, 0.1% zinc sulfate; C₇, 0.2% zinc sulfate; C₈, 0.3% zinc sulfate; and C₉, 0.4% zinc sulfate. All micronutrient concentrations were expressed as weight per volume (w/v). Foliar application was performed at bud initiation, flowering and pod formation; the control received no micronutrient spray.

2.3 Harvesting and laboratory evaluations

The crop was harvested when more than 95% of pods had turned brown, and most leaves had abscised. Harvested material was segregated by treatment, sun-dried on a clean surface and threshed after adequate drying. Treatment-wise, seed samples were collected for laboratory assessment of seed-coat thickness, hardness, total phenolic content, total flavonoid content, proanthocyanidins, tannins and peroxidase activity. SEM was used to examine seed-coat microstructure, while EDS was employed to characterize elemental composition. These assessments enabled comparison of the physical, biochemical and structural characteristics of seed coats following foliar micronutrient application.

2.4 Measurement of seed-coat thickness

Seed-coat thickness was determined using a digital caliper and expressed in millimetres (mm). For each treatment within each of the three replications, 25 seed coats were evaluated. Each seed coat was positioned between the external measuring jaws of the caliper, with the jaws oriented perpendicular to the seed-coat surface. The jaws were gently closed until they made light contact with the sample, thereby minimizing compression during measurement. Individual readings were recorded in millimetres, and the mean value was calculated for each treatment in each replication for subsequent statistical analysis.

2.5 Measurement of seed hardness

Seed hardness was evaluated using a Stable Micro Systems TA-HD plus texture analyser equipped with an aluminium P/2N needle probe (Figure 1). Twenty-five seeds from each treatment in each of the three replications were tested. Individual seeds were positioned on the instrument platform, and the probe was advanced until contact with the seed surface was detected. Compression was continued until rupture of the seed coat, and the maximum force recorded at rupture was considered the seed-coat rupture force and expressed in newtons (N). The analyser was operated in compression mode with pre-test, test and post-test speeds of 2, 0.5 and 10 mm s⁻¹, respectively. The test distance was set at 8 mm from the top of the probe, with automatic force triggering. The return distance, return speed and contact force were set at 10 mm, 20 mm s⁻¹ and 15 g, respectively. The resulting measurements were used to compare seed mechanical resistance among treatments.

2.6 Preparation of methanolic seed-coat extracts

Seed coats were manually separated from seeds collected from each treatment, ground into a fine powder using a pestle and mortar, and stored under refrigerated conditions until extraction. For methanolic extraction, 0.5 g of powdered seed-coat material from each experimental replicate was transferred into a centrifuge tube containing 25 mL of HPLC-grade methanol. The suspension was agitated using an orbital shaker for 3 h and subsequently incubated in darkness overnight to facilitate the extraction of soluble constituents. The resulting mixture was centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was collected and filtered through Whatman No. 1 filter paper. The clarified extracts were stored under refrigerated conditions until further biochemical analyses.

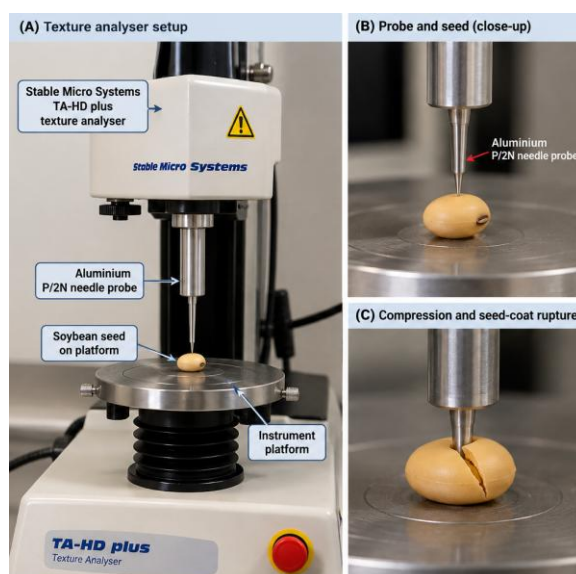


Figure 1. Measurement of soybean seed-coat hardness using a Stable Micro Systems TA-HD plus texture analyser equipped with an aluminium P/2N needle probe. (A) Texture analyser setup; (B) close-up of the probe positioned above the soybean seed; and (C) compression of the seed until seed-coat rupture.

2.7 Determination of proanthocyanidins

Proanthocyanidin content in the methanolic seed-coat extracts was determined using a modified vanillin colorimetric assay, following Zhang *et al.* (2016). Briefly, 1 mL of methanolic extract was mixed with 2.5 mL of 1% (w/v) vanillin solution prepared in methanol and 2.5 mL of 20% (v/v) sulfuric acid in methanol. The reaction mixture was incubated at 30°C for 15 min to facilitate colour development. Absorbance was subsequently measured at 500 nm using methanol as the blank. Catechin was used to construct the calibration curve, and proanthocyanidin content was calculated from the resulting standard curve and expressed as milligrams of catechin equivalents per gram of sample (mg CE g⁻¹).

