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RESEARCH ARTICLE

Impact of seed hardness on quality in variety VBN- 8 of black gram (*Vigna mungo* L. Hepper) during maturation

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Abstract

The present study was performed to record the formation of hard seeds during the development and maturation of Blackgram variety VBN-8 under field conditions, which were then assessed and evaluated under laboratory conditions. It also focused on analyzing the biochemical properties, seed quality parameters and structural characteristics using Scanning Electron Microscopy (SEM). The findings could help develop strategies to improve germination rates and overcome seed dormancy. The present study on complex seed formation in black gram variety VBN-8 during the development and maturation stage was carried out using seed samples collected from standing crops 3 to 38 Days after Anthesis. The formation of hard seeds was assessed and evaluated from the seed sample taken from the different stages of development and maturation (3 to 38 DAA) of the black gram variety VBN-8. The physical and physiological parameters were studied as per the ISTA rules. The biochemical properties were estimated using the fractionation method and histological studies were conducted using SEM analysis. The present investigation revealed that a higher percentage of complex seed formation was observed at 33 DAA when the seed attained physiological maturity. The biochemical analyses showed that hard seeds have much higher quantities of pectin (4.68%), cellulose (4.27%), hemicellulose (2.55%), lignin (2.30%) and phenolic contents (5.78%) as compared to non-hard seeds of lower quantities of pectin (2.01%), cellulose (1.27%), hemicellulose (1.87%), lignin (0.54%) and phenolic contents (3.68%). The SEM analysis revealed that hard seeds have a thick cuticle (60.96 µm), a waxy layer, a compartmentalized pattern, amorphous deposits and a rough texture, all hindering water uptake.

In contrast, non-hard seeds exhibit a smoother surface with fewer deposits and thinner cuticles (33.81 µm), enhancing their permeability. Additionally, in hard seeds, water entry points like the micropyle and hilum are often obscured or blocked, making water absorption difficult. Conversely, non-hard seeds feature a more visible hilum and an open micropyle, readily allowing water to enter. Understanding these changes in seed coat qualities can help agricultural experts regulate dormancy, provide suitable germination conditions and plan for sowing seed storage and crop management techniques.

Keywords

black gram; biochemical component; hard seed; non-hard seed; SEM analysis

Introduction

Black gram (*Vigna mungo* L. Hepper), often known as urdbean, is India's third most important pulse crop. It is native to India and belongs to the Leguminosae family, with a chromosomal number of 2n=2x=22 (1). Black gram, also known as dal, can be used as a green manure crop to fix atmospheric nitrogen and restore soil fertility (2). It is the most preferred pulse and known as the king of Pulses (3), and black gram is a highly-priced pulse with a high phosphorus content. India is the world's largest producer of black gram, contributing over 70% of global production. It is also the leading producer, consumer and importer of pulses worldwide (4). During the 2022-23, India produced 26.05 million tonnes of pulses (5). Chickpeas, pigeon peas and lentils are more commonly consumed in Northern India, while Southern states prefer black gram (6). The per capita net availability of pulses has increased recently, from 15.5 kg per year in 2018-19 to 19.6 kg in 2021-22 (7). The most significant black gram (*Kharif*) growing states are Tamil Nadu (2.74 lakh ha), Andhra Pradesh (2.55 lakh ha), Odisha (2.00 lakh ha), Telangana (0.178 lakh ha), Chhattisgarh (0.14 lakh ha) and West Bengal (0.18 lakh ha). It is highly nutritious with an excellent combination of 25-26 per cent protein, 60 per cent carbohydrates and 1.5 per cent fat. As per the available studies, there is no existing literature/ report on the VBN 8 varietal influence on complex seed development and its impact on resultant seed quality. This study was conducted based on the problem faced by farmers in Tamil Nadu.

Hard seed is the leading cause of dormancy in most species of Leguminosae (8). However, physical dormancy is challenging in agricultural practice as it hinders timely and uniform seed germination (9). The phenomenon of dormancy refers to the inability of mature seeds to germinate even under favourable environmental conditions (10) quickly. Hard seediness poses significant challenges, hindering the use of wild germplasm for cultivar improvement and negatively impacting germination rates, seed viability and the quality of soybeans, as reported by (11). In black gram, hard seeds are primarily due to the impermeability of the seed testa. Notably, black gram (MBG 1050) has a higher percentage (43%) of hard seeds (physical dormancy) than many other pulse crops (12). Similarly, a hard seed coat in legumes such as *Teramnus labialis* has been associated with poor water permeability (13). The regulation of dormancy and the initiation of germination are crucial for allowing plants to survive in unfavourable environmental conditions (14).

Several factors, such as genetic features, environmental conditions during seed development and maturity and post-harvest handling procedures, influence the formation of hard seeds in black gram. Often referred to as the parental or maternal environment effect, the environmental conditions plants undergo during seed maturation influence the degree of dormancy and timing of germination, as recorded by Klupczyńska and Pawłowski (15). (16) emphasizes the importance of environmental variables such as temperature, moisture and soil conditions in determining the level of hard seediness in leguminous crops. Hard seededness may promote planting by maintaining seed material for years (17). Seed maturation is an essential stage in a plant's lifecycle because it allows for the spatial and temporal distribution of offspring. Storage reserve deposition, desiccation, dormancy induction, seed coat construction and protective chemical production occur during maturity (18). After seed maturation, the hilum is considered a hygroscopically activated valve within the impermeable epidermis of the seed coat, playing a crucial role in seed dormancy, as reported (11).

This present study assessed and evaluated the occurrence of hard seeds in the black gram variety VBN-8 during seed development and maturation. Despite the lack of existing literature on this varietal study, VBN-8 was chosen based on farmers' reports of issues with hard seeds. Physical dormancy poses a significant challenge to farmers and seed dealers by disrupting uniform germination and maturity rates. This understanding will help develop appropriate management practices to improve germination and enhance crop productivity in black gram cultivation. Additionally, the physical, physiological, biochemical and histological properties associated with hard seeds were thoroughly investigated to gain deeper insights into the nature of hard seeds.