2.8 Determination of total flavonoid content

Total flavonoid content in the methanolic seed-coat extracts was determined using the sodium nitrite–aluminium chloride (NaNO₂–AlCl₃) colorimetric assay. Briefly, 1 mL of diluted extract was mixed with 4 mL of distilled water and 0.3 mL of 5% (w/v) sodium nitrite solution. After 5 min of incubation, 0.3 mL of 10% (w/v) aluminium chloride solution was added, followed by a further incubation period of 6 min. Subsequently, 2 mL of 1 M sodium hydroxide was added, and the reaction mixture was made up to a final volume of 10 mL with distilled water. The absorbance was measured at 510 nm using a spectrophotometer. Rutin was used as the reference standard for calibration, and total flavonoid content was calculated from the standard curve and expressed as milligrams of rutin equivalents per gram of sample (mg RE g⁻¹).

2.9 Determination of total phenolic content

Total phenolic content in the seed-coat extract was determined using the Folin–Ciocalteu colorimetric method. Briefly, 0.5 mL of the extract was mixed with 2 mL of Folin–Ciocalteu reagent diluted 1:10 (v/v) with distilled water. After 5 min, 4 mL of 7.5% (w/v) sodium carbonate solution was added, and the reaction mixture was thoroughly homogenized and incubated at room temperature for 30 min. The absorbance was then measured at 765 nm using a spectrophotometer. Gallic acid was used as the reference standard to construct a calibration curve, and the total phenolic content was calculated and expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE g⁻¹).

2.10 Determination of peroxidase activity

Peroxidase activity in seed-coat extracts was determined following the method described by Sadasivam and Manickam (2008). Enzyme extraction was performed by homogenizing 1 g of seed-coat powder in 1 mL of 0.1 M phosphate buffer (pH 7.0). The homogenate was maintained at 4°C for 4 h and subsequently centrifuged at 10,000 rpm for 15 min at 4°C. The resulting supernatant was collected and kept chilled until analysis. For the enzyme assay, the reaction mixture consisted of 2 mL of 0.1 M phosphate buffer (pH 7.0), 200 µL of guaiacol and 200 µL of 12.3 mM hydrogen peroxide (H₂O₂). The mixture was equilibrated at 25°C, and the reaction was initiated by adding 100 µL of enzyme extract. Absorbance was monitored at 450 nm at 30-s intervals for 3 min using a spectrophotometer, with water used as the blank. In accordance with the original reporting convention, peroxidase activity was expressed as absorbance at 450 nm (A₄₅₀).

2.11 Determination of tannin content

Tannin content in the seed-coat extracts was determined using the vanillin–HCl colorimetric method described by Price *et al.*, 1978. The methanolic extracts and reagents were equilibrated at 30°C before analysis. Briefly, 1 mL of methanolic extract was mixed with 5 mL of vanillin reagent containing 0.5% (w/v) vanillin and 8% HCl in methanol. The reaction mixture was incubated at 30°C for 20 min, and absorbance was measured at 500 nm using a spectrophotometer. Catechin was used as the reference standard to construct a calibration curve, and tannin content was expressed as milligrams of catechin equivalents per gram of sample (mg CE g⁻¹).

2.12 SEM–EDS analysis of seed-coat structure and elemental composition

Cracked and uncracked seed-coat tissues were sectioned using a Thermo Scientific HM 550 OVP-D cryostat. The samples were embedded in cryostat matrix gel, mounted on specimen holders, and sectioned at a thickness of 6 μm using the motorized sectioning system and anti-roll plate. The sections were maintained under cool, dry conditions until further analysis. For scanning electron microscopy and energy-dispersive X-ray spectroscopy (SEM–EDS), the sections were mounted on SEM stubs using conductive carbon tape and coated with a thin layer of gold by sputter coating. Microscopic examination was performed at the Indian Institute of Science, Bengaluru, using an Ultra55 field-emission scanning electron microscope (FE-SEM; Carl Zeiss) equipped with an Oxford 10-mm silicon drift detector. SEM was used to examine seed-coat microstructure and anatomical features, whereas EDS was employed to characterize the elemental composition, with particular emphasis on potassium (K), calcium (Ca), and magnesium (Mg). Anatomical features were interpreted with reference to established descriptions of soybean seed-coat structure by Wolf and Baker (1972) and Wolf *et al.* (1981).

2.13 Statistical analysis

The field experiment was conducted using a randomized block design (RBD) with nine treatments and three replications. The experimental data were subjected to analysis of variance (ANOVA) appropriate for the design, following the procedures described by Gomez and Gomez (1984). Statistical significance was assessed at the 5% probability level ($P \leq 0.05$). When the treatment effect was significant, treatment means were compared using the critical difference (CD) at the 5% significance level. The standard error of the mean ($\text{SEm} \pm$) and coefficient of variation (CV, %) were reported to indicate the precision and variability of the experimental results.

3. RESULTS

3.1. Seed-coat characteristics

Foliar nutrient management substantially differentiated the seed-coat characteristics of soybean, with significant effects on both thickness and rupture force (Table 1). The highest values for these traits were associated with C_7 , in which 0.2% ZnSO_4 application resulted in a seed-coat thickness of 0.83 mm and a rupture force of 69.59 N. The next-best response was recorded under C_3 (0.2% FeSO_4), yielding corresponding values of 0.80 mm and 68.11 N. In contrast, seeds from untreated plants exhibited the lowest thickness (0.63 mm) and rupture force (58.60 N). Relative to the untreated control, the ZnSO_4 treatment increased seed-coat thickness by approximately one-third (31.75%) and rupture force by 18.75%. The other treatments occupied intermediate positions, reflecting differences in the magnitude of response to the applied nutrients. The observed treatment pattern establishes C_7 as the leading treatment for both evaluated traits and indicates that foliar application of 0.2% ZnSO_4 was associated with greater seed-coat thickness and higher resistance to rupture under the conditions of the study.

Table 1. Effect of foliar FeSO_4 and ZnSO_4 on seed-coat properties of vegetable soybean

Treatment	Seed-coat thickness (mm)	Rupture force (N)
C_1	0.63	58.60
C_2	0.75	65.31
C_3	0.80	68.11
C_4	0.68	61.05
C_5	0.73	62.96
C_6	0.71	62.51
C_7	0.83	69.59
C_8	0.76	66.04
C_9	0.78	67.37
Mean	0.74	64.61
SEm $\pm$	0.02	1.48
CD ($P = 0.05$)	0.05	4.40
CV (%)	3.74	3.97

3.2. Biochemical components of the seed coat

Foliar nutrient treatments altered the biochemical composition of soybean seed coats, with the most pronounced responses generally observed following application of 0.2% ZnSO_4 (C_7) (Tables 2). This treatment produced the highest total phenolic concentration (0.39 mg g^{-1}), exceeding the values recorded under 0.2% FeSO_4 (C_3 ; 0.37 mg g^{-1}) and the untreated control (C_1 ; 0.19 mg g^{-1}) by 5.41% and 105.26%, respectively, when calculated relative to the respective comparison treatments. The response was more pronounced for flavonoids, which reached 0.64 mg g^{-1} under C_7 compared with 0.41 mg g^{-1} under C_3 and 0.10 mg g^{-1} under C_1 . C_7 also recorded the highest proanthocyanidin and tannin concentrations, at 0.35 and 0.41 mg g^{-1} , respectively, representing increases of 250% and 272.73% over the control. In comparison, the corresponding minimum concentrations were observed in C_1 , at 0.10 mg g^{-1} for proanthocyanidins and 0.11 mg g^{-1} for tannins. The peroxidase-assay response exhibited a similar overall treatment pattern but differed in the intermediate ranking: C_7 recorded the maximum A_{450} (2.77), followed by C_9 (2.43) and C_3 (2.33), whereas C_1 had the minimum value (0.73). The C_7 absorbance was 279.45% greater than that of the control. Taken together, these findings establish C_7 as the treatment associated with the highest

values across all evaluated biochemical parameters. The results also indicate that the magnitude of the response varied among biochemical constituents, with flavonoids showing the largest proportional increase over the untreated control.

Table 2. Effect of foliar FeSO₄ and ZnSO₄ on biochemical constituents of vegetable soybean

Treatment	Phenolics (mg g ⁻¹)	Flavonoids (mg g ⁻¹)	Proanthocyanidins (mg g ⁻¹)	Tannin assay (mg g ⁻¹)	Peroxidase absorbance at 450 nm (A ₄₅₀)
C ₁	0.19	0.10	0.10	0.11	0.73
C ₂	0.28	0.22	0.18	0.25	1.21
C ₃	0.37	0.41	0.27	0.37	2.33
C ₄	0.20	0.15	0.12	0.15	1.01
C ₅	0.25	0.18	0.15	0.22	1.35
C ₆	0.23	0.16	0.13	0.18	1.17
C ₇	0.39	0.64	0.35	0.41	2.77
C ₈	0.30	0.28	0.21	0.29	1.68
C ₉	0.31	0.37	0.24	0.34	2.43
Mean	0.28	0.28	0.19	0.26	1.63
SEm ±	0.01	0.01	0.01	0.01	0.03
CD (P = 0.05)	0.02	0.02	0.02	0.02	0.10
CV (%)	4.43	4.70	4.77	4.64	3.71

3.3. Seed-coat anatomy and elemental composition: SEM–EDS analysis

The SEM and EDS observations provided complementary information on the microstructural features and localized elemental composition of soybean seed coats under different foliar nutrient treatments. SEM examination identified the characteristic tissue arrangement of the seed coat, comprising the cuticle, palisade layer, hourglass-cell region and underlying parenchymatous tissue (Figure 2). Cryostat sectioning produced specimens approximately 6 µm thick for microscopic analysis.