Materials and Methods

The chemicals and reagents used for the estimation of phenolic content and fractionation methods are listed below

S.No	Chemical and reagents	Company
1	Sodium dodecyl sulfate	
2	Ethanol	
3	Ammonium oxalate	
4	Potassium hydroxide	Sri Sastha Chemicals
5	Folin-Ciocalteu reagent	
6	Sodium carbonate	
7	Gallic acid	

Seed collection and preparation

Fresh breeder seeds of the variety VBN-8 were obtained from the National Pulses Research Centre (NPRC), and the field was established during *Kharif* 2023 at NPRC, Vamban, Pudukkottai, Tamil Nadu (19). Following the recommended practice package, seeds for raising a bulk crop were sown under field conditions at NPRC, Vamban. The experimental plot size used was 4x3 m, with a spacing of 30x10 cm. Plants of uniform height were randomly selected and 2000 individual flowers were tagged at the time of anthesis. Individual flowers were tagged using jewel tags and labelled with information such as numbering, date of flowering and season. 200 to 250 individual pod samples were collected from 3rd day after anthesis (DAA) until seeds reached physiological maturity at five-day intervals. The pods were grouped into eight stages of development, viz., 3 DAA, 8 DAA, 13 DAA, 18 DAA, 23 DAA, 28 DAA, 33 DAA and 38 DAA, respectively (Fig. 1).

Fig 1. Pod and seed characteristics during different development stages in Blackgram VBN 8 (3 DAA to 38 DAA stages)

Weather parameters

Weather data of the experimental farm for rainfall, maximum temperature, minimum temperature and Relative humidity during the entire cropping period (July 2023-Oct 2023) were collected from Metrological observatory at Krishi Vigyan Kendra, Vamban, Pudukkottai, Tamil Nadu (Table 2).

Soil nutrient analysis

Soil samples were collected from the experimental field before sowing using the auger hole method (20). The field was divided into homogenous plots based on visual observation and experience. Surface litter was removed from the sampling spot and an auger or a spade was used to take soil samples from a depth of 15 cm. A total of 10 to 15 samples were collected from the plot mix thoroughly and foreign materials were used. Reduce the bulk to about half to one kilogram by either quartering or compartmentalization, ensuring a uniform and representative sample. The final sample was kept in a clean cloth or polythene bag and labelled with the detailed farm location, survey number, previous and present crops, collection date and sampler name. The soil sample was tested at the Department of Soil Science laboratory at Agricultural College and Research Institute (AC & RI), Tamil Nadu Agricultural University (TNAU), Madurai, Tamil Nadu. The soil sample analysis methods include soil texture analysis (feel method) (21), estimation of calcareous content (acid titration method) (22), measurement of pH (potentiometry) (23) and assessment of EC (conductometry) (24). Available nitrogen is determined using the alkaline permanganate method (25), while available phosphorus is analyzed using colourimetry (26). Available potassium is measured using the Olsen method (27), and organic carbon content is estimated using the Walkley-Black method (28). For micronutrient analysis, available iron, manganese, zinc and copper are assessed using the DTPA method (29) (Table 1).

Table 1. Soil samples analysis on the experimental field during *Kharif* season 2023

Parameter	Unit	Value	Interpretation
Texture (Feel method)	-	-	Sandy clay loam
Calcaereous	-	-	Nil
pH	-	6.55	Slightly acid
Ec	dS m^{-1}	0.10	Normal
Available Nitrogen	Kg ha^{-1}	112	Low
Available phosphorous	Kg ha^{-1}	59	High
Available potassium	Kg ha^{-1}	217	Medium
Organic carbon	g Kg^{-1}	4.50	Low
Available iron	ppm	11.00	Sufficient
Available manganese	ppm	5.56	Sufficient
Available zinc	ppm	0.75	Deficient
Available copper	ppm	0.67	Deficient

Physiological Parameters

The seeds were collected from the different stages and investigated for the following quality parameters viz., seed moisture content (%), germination (%), hard seed (%), abnormal seedling (%), Dead seed (%), root length (cm), shoot length (cm), dry matter production (g/10 seedlings). The vigour index was also computed using the above parameters.

Seed moisture content

The procedure estimated seed moisture content per the ISTA rules (30). Five grams of seed were grounded and transferred to a pre-weighed moisture bottle. The moisture bottle and seed sample were placed in a hot air oven at 130 ± 2°C for one hour. After drying, the moisture bottle was placed in a desiccator for 30 minutes to cool down. The moisture content was calculated using the following formula and expressed in percentages.

$$\text{Moisture content (\%)} = \frac{M_2 - M_3}{M_2 - M_1} \times 100$$

Where,

M1 = weight of the bottle (g)

M2 = weight of the bottle with sample before drying (g)

M3 = weight of the bottle with sample after drying (g)

Table 2. Weather conditions during the Black gram experiment (*Kharif* season 2023).

Day after anthesis (DAA)	Rainfall (mm)	Maximum temperature (Max. T)	Minimum Temperature (Min. T)	Humidity (RH)
3 DAA	0.00	31.00	23.00	81.00
8 DAA	14.50	34.00	24.00	91.00
13 DAA	65.00	33.00	24.00	80.00
18 DAA	19.00	27.00	21.00	70.00
23 DAA	0.00	31.00	21.00	80.00
28 DAA	19.00	35.00	21.00	85.00
33 DAA	17.50	35.40	20.00	73.00
38 DAA	44.50	36.50	22.50	70.00

Germination

The standard germination test was conducted per the ISTA rules (30). Four replicates of a hundred seeds each were kept for germination in the roll towel method at 25 ±2°C and relative humidity of 95 ±3% in a germination room for seven days. After the period, the seedlings were evaluated and a number of normal seedlings were taken for the germination calculation, which was worked out in percentage. The germination percentage, including germinated and hard seeds, is considered germination percentage (including hard seed %). Dead seeds (%) are typically calculated at the final count during the germination test period.