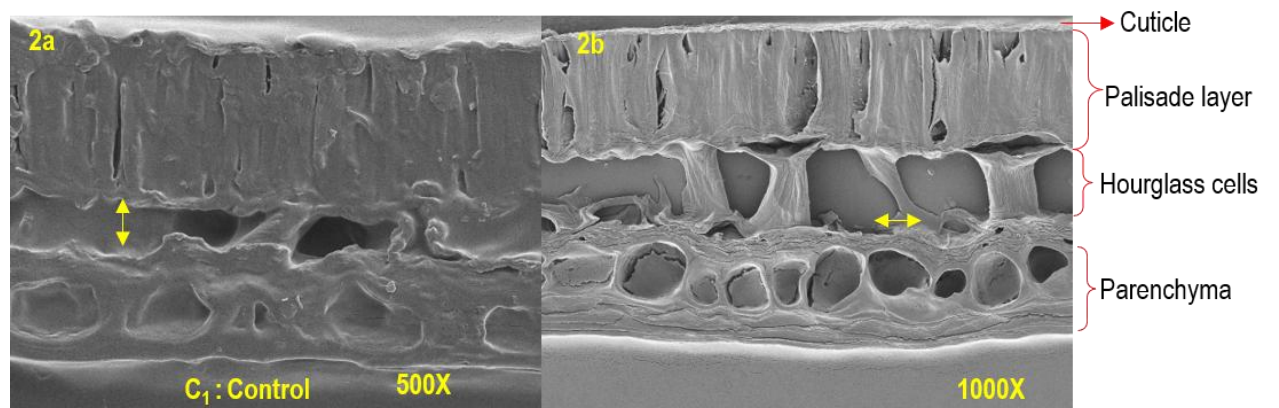


Fig. 2a. Cross sectional view of the separation of the epidermal (palisade cells) and hypodermal (hourglass cells) tissues from parenchyma. 2b. Week hourglass cells

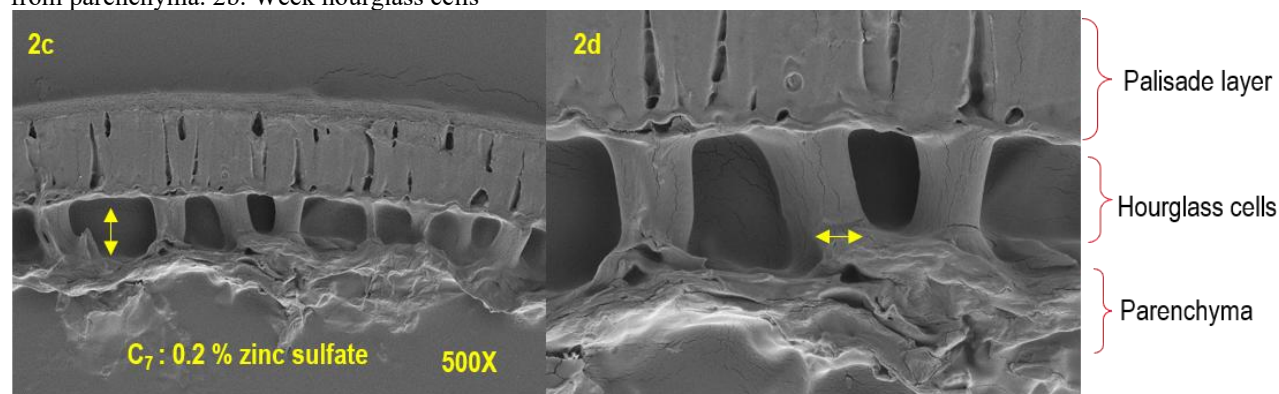


Fig. 2c. Intact epidermal (palisade cells) and hypodermal (hourglass cells) tissues from parenchyma. 2d. Strong hourglass cells

Figure. 1: SEM imaging for seed coat cracking in soybean cv. Karune. In C₁ there is a separation of the epidermal (palisade cells) and hypodermal (hourglass cells) tissues from parenchyma compared to C₇ which has an intact epidermal (palisade cells) and hypodermal (hourglass cells) tissues from parenchyma.

The untreated control (C₁) showed discernible separation among the major tissue regions, particularly at the interfaces between the palisade layer, hourglass-cell region and underlying parenchyma (Figure 2). In contrast, C₇ (0.2% ZnSO₄) displayed comparatively greater apparent continuity and fewer visible discontinuities. These observations suggest differences in the structural appearance of the sampled seed coats, although objective image analysis would be necessary to quantify such differences. The corresponding EDS profiles revealed variation in K₂O-, CaO- and MgO-equivalent proportions between cracked and uncracked tissues (Table 3). In C₁, K₂O was higher in cracked tissue (1.78%) than in uncracked tissue (1.11%), whereas CaO showed little variation, at 0.12 and 0.14%, respectively. MgO was not detected in either control sample. Under C₇, the uncracked tissue exhibited 3.12% K₂O, 0.35% CaO and 0.26% MgO, compared with 2.74, 0.27 and 0.18%, respectively, in cracked tissue. These values were higher than those of the corresponding uncracked control for all three reported components. Nevertheless, treatment-specific maxima varied by tissue condition: C₃ (0.2% FeSO₄) recorded the highest K₂O proportion in cracked tissue (2.75%) and the highest CaO proportion in uncracked tissue (0.36%). MgO was detected in multiple treated samples but remained below the detection limit in the control. Taken together, the observations indicate treatment-associated differences in the visual continuity and localized elemental composition of the seed coat. However, confirmation of a relationship between these features and mechanical performance requires quantitative image analysis, appropriate replication and direct measurements of seed-coat mechanical properties.

Table 3. EDS-reported oxide-equivalent composition of cracked and uncracked seed-coat tissues of soybean cv. Karune.

Treatment	K ₂ O uncracked (%)	K ₂ O cracked (%)	CaO uncracked (%)	CaO cracked (%)	MgO uncracked (%)	MgO cracked (%)
C ₁	1.11	1.78	0.14	0.12	–	–
C ₂	2.01	2.38	0.15	0.16	–	–
C ₃	2.67	2.75	0.36	0.25	0.19	0.14
C ₄	1.89	2.44	0.27	0.13	0.20	0.15
C ₅	1.08	1.91	0.23	0.11	0.15	0.11
C ₆	2.04	2.68	0.29	0.19	0.17	0.13
C ₇	3.12	2.74	0.35	0.27	0.26	0.18
C ₈	1.87	2.11	0.25	0.23	0.14	0.12
C ₉	1.96	2.19	0.29	0.22	0.21	0.16

4. DISCUSSION

4.1. Seed-coat characteristics

4.1.1. Seed-coat thickness and mechanical resistance

Seed-coat thickness and rupture force are important physical characteristics associated with the protective function of the testa. The present study showed that foliar application of 0.2% ZnSO₄ (C₇) resulted in the greatest seed-coat thickness (0.83 mm) and rupture force (69.59 N), whereas the untreated control recorded the lowest values for both traits. The corresponding increases of 31.75% in thickness and 18.75% in rupture force suggest that the C₇ treatment was associated with a seed coat that was both thicker and more resistant to rupture under the test conditions.

The parallel response of these two characteristics is noteworthy because thickness alone does not necessarily determine mechanical resistance. The arrangement, compactness and continuity of the seed-coat tissues, together with their chemical composition, may also contribute to resistance against mechanical damage. The seed coat forms a protective interface between the embryo and the external environment and plays an important role in seed integrity and protection (Souza and Marcos-Filho, 2001; Black and Halmer, 2006). In soybean, the structural organization of the palisade and hourglass-cell regions is relevant to the physical behaviour of the seed coat and its susceptibility to cracking (Wolf and Baker, 1972; Wolf *et al.*, 1981).

The higher rupture force observed under C₇, accompanied by greater thickness, is consistent with improved mechanical resistance. However, the present measurements do not establish whether the increase in rupture force resulted directly from greater thickness, altered tissue organization, changes in biochemical composition, or a combination of these factors. The SEM observations and biochemical measurements provide complementary evidence for interpreting this response, but additional quantitative anatomical and mechanical analyses would be needed to establish the underlying relationships.

4.1.2. Seed-coat anatomy and structural integrity

SEM examination revealed apparent differences in the continuity of seed-coat tissues between the control and C₇ treatment. The control showed visible separation between the outer palisade region, the hourglass-cell region and the underlying parenchyma, whereas the C₇ sample appeared comparatively continuous. These qualitative observations provide anatomical context for the higher thickness and rupture-force measurements recorded under C₇.

The soybean seed coat consists of differentiated tissue layers whose organization contributes to its protective and mechanical functions. The palisade layer provides an outer structural component, while the underlying hourglass cells and parenchymatous tissues contribute to the organization of the seed coat. Previous work on soybean seed-coat structure has

examined the relationship between tissue organization, cracking and physical integrity (Wolf and Baker, 1972; Wolf *et al.*, 1981). Differences in tissue continuity may therefore be relevant to the mechanical response observed in the present study. The SEM images suggest that the C₇ treatment was associated with fewer visible separations in the examined sections. Nevertheless, SEM images from selected sections cannot independently demonstrate that tissue cohesion improved throughout the seed population or that cracking incidence declined significantly. Such conclusions require replicated image analysis, quantitative measurements of tissue separation and cracking frequency, and statistical comparison among treatments. Accordingly, the present anatomical observations support—but do not independently prove—the interpretation that C₇ was associated with better seed-coat structural integrity.

4.2. Biochemical components of the seed coat

4.2.1. Total phenolics and flavonoids

The highest total phenolic and flavonoid contents were recorded under C₇, followed by C₃, whereas the control exhibited the lowest values. This pattern indicates that the response of these biochemical constituents was associated with the nutrient source and concentration. In particular, the marked increase in flavonoid content under C₇ suggests that the 0.2% ZnSO₄ treatment was associated with a substantial change in the accumulation of measured flavonoid compounds in the seed coat. Phenolic compounds and flavonoids are involved in several aspects of plant defence and oxidative-stress responses. Their accumulation can be influenced by nutrient status, developmental processes and environmental conditions. Earlier studies have reported associations between foliar zinc application and changes in phenolic and flavonoid accumulation (Song *et al.*, 2015; Lamia *et al.*, 2017; Rana *et al.*, 2017). Zinc also has important roles in numerous physiological processes, including the maintenance of cellular function and protection against oxidative stress (Cakmak, 2000; Broadley *et al.*, 2007). The present findings are consistent with the possibility that foliar ZnSO₄ application influenced seed-coat secondary metabolism. However, the measured increases do not establish that zinc directly stimulated specific phenolic biosynthetic genes or enzymes, because gene expression and pathway-enzyme activities were not reported in the supplied results. The biochemical response should therefore be interpreted as an association with treatment rather than proof of a specific molecular mechanism.

The concurrent increase in phenolic and flavonoid contents and the higher rupture force observed under C₇ is also relevant. These biochemical constituents may contribute to seed-coat defence and chemical properties, but their direct contribution to mechanical resistance cannot be established from treatment means alone. Further analyses linking individual biochemical constituents to seed-coat mechanical properties would help clarify their functional significance.

4.2.2. Proanthocyanidins, tannins and seed-coat integrity

Proanthocyanidin and tannin contents followed a pattern similar to that of total phenolics and flavonoids, with the highest values recorded under C₇ and the lowest under the control. The increased accumulation of these compounds under C₇ suggests that the treatment was associated with changes in the phenolic-related biochemical profile of the seed coat.