Hard seed

Hard seeds were counted during the final count of the germination period. The number of seeds that did not absorb water and remained hard after a specific period of germination and the total number of seeds subjected to the germination period. The formula was used to calculate the percentage of hard seeds (30).

$$\text{Hard seed (\%)} = \frac{\text{Number of hard seed}}{\text{Total number of seeds sown}} \times 100$$

According to ISTA rules, hard seeds are included in the germination percentage, which is reported as the germination percentage (including hard seed %). Hard seeds are counted because they have the potential to germinate under favourable conditions. The rate of dead seeds is determined during the final count of the germination test.

Abnormal seedlings

The formula for calculating the percentage of abnormal seedlings is provided below.

$$\text{Abnormal seedlings (\%)} = \frac{\text{Number of abnormal seedling}}{\text{Total number of seeds sown}} \times 100$$

Root length

The root length was measured centimetres from the collar region to the tip of the longest root from randomly selected ten normal seedlings (30).

Shoot length

The distance from the collar region to the shoot apex of randomly selected ten normal seedlings was measured in centimetres (30).

Dry matter production

The ten normal seedlings used for root and shoot length were taken after removing the cotyledon and seed coat and kept for drying under shade for 24 h, followed by hot air oven drying at 85 ± 2°C for 24 h. The dry weight of the seedlings was weighed in an electronic balance after cooling in a desiccator for 30 min and expressed in g 10 seedlings-1 (30).

Vigour index

The vigour index was calculated with germination and total seedling length using the following formula (30) and expressed in whole numbers.

Vigour index = Germination (%) x Total seedling length (cm)

Biochemical parameters

Biochemical components viz., pectin, hemicellulose, cellulose, lignin and phenols were estimated from the seed coat of freshly harvested seeds at the physiological maturity (33 DAA). Freshly harvested seeds with a moisture content of 12.47 % were grouped into three parts: hard, non-hard and bulk. Mature seeds were soaked in water for 1 hour for imbibition to separate the hard seeds and non-hard seeds. After one hour of imbibition, firm seeds were divided into hard seeds. After the separation of hard seeds, the remaining imbibed seeds were dried under shade followed by sunlight to their original moisture content (12.37%) and considered non-hard seeds. Bulk seed was taken without separating hard seed and non-hard seed, i.e. without the imbibition process.

A study (31) detailed a method for separating the seed coat from the cotyledons. The seed coats from hard, non-hard and bulk seeds were labelled as T_1, T_2 and T_3, respectively. To facilitate the separation process, the black gram seeds were first plunged into liquid nitrogen until boiling subsided, then drained and dried overnight in a hot air oven (Model no: HTLP-013, Company: Hi-Tech Lab Products and headquartered in Mumbai) at 60°C. The seeds were placed between sheets of paper and broken into small pieces using a hammer. The seed coat was manually separated from the cotyledons and stored in screw-capped vials at 4°C.

Fig. 2 outlined the protocol for estimating pectin, hemicellulose, lignin and cellulose using the fractionation method (31). This process was used to fractionate finely crushed seed coat material. The weight of the materials collected from each fraction was documented. Using the fractionation method, pectin content was calculated as the sum of fractions 1 and 2, while hemicellulose content was measured as the sum of fractions 3a, 3b, 3c, 4a and 4b. Cellulose was computed as the difference between glucose measured by 12 M and 1 M acid hydrolysis of fragment 5. The residue from fraction 5 - 12 M sulfuric acid hydrolysis was washed three times with distilled water before being dried and weighed to estimate the Klason lignin concentration (32). The phenolic content was calculated using the Folin-Ciocalteu method (33). Weigh 0.5 grams of seed coat, then dilute it with water to approximately 7 ml in a 10-ml test tube. Add 0.50 ml of Folin-Denis reagent to the diluted sample to react with the phenolic compounds, forming a blue-coloured complex. Shake the mixture thoroughly to ensure proper mixing and allow it to respond for 3 minutes. Then, 1.0 ml of saturated sodium carbonate solution was added to neutralize the reaction, stabilizing the colour of the complex. Dilute the mixture to a final volume of 10 ml with additional water. Allow the solution to develop for 1 hour, then measure the absorbance at 725 nm using a spectrophotometer (Model no: Double Beam UV-VIS Spectrophotometer 2205, Company: SYSTRONICS and headquartered in Ahmedabad) to obtain a quantitative indication of the total phenol concentration in the sample.

$$\text{Phenolic Content (mg GAE/g sample (\%)} = \frac{\text{Absorbance x Total Volume (ml)}}{\text{Molar absorptivity of Gallic Acid x Weigh of Sample (g)}}$$

Scanning Electron Microscope (SEM)

Histological analysis of seed coat of hard and non-hard seeds was done using a Scanning Electron Microscope (Model no: Quanta 250, Company: FEI, Czech Republic and headquartered in Oregon) at the Department of Nanotechnology, Tamil Nadu Agricultural University, Coimbatore. Dry seeds were mounted on stubs and placed directly into a TUSCAN WEGA 3 software. Scanning Electron Microscope at 10 kv, with magnification ranging from 34 X to 1.27 kX for viewing and photography (34).

Statistical analysis

The variance analysis for all characters was conducted by (35). Before actual statistical analysis, the percentage values of germination were converted to angular (arcsine) values when appropriate. The critical difference (CD) was calculated at a 5 per cent probability level.

Results

Hard seed is a physical dormancy phenomenon in which seeds do not germinate immediately after harvest. Thus, the presence of hard seeds causes difficulties in cultivation and during food consumption. A study (36) highlights those hard seeds with their water-impermeable pose significant challenges in both cultivation and consumption. These hard -to-cook legumes (HTCL) complicate agricultural practices and food preparation by affecting seed germination and cooking efficiency. Variations in seed physical properties and chemical compositions contribute to these cultivation difficulties, while the hard texture of the seeds hinders their softening during cooking. Better knowledge of the formation of hard seeds and their development in the mother plants is essential to develop suitable strategies to overcome hard seedness. Seed maturation is a main factor in seed quality and a prerequisite for successful germination and emergence (37). The results of the present investigation are discussed here under.

Seed Quality parameters during seed development and maturation at different stages

The physical parameters like seed moisture content and physiological parameters like germination per cent, root length (cm), shoot length (cm), dry matter production and vigour index during seed development and maturation at different stages were evaluated. Germination per cent was statistically influenced by various stages of seed development. At 3 DAA, the seed moisture content was high at 46.20 %; at this early stage, no seed had germinated (0.00%). No abnormal seedlings (0.00%) were observed and no root or shoot growth was observed. Consequently, dry matter production was non-existent (0.00 g/10 seedlings), and the vigour index was 0.00, reflecting the complete lack of seedling development at this stage. In the initial stage, known as histo-differentiation or embryogenesis (38), seeds could not germinate. By 8 DAA, the seed moisture content had increased to 57.48 %. Germination had begun, with 20.00 % of seeds sprouting. However, 2.67 % of the seedlings were abnormal, and 72.00 % of the seeds remained dead seed. The root length was measured at 9.89 cm, while the shoot length reached 13.23 cm. Despite the early development, dry matter production was minimal at 0.008 g/10 seedlings. The vigour index was 575, indicating initial but still limited seedling vigour. During the early stages of development after fertilization, seed moisture content increases and then begins to fall until it reaches equilibrium with the environment (37).

At 13 DAA, seed moisture content increased significantly to 53.89 %. Germination improved slightly to 25.00 %, but a high percentage of abnormal seedlings (28.00%) was observed. The rate of dead seed seeds decreased to 25.33 per cent. Root and shoot growth continued, with roots reaching an average length of 14.19 cm and shoots of 16.03 cm. Dry matter production reached 0.042 g/10 seedlings, while the vigour index increased to 610, indicating moderate seedling development. At 18 DAA, seed moisture content decreased to 43.52 per cent. The germination percentage increased significantly to 60.00 per cent, although 14.67 per cent of the seedlings were abnormal. The rate of dead seed seeds dropped to 38.67 per cent. Root length averaged 14.78 cm and shoot length reached 16.94 cm. Dry matter production further increased to 0.078 g/10 seedlings. The vigour index significantly improved, reaching 1416, indicating stronger seedling vigour. (39) indicated that shoot and root lengths are the vigour indicators, reflecting the seed's performance under various environmental conditions.

Seed moisture content significantly decreased from 28.12% at 28 DAA to 20.54% at 33 DAA, reaching its lowest value of 16.01% at 38 DAA. This reduction in moisture content is critical, as it indicates physiological maturity when seeds have achieved their maximum dry weight and nutrient storage, making them ready for harvest. Now, seeds are fully developed and capable of germinating under suitable conditions. As seeds mature, the decline in moisture content is essential for preventing physiological disorders, fungal infections and seed deterioration associated with high moisture levels. Fungi that invade seeds before or after harvest can significantly reduce seed quality and longevity (40). Seeds that reach physiological maturity with optimal moisture content are generally more stable during storage. Excess moisture can lead to mould growth and other issues that compromise seed viability, while properly matured seeds can be stored for extended periods without significant loss in quality. During physiological maturity, seeds draw on the plant's reserves to store carbohydrates, proteins and lipids, which are crucial for germination and early seedling growth. Similar studies reported the transfer of compounds from the mother plant to seeds, including carbohydrates,

proteins and lipids, which are significant reserves necessary for seed development and subsequent germination (41). Additionally, adequate moisture content is required to accumulate nutrients and metabolites effectively. Monitoring seed moisture content is vital for determining the optimal harvest time, as harvesting too early, when moisture levels are high, can result in poor seed quality, while harvesting too late increases the risk of seed loss due to shattering or degradation.

Germination percentage improved significantly from 81.00% at 28 DAA to 99.00% at 33 DAA, slightly declining to 97.00% at 38 DAA. A high germination percentage, such as the 99.00% observed at 33 DAA, indicates that most seeds are viable and capable of developing into healthy seedlings. This reflects adequate seed maturation and is crucial for successful plant establishment. High germination percentages are a vital indicator of seed quality and potential crop performance, ensuring effective resource use, improved yield potential and robust seedling development (42). A decrease in abnormal seedlings (from 9.33% at 28 DAA to 1.33% at 33 DAA) and high germination rates suggest that seedlings usually develop effectively.

Root length increased steadily, reaching 20.31 cm at 38 DAA, while shoot length peaked at 21.93 cm at the same stage. Dry matter production (DMP) increased from 0.178 g/10 seedlings at 28 DAA to a maximum of 0.371 g/10 seedlings at 33 DAA before slightly decreasing to 0.318 g/10 seedlings at 38 DAA. Physiological maturity is when seeds reach their maximum dry weight and associated physical characteristics (43). After this stage, a slight decrease in dry matter production is attributed to internal mechanisms that cause the disorganization of cell organelles following physiological maturity (44). Physiological maturity represents the point at which seeds reach their maximum dry weight (45) and (46).

The vigour index peaked at 4108 at 38 DAA, reflecting optimal seedling vigour and development at this stage. The significant increase in the vigour index to 2910 at 28 DAA, followed by a peak of 4017 at 33 DAA, indicates robust seedling development and overall high vigour. Seed vigour is a crucial aspect of seed quality, with the ISTA Vigour Committee promoting research on this trait across a broad range of species. Within seed laboratories and the seed industry, it is essential to develop validated vigour tests that are straightforward, practical and reliably linked to field performance or seed longevity (47).