Proanthocyanidins are flavonoid-derived compounds associated with seed-coat pigmentation and development. Their accumulation and oxidation during seed maturation contribute to testa colour, while their distribution within seed-coat tissues has been described in studies of seed-coat development (Debeaujon *et al.*, 2001, 2003). Proanthocyanidins and other phenolic compounds may also interact with proteins and other structural constituents, potentially influencing the physicochemical properties of seed tissues. These roles provide a plausible basis for examining their relationship with seed-coat integrity.

The higher proanthocyanidin and tannin contents recorded under C₇ coincided with greater seed-coat thickness and rupture force. This correspondence suggests a possible relationship between phenolic-related constituents and the physical characteristics of the seed coat. Nevertheless, the data do not demonstrate that increased proanthocyanidin or tannin content directly caused the higher rupture force. Nor can the individual contributions of these compounds be separated from those of other structural or biochemical factors. Further studies involving biochemical profiling, tissue localization and correlation analysis would be needed to determine the strength and nature of these relationships.

4.2.3. Peroxidase-assay response and physiological interpretation

The highest peroxidase-assay absorbance at 450 nm was observed under C₇, followed by C₉ and C₃, while the control recorded the lowest value. This response indicates that the assay signal differed among treatments and was greatest under 0.2% ZnSO₄.

Peroxidases are a diverse group of enzymes involved in plant redox regulation and, depending on the enzyme and substrate, can participate in the metabolism of reactive oxygen species and the modification of cell-wall components. Zinc is an essential micronutrient involved in multiple metabolic and protective processes, including the maintenance of enzyme function and cellular redox balance (Cakmak, 2000; Broadley *et al.*, 2007). These physiological roles provide context for investigating whether zinc nutrition is associated with changes in the seed-coat peroxidase response.

However, zinc should not be described as a direct cofactor of all peroxidases, and the observed absorbance difference alone does not demonstrate enhanced antioxidant protection or cell-wall reinforcement. Because the supplied results report optical density rather than a calculated enzyme-activity rate, the response must be interpreted cautiously. Confirmation would require details of the assay protocol, reaction duration, blank correction, normalization basis and calculation of enzyme activity. Subject to these limitations, the higher A₄₅₀ under C₇ represents a treatment-associated biochemical response that merits further investigation.

4.2.4. Integration of biochemical and physical responses

A notable feature of the results is the consistent ranking of C_7 for seed-coat thickness, rupture force, total phenolics, flavonoids, proanthocyanidins and tannins. C_3 , corresponding to 0.2% FeSO_4 , generally ranked second for these measured traits. This agreement across physical and biochemical measurements suggests that the observed seed-coat response was associated with both nutrient source and application concentration.

The coordinated pattern is biologically relevant because the seed coat is a complex tissue whose mechanical behaviour can be influenced by anatomy and composition. The biochemical results provide evidence of differences in measured secondary metabolites, while the physical measurements quantify thickness and rupture force. SEM provides qualitative information about tissue organization, and EDS provides localized elemental-composition measurements. Considered together, these independent types of observations offer a broader description of the treatment response than any single measurement alone. Nevertheless, the agreement among treatment rankings should not be interpreted as proof of a causal chain in which zinc increased phenolic accumulation, which then strengthened seed-coat tissues and raised rupture force. Establishing such a mechanism would require statistical analysis of relationships among individual observations, alongside targeted biochemical, anatomical and molecular measurements. The present findings support an integrated interpretation of the response under C_7 while leaving the mechanisms open for further validation.

4.3. SEM-EDS analysis and elemental composition

4.3.1. Elemental variation in cracked and uncracked seed-coat tissues

EDS revealed differences in the reported K_2O , CaO and MgO proportions among treatments and between cracked and uncracked seed-coat samples. Under C_7 , the uncracked tissue exhibited higher reported values of all three components than the control. However, the elemental pattern was not uniform across all treatments: C_3 recorded the highest K_2O value in cracked tissue and the highest CaO value in uncracked tissue. Thus, C_7 did not produce the maximum value for every measured elemental component.

These findings indicate that localized elemental composition varied with treatment and tissue condition. Calcium is commonly associated with cell-wall structure and membrane function, while potassium and magnesium have important roles in plant metabolism. These general physiological functions provide context for considering the measured elements, but the EDS data do not demonstrate that the detected elements were incorporated into specific structural components of the soybean seed coat.

EDS is a localized, generally semi-quantitative technique. Its output depends on the sampled region, specimen preparation, instrument settings, matrix effects and the method used to express elemental composition. In particular, oxide-equivalent percentages should not automatically be interpreted as direct measurements of the concentration of free oxides or as evidence of whole-seed nutrient accumulation. The detection of MgO in treated samples but not in the control should also be interpreted in relation to the instrument's detection limits and analytical conditions.

Consequently, the higher reported elemental values under C_7 in uncracked tissue are descriptive evidence of compositional differences in the analysed regions. They cannot, on their own, establish increased nutrient uptake, enhanced cell-wall stability or reduced cracking. These interpretations would require complementary chemical analyses and quantitative measurements of seed-coat damage.

4.3.2. Integrating SEM and EDS observations with seed-coat characteristics

The SEM and EDS observations complement the physical measurements by describing tissue morphology and localized elemental composition. The apparent continuity of seed-coat layers in the C_7 sample coincided with greater measured thickness and rupture force. The EDS results further showed differences in the reported elemental composition between the C_7 and control samples, particularly in uncracked tissue.