3.2 Hard seed formation at the stage from 33 DAA and 38 DAA

The data obtained in the present study revealed that the hard seed was not developed until 28 DAA. Hard seed was formed at 33 DAA (16.00%). The maximum rate of hard seeds (17.67%) was observed at 38 DAA (Fig. 2). The hard seeds were identified during the germination test. The seeds that did not imbibe water at the end of the germination period were considered hard seeds (Fig. 3). Hard seeds have an impermeable seed coat, preventing water absorption by the imbibition process during germination. Experiments showed that hard seed (dormant seed) is highly related to

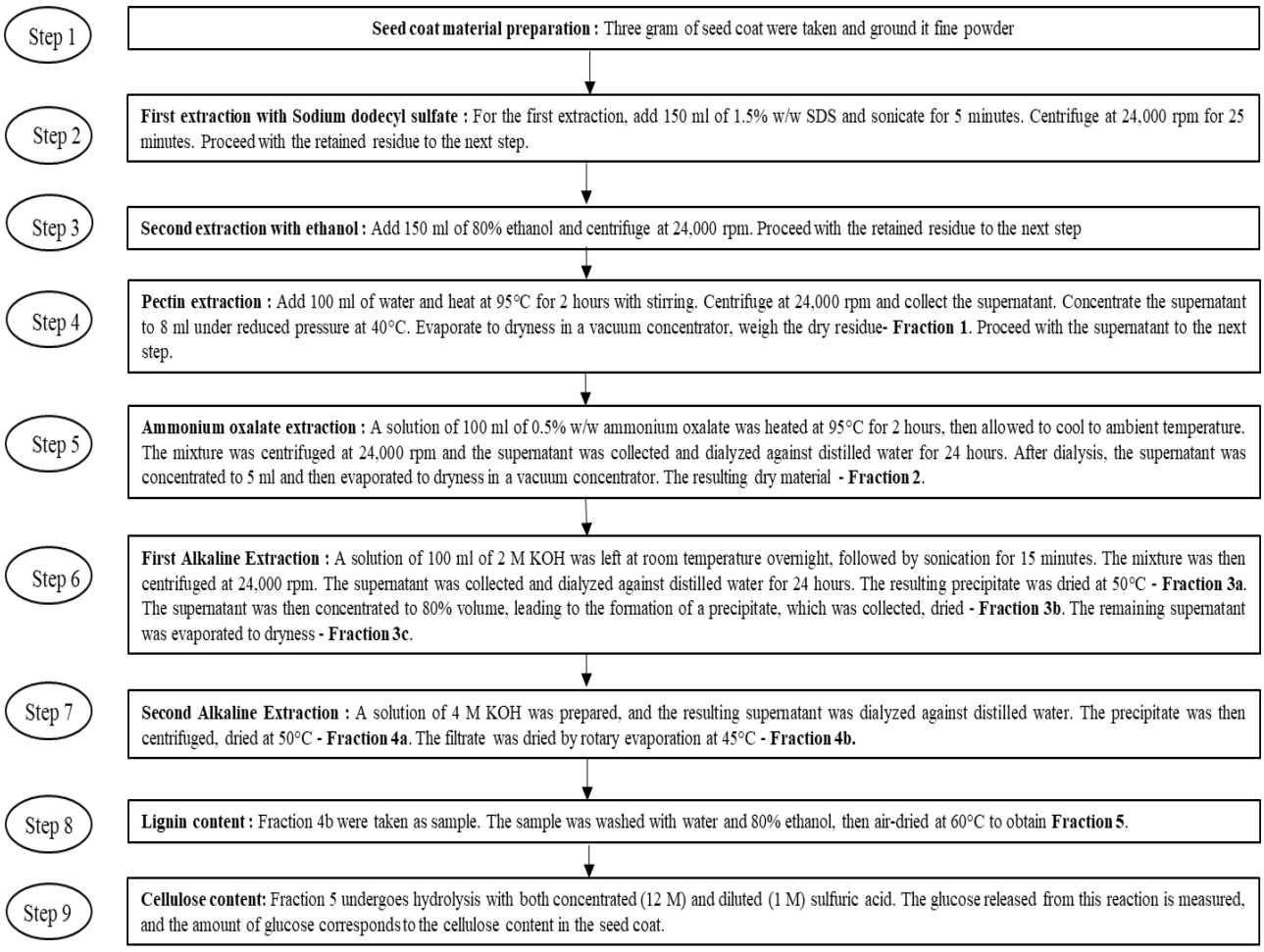

Fig 2. Protocol for the Determination of Pectin, Cellulose, Hemicellulose and Lignin

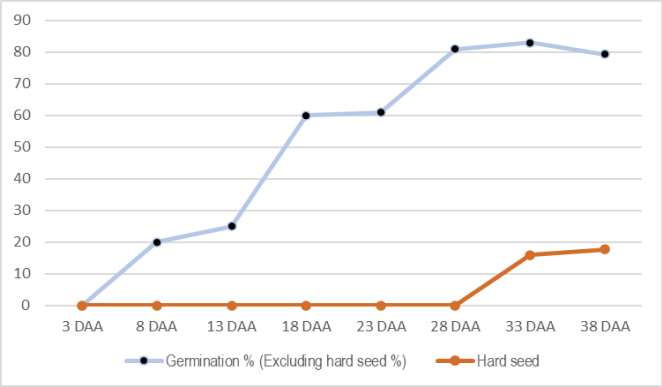

Fig 3. Induction of hard seed and germination during seed development and maturation in black gram

the dehydration process, which occurs at maturity. Hard seeds do not readily absorb water when soaked, even for an extended period, restricting germination. Hard seeds have no pores in the epidermis layer and an impermeable seed coat is made of suberin layers. These characteristics are linked to environmental factors, seed size and heredity (48). The hard seed advantage for seed plants is that they can remain in the soil, expand germination across time and establish plant life cycles. Hard seeds tend to reduce consumer acceptance of food products, presenting a significant issue for legume crops. Seed dormancy is a physiological adaptation to environmental diversity and a key factor influencing the dynamics of natural populations (38).

In many seeds, dormancy is caused by a hard seed coat, a phenomenon identified long ago. In this study, hard seed formation was observed at 33 DAA and 38 DAA, with a notable increase in the percentage of hard seeds in the *kharif* season. A study (49) observed that seeds grown during the *kharif* season encompassed a more significant percentage of hard seeds than in summer (11.00 to 33.00%). This suggests that hard seededness is affected by both genetics and environment. The germination percentage (excluding hard seeds) remained high at 33 DAA. Still, it showed a slight decrease by 38 DAA, likely due to the increasing proportion of hard seeds that were unable to germinate (Fig. 2). Chinnasamy and Bal (50) reported that in beach pea (*Lathyrus maritimus*), hard seeds were present at the S5 and S6 stages of development, preventing germination due to the formation of a hard seed coat. In grass pea (*Lathyrus sativus*), hard seeds were found at the S6 stage, marking it as the physiological maturity stage. The recorded weather parameters indicated a high temperature of 35.40°C/20°C 33 days after anthesis (DAA). The maximum temperature reached a high of 35.40°C followed by 44.50°C during 38 DAA, while the minimum temperature dropped 20.00°C followed by 22.50°C (38 DAA). Relative humidity was lower at (73%), followed by 70% (38 DAA). Table 2 shows weather parameter analysis 28 days after anthesis indicates high temperatures. The research by (41) found that high day/night temperatures (35/28°C, 31/24°C, or a constant 27 and 24°C) increased seed hardness and reduced seed moisture content at the harvest. As per Table 1, The soil sample analysis shows a low available nitrogen content of 112 kg ha-1 and deficiencies in zinc (0.75 ppm) and copper (0.67 ppm). These nutrient shortages, high temperatures, and low relative humidity may contribute to hard seed formation. A study showed that light quality, temperature fluctuations and nitrate levels significantly influence seed dormancy (38). Addressing these nutrient and environmental issues is essential, as they can lead to complex seed formation. Further research is needed to better understand the effects of nutrient deficiencies and ecological conditions on hard seed formation, especially given the limited existing literature on the influence of weather parameters and soil nutrients.

3.3 Biochemical parameters

The biochemical components like pectin, hemicellulose, cellulose, lignin and phenols were estimated in the seed coat of black gram seeds. In fractionation methods, components are measured in the seed coat of hard seed (T_1), Non-hard seed (T_2) and Bulk seed (T_3). In Table 4, fractions 1 and 2 represent pectin content and 3a, 3b,3c, 4a, and 4b represent hemicellulose.

In this study, hard seed recorded higher fraction 1, 2, 3a, 3b,3c, 4a and 4b, respectively. The sum of fractions 1 (3.12%) and 2 (1.56%) was recorded higher on hard seed coat material (Pectin content). A sum of fractions 3a (1.31%), 3b (0.035%), 3c (0.59%), 4a (0.17%) and 4b (0.45%) recorded higher on hard seed coat material (Hemicellulose

Table 3. Seed quality and growth parameters at different days after anthesis (DAA) in Blackgram during the *Kharif* season 2023

Day after anthesis (DAA)	Seed moisture content (%)	Germination % (Including hard seed %)	Abnormal seedling (%)	Dead seeds (%)	Root length (cm)	Shoot length (cm)	Dry matter production (g/10 seedlings)	Vigour Index
3 DAA	46.20	0.00	0.00	100.00	0.00	0.00	0.00	0.00
8 DAA	57.48	20.00	2.67	72.00	9.89	13.23	0.008	575
13 DAA	53.89	25.00	28.00	48.00	14.19	16.03	0.042	610
18 DAA	43.52	60.00	14.67	38.67	14.78	16.94	0.078	1416
23 DAA	31.88	61.00	10.67	25.33	14.81	18.31	0.145	1936
28 DAA	28.12	81.00	9.33	9.33	17.24	18.51	0.178	2910
33 DAA	20.54	99.00	1.33	0.00	20.26	20.48	0.371	4017
38 DAA	16.01	97.00	3.00	0.00	20.31	21.93	0.318	4108
Mean	34.71	55.37	8.83	36.67	13.94	15.68	0.163	1947
S.Ed	1.0132	1.404	0.2716	0.9832	0.3100	0.4450	0.0036	56.7937
CD (p=0.05)	2.148**	2.977**	0.575**	2.084**	0.657**	0.943**	0.0077**	120.399**

Table 4. Recovery of Fractions as Percent Weight of Starting Material in Blackgram

Treatment	Pectin	Hemicellulose					5	Total
	1	2	3a	3b	3c	4a	4b		
T_1 - Hard seed	3.12	1.56	1.31	0.035	0.59	0.17	0.45	7.08	14.31
T_2 - Non-hard seed	0.59	1.42	0.97	0.026	0.43	0.12	0.33	6.53	10.90
T_3 - Bulk	1.98	1.23	1.21	0.032	0.54	0.15	0.41	5.21	10.30
Mean	1.90	1.40	1.17	0.031	0.52	0.15	0.40	6.27	11.84
S.Ed	0.041	0.019	0.022	0.0006	0.009	0.002	0.002	0.108	0.205
CD (p=0.05)	0.087**	0.041**	0.047**	0.001**	0.019**	0.005**	0.005**	0.230**	0.437**

content) when compared to non-hard and bulk seed coat (Table 4). Comparing the chemical composition of the hard, non-hard, and bulk seed coats offers significant insight into the underlying mechanisms controlling seed impermeability. The results demonstrated that hard seeds have the highest concentration of phenolic content, lignin, pectin and cellulose (Table 5).

Hard seeds (T_1) were found to have higher levels of pectin (4.68%) compared to T_2 (2.01%) and T_3 (3.21%) Table 5. Pectin is known for its role in cell adhesion and wall porosity, which could limit water absorption and penetration. This suggests that pectin is key in making the seed coat impermeable, contributing to seed dormancy. The water-repellent properties of phenols and pectin, as reported by (51) and (52), support the idea that these substances may help form a barrier preventing water from entering the seed, aiding in seed dormancy and preservation. The exact location of impermeability within the seed coat is still debated. A study by (53), (54) and (55) suggests that impermeability likely occurs below the cuticle, particularly in the osteosclereid and palisade cells. However, some evidence indicates that pectin and phenols might also be present in the cuticle. This suggests that deeper structural elements in the seed coat, rather than just the surface features like the cuticle, may be responsible for impermeability.