This convergence of observations provides a reasonable basis for identifying C_7 as the most promising treatment among those evaluated for the measured seed-coat characteristics. However, SEM and EDS provide different types of evidence: SEM describes morphology, whereas EDS characterizes elemental signals from selected regions. Neither technique alone establishes the mechanism responsible for the observed physical response. In addition, because the EDS data are descriptive and no inferential statistics were provided for these measurements, the differences should not be described as statistically significant.

Future studies should combine replicated SEM imaging and quantitative crack analysis with standardized EDS measurements, independent mineral analysis, and seed-coat mechanical testing. Such an approach would help determine whether the observed morphological and elemental differences are reproducible and whether they are statistically associated with mechanical resistance and seed quality.

5. CONCLUSION

This study identified foliar application of 0.2% ZnSO_4 (C_7) as the most promising treatment for modifying the measured physical, biochemical and anatomical characteristics of the soybean seed coat. The treatment recorded the highest seed-coat thickness and rupture force, the greatest concentrations of the analyzed phenolic constituents, and comparatively greater tissue continuity in SEM observations. EDS analysis revealed additional variation in localized elemental composition, providing complementary information on the characteristics of the examined seed-coat regions. The convergence of these observations supports further investigation of foliar zinc application as a potential strategy for managing seed-coat quality in soybean. Its practical relevance lies in the possibility of developing seed production practices that improve resistance to

mechanical damage and help maintain seed quality throughout harvesting, processing, storage and distribution. However, these benefits cannot be inferred from seed-coat measurements alone. Validation should include direct mechanical damage tests, germination and vigour assessments, storage experiments, replicated field trials and an economic analysis of treatment costs and returns. Establishing these relationships will clarify whether the observed responses translate into measurable improvements in commercial seed quality and support evidence-based recommendations for soybean seed production.

ACKNOWLEDGMENT

The authors gratefully acknowledge the invaluable technical assistance and infrastructural facilities provided by the All India Coordinated Research Project (AICRP) on Soybean, Zonal Agricultural Research Station, and the Department of Crop Physiology, University of Agricultural Sciences, Bengaluru, Karnataka, India, which greatly contributed to the successful execution of this study.

Author Contributions

Nishchala, S.R.: performed the experimental work, data collection, and preliminary analysis; Radha, B. N.: conceived the study, designed the methodology, and provided overall technical supervision; Nagaraj hullur: assisted in statistical analysis; Basavaraja, B.: Facilitated biochemical investigations through laboratory support and infrastructure. C. S. Shantharaja: Provided technical assistance for laboratory analysis;

Funding: This study was not funded by any source.

Consent to participate

All authors consented to participate in the article (Radha BN -on behalf of all coauthors).

Consent to publish

All authors gave their consent for the article to be published if it was accepted (Radha BN, on behalf of all coauthors).

Disclosure statement

No potential conflict of interest was reported by the authors.

REFERENCES

1. Abd El-Kader, M. G. (2013). Effect of sulfur application and foliar spraying with zinc and boron on yield, yield components, and seed quality of peanut (*Arachis hypogaea* L.). Research Journal of Agriculture and Biological Sciences, 9(4), 127–135. <https://www.aensiweb.net/AENSIWEB/rjabs/rjabs/2013/127-135.pdf>
2. Marichali, A., Dallali, S., Ouerghemmi, S., Sebei, H., & Hosni, K. (2014). Germination, morpho-physiological and biochemical responses of coriander (*Coriandrum sativum* L.) to zinc excess. Industrial Crops and Products, 55, 248–257. <https://doi.org/10.1016/j.indcrop.2014.02.033>
3. Black, M., Bewley, J. D., & Halmer, P. (Eds.). (2006). The Encyclopedia of Seeds: Science, Technology and Uses. CABI Publishing, Wallingford, UK. 828 pp. ISBN: 978-0-85199-723-0.
4. Broadley, M. R., White, P. J., Hammond, J. P., Zelko, I., & Lux, A. (2007). Zinc in plants. New Phytologist, 173(4), 677–702. <https://doi.org/10.1111/j.1469-8137.2007.01996.x>
5. Burchett, C. A., Schapaugh, W. T., Jr., Overley, C. B., & Walter, T. L. (1985). Influence of etched seed coats and environmental conditions on soybean seed quality. Crop Science, 25(4), 655–660. <https://doi.org/10.2135/cropsci1985.0011183X002500040018x>
6. Cakmak, I. (2000). Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. New Phytologist, 146(2), 185–205. <https://doi.org/10.1046/j.1469-8137.2000.00630.x>
7. Debeaujon, I., Peeters, A. J. M., Léon-Kloosterziel, K. M., & Koornneef, M. (2001). The TRANSPARENT TESTA12 gene of Arabidopsis encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. The Plant Cell, 13(4), 853–871. <https://doi.org/10.1105/tpc.13.4.853>
8. Debeaujon, I., Nesi, N., Perez, P., Devic, M., Grandjean, O., Caboche, M., & Lepiniec, L. (2003). Proanthocyanidin-accumulating cells in Arabidopsis testa: Regulation of differentiation and role in seed development. The Plant Cell, 15(11), 2514–2531. <https://doi.org/10.1105/tpc.014043>
9. He, F., Pan, Q.H., Shi, Y., & Duan, C.Q. (2008). Biosynthesis and genetic regulation of proanthocyanidins in plants. Molecules, 13(10), 2674–2703. <https://doi.org/10.3390/molecules13102674>
10. Gomez, K. A., & Gomez, A. A. (1984). Statistical procedures for agricultural research (2nd ed.). John Wiley & Sons, New York, USA. 704 pp. ISBN: 978-0-471-87092-0
11. Jackson, M. L. (1973). Soil Chemical Analysis. Prentice-Hall of India Private Limited, New Delhi, India. 498 p.
12. Maedza, V. K., Nkomo, M., Ngezimana, W., & Mudau, F. N. (2016). Effects of foliar spray application of selected micronutrients on the quality of bush tea. HortScience, 51(7), 873–879. <https://doi.org/10.21273/HORTSCI.51.7.873>
13. Piper, C. S. (1966). Soil and Plant Analysis: A laboratory manual of methods for the examination of soils and the determination of the inorganic constituents of plants. Hans Publishers, Bombay, India, 368 pp.
14. Price, M. L., Van Scoyoc, S., & Butler, L. G. (1978). A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. Journal of Agricultural and Food Chemistry, 26(5), 1214–1218. <https://doi.org/10.1021/jf60219a031>
15. Vojodi Mehrabani, L., Valizadeh Kamran, R., Hassanpouraghdam, M. B., & Pessarakli, M. (2017). Zinc sulfate foliar application effects on some physiological characteristics and phenolic and essential oil contents of *Lavandula stoechas* L.