Hard seeds (T_1) demonstrated significantly higher levels of hemicellulose (2.55%), lignin (2.30%), cellulose (4.27%) and phenolic content (5.78%) compared to T_2 and T_3 (Table 5). These findings support previous studies, such as those by (31) and (38), which highlight the role of hemicelluloses, especially xylans, in contributing to seed hardness by enhancing the water-repellent properties of the seed coat. Lignin, known for strengthening cell wall

Table 5. Macro constituents of Blackgram Seed Coat

Treatment	Pectin	Hemicellulose	cellulose	Lignin	Phenols
T_1-Hard seed	4.68	2.55	4.27	2.30	5.78
T_2-Non-hard seed	2.01	1.87	1.27	0.54	3.65
T_3-Bulk	3.21	2.34	2.80	1.25	4.78
Mean	3.30	2.25	2.78	1.36	4.73
S.Ed	0.061	0.147	0.056	0.032	0.1087
CD (p=0.05)	0.132**	0.314**	0.119	0.069**	0.245**

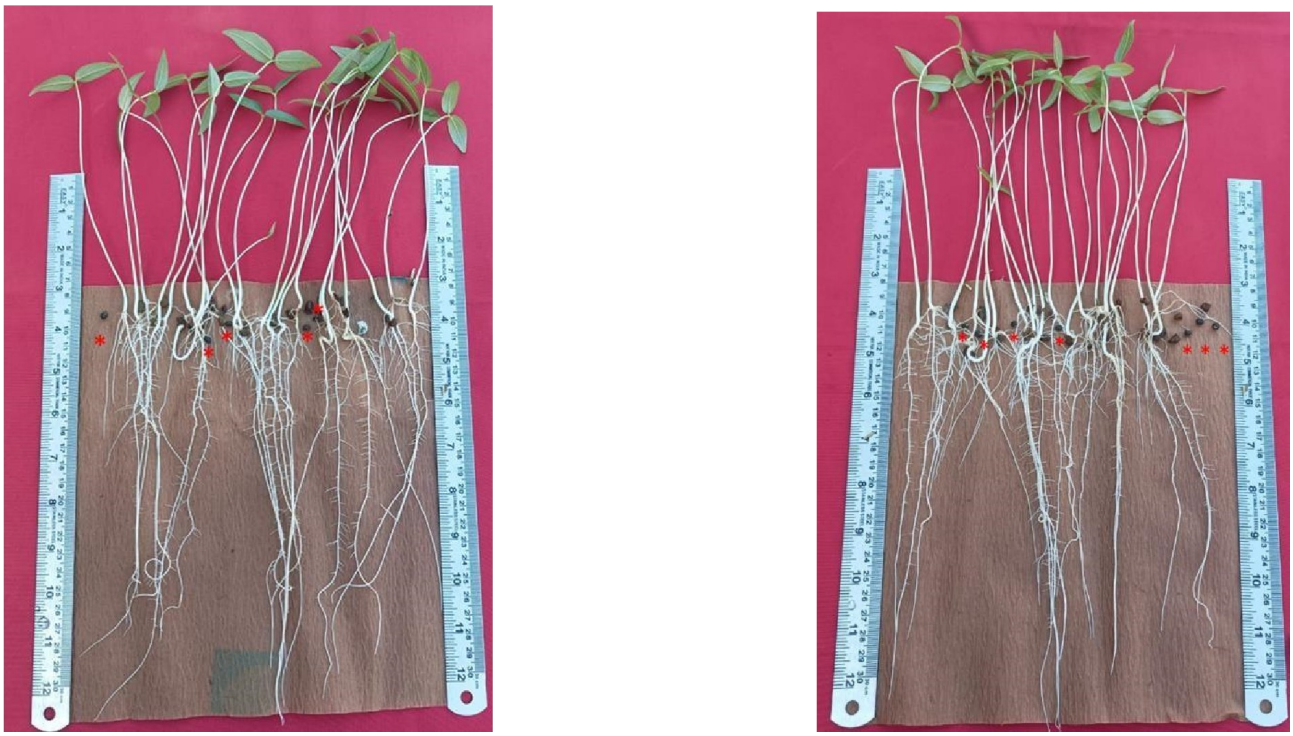

Fig 4. Presence of hard seed during germination test at 33 DAA and 38 DAA

*Indicates the number of hard seeds present during germination using the roll towel method at the stage 33 DAA & 38 DAA stage

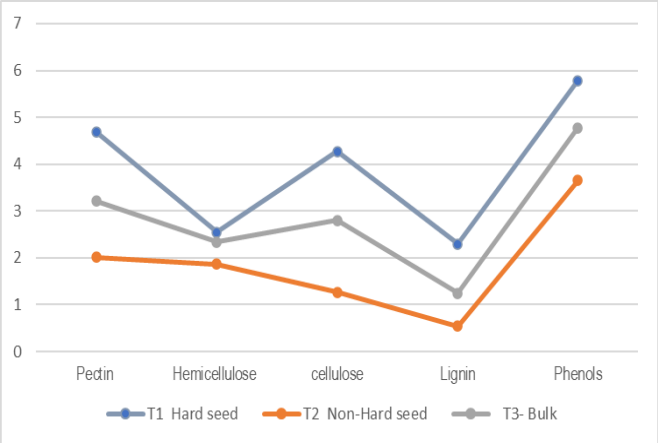

Fig 5. Macro constituents of Blackgram Seed Coat

(Figures represent T_1- Hard seed, T_2- Non-Hard seed and T_3- Bulk)

polysaccharides like cellulose and hemicellulose, was also higher in hard seeds, as observed in studies on impermeable soybean seeds. This increased lignin content fortifies the seed coat structure, making it more impervious to water. The higher cellulose and lignin content in hard seeds further strengthens the seed coat, promoting seed hardness by thickening secondary cell walls. This is consistent with findings by (56) that highlight the role of these compounds in seed hardness.

Moreover, the phenolic content in hard seeds (5.78%) was notably higher than in T_2 and T_3. Studies on *Cajanus cajan* have shown that high phenolic compound concentrations in the seed coat contribute to dormancy and impermeability by forming barriers that restrict water and gas entry. This aligns with our findings, which show that phenolic compounds are critical in hard seedness. As noted by (57), these compounds, particularly in the hilum area, are crucial to inducing dormancy by delaying germination. The role of peroxidase and phenolic compounds in developing seed coat impermeability, as seen in *Prickly sida* (58), further supports this association. These findings indicate that hemicelluloses, lignin, cellulose and phenolic compounds are key contributors to seed coat impermeability and dormancy in hard seeds.