- under sodium chloride (NaCl) salinity conditions. *Communications in Soil Science and Plant Analysis*, 48(16), 1860–1867. <https://doi.org/10.1080/00103624.2017.1406105>
16. Ravishankar, M., Pan, R. S., Kaur, D. P., Giri, R. R., Anil Kumar, V., Rathore, A., Easdown, W., & Nair, R. M. (2016). Vegetable soybean: A crop with immense potential to improve human nutrition and diversify cropping systems in Eastern India—a review. *Soybean Research*, 14(2), 1–13. <https://ssrd.co.in/wp-content/uploads/2021/01/Soybean-Research-142-2016.pdf>
 17. Sadasivam, S., & Manickam, A. (1996). *Biochemical Methods* (2nd ed.). New Age International Publishers, New Delhi, India
 18. Shanmugasundaram, S., Nair, R. M., Yan, M.-R., & Palada, M. C. (2015). Vegetable soybean (Edamame). In K. V. Peter and P. Singh (Eds.), *Handbook of Vegetables*, 3, pp. 521–555. Studium Press LLC, Houston, Texas, USA.
 19. Shi, P., Song, C., Chen, H., Duan, B., Zhang, Z., & Meng, J. (2018). Foliar applications of iron promote flavonoids accumulation in grape berry of *Vitis vinifera* cv. Merlot grown in the iron deficiency soil. *Food Chemistry*, 253, 164–170. <https://doi.org/10.1016/j.foodchem.2018.01.109>
 20. Song, C. Z., Liu, M. Y., Meng, J. F., Chi, M., Xi, Z. M., & Zhang, Z. W. (2015). Promoting effect of foliage sprayed zinc sulfate on accumulation of sugar and phenolics in berries of *Vitis vinifera* cv. Merlot growing on zinc deficient soil. *Molecules*, 20(2), 2536–2554. <https://doi.org/10.3390/molecules20022536>
 21. Souza, F. H. D. de, & Marcos-Filho, J. (2001). The seed coat as a modulator of seed-environment relationships in Fabaceae. *Brazilian Journal of Botany*, 24(4), 365–375. <https://doi.org/10.1590/S0100-84042001000400002>
 22. Wada, S., Kennedy, J. A., & Reed, B. M. (2011). Seed-coat anatomy and proanthocyanidins contribute to the dormancy of *Rubus* seed. *Scientia Horticulturae*, 130(4), 762–768. <https://doi.org/10.1016/j.scienta.2011.08.034>
 23. Wang, Z. Q., Senga, E. F. B., & Wang, D. Y. (2005). Vegetable soybean (*Glycine max* (L.) Merrill) from production to processing. *Outlook on Agriculture*, 34(3), 167–172. <https://doi.org/10.5367/000000005774378766>
 24. Wolf, W. J., & Baker, F. L. (1972). Scanning electron microscopy of soybeans. *Cereal Science Today*, 17, 124–130
 25. Wolf, W. J., Baker, F. L., & Bernard, R. L. (1981). Soybean seed-coat structural features: Pits, deposits and cracks. *Scanning Electron Microscopy*, 1981(III), 531–544.
 26. He, Y., Luo, Y., Wang, Q., Sun, Y., Duan, N., Chen, Z., & Zeng, H. (2021). Spray treatment of leaves with Fe²⁺ promotes procyanidin biosynthesis by upregulating the expression of the F3H and ANS genes in red rice grains (*Oryza sativa* L.). *Journal of Cereal Science*, 100, Article 103231. <https://doi.org/10.1016/j.jcs.2021.103231>
 27. Zhang, Y., Zhou, X., Tao, W., Li, L., Wei, C., Duan, J., Chen, S., & Ye, X. (2016). Antioxidant and antiproliferative activities of proanthocyanidins from Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) leaves. *Journal of Functional Foods*, 27, 645–654. <https://doi.org/10.1016/j.jff.2016.10.004>