Scanning electron microscope analysis

The SEM investigation performed in this study gave valuable insights into the structural properties of the black gram seed coat, hard seed and non-hard seed. Hard seeds were shown to have a thick cuticle layer recorded (60.96 µm) (Fig. 6d), waxy layer, compartmentalized pattern, amorphous deposits and a rough seed coat texture (Fig. 6b), as opposed to non-hard seeds which had a smoother surface and no surface deposits (Fig. 6a). Sun and Yuan in soybeans, seed composition, texture, and food reserves are also influenced by the seed coat. The outermost layer of the seed coat, a waxy cuticle, serves as a barrier to imbibition (11). Seed coat characteristics, such as size, colour, porosity, chemical composition and morphology, have all been linked to the seed's ability to absorb water (59). Furthermore, the presence of amorphous deposits and a rough seed coat texture in hard seeds (Fig indicates the deposition of lipids, phenolic chemicals and other secondary metabolites that provide impermeability and hardness to the seed coat. These deposits may contribute to the hydrophobic nature of the seed coat surface, thus reducing water uptake and promoting seed dormancy, as reported by (60).

In contrast, non-hard seeds have a smooth surface texture and no surface deposits, indicating a thinner cuticle layer recorded (33.81 µm) (Fig 6c) and less secondary metabolite. The absence of amorphous deposits, as well as the lack of them in the higher portions of the seed coat indicate a lower level of impermeability and mechanical resistance than hard seeds (Fig. 6). A study by (60) found that the presence of significant cuticle ruptures on the seed coat surface correlates with the permeability of wild soybean seed coats. The structure and composition of seed coat cells and the presence of biochemical and

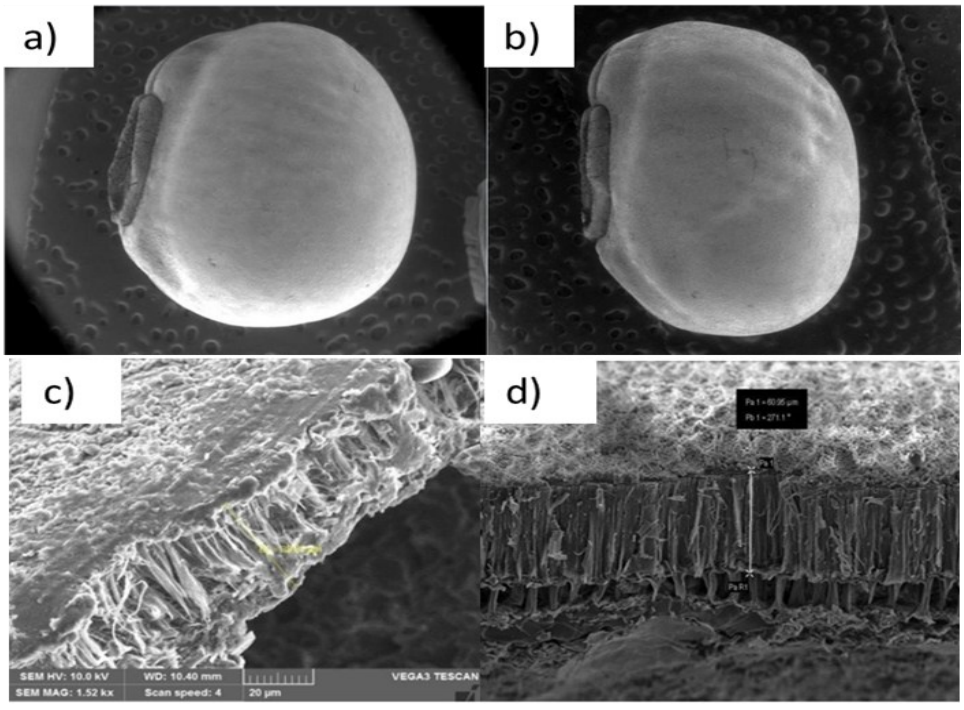

Fig 6. Pictures represent a) Non-hard seed and b) Hard seed and Cross section of the seed coat of Blackgram, c) Non-hard seed and d) Hard seed

impermeable materials are important factors. Their surface morphology, observed through SEM, shows a compact, non-porous structure with few or no visible cracks on hard seeds (Fig 7a). In contrast, non-hard seeds have a permeable seed coat with a more porous surface, allowing water to penetrate quickly and facilitating ready germination under suitable conditions (Fig 7b). The seed coat acts as a barrier between the embryo and its environment, controlling seed permeability and resistance to seed-borne diseases largely dependent on the seed coat and its related tissues (61). These differences significantly impact agricultural practices, as hard seeds can lead to uneven germination and crop establishment (62), while non-hard seeds are preferred for uniform and reliable crop growth.

Conclusion

This study reveals the complex relationship between the stages of seed development and the formation of hard seeds in black gram (*Vigna mungo*). The findings demonstrate significant changes in seed moisture content, germination rates and seedling vigour throughout development, with hard seed formation commencing at 33 DAA and peaking at 38 DAA, associated with reduced seed moisture and increased hard seed percentages. Biochemical analyses indicate that hard seeds have higher concentrations of pectin, hemicellulose, lignin and phenolic compounds, contributing to their impermeability and dormancy. Scanning Electron Microscope (SEM) analysis corroborates these results, revealing thicker cuticle layers and amorphous deposits in hard seeds that enhance their water-repellent properties. These insights highlight the critical role of seed coat composition and environmental factors in managing seed dormancy and optimizing crop cultivation practices.

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Authors' contributions

SP was responsible for conceptualizing the research, gathering relevant literature and preparing the original manuscript draft. MC contributed to the conceptualization, developed the research layout, took part in writing and editing, and provided overall supervision throughout the study. YA provided supervision, offered suggestions and made corrections. MD interpreted the results and contributed to writing the paper. RK provided suggestions, comments and corrections. VC also offered valuable suggestions, comments and corrections.

Compliance with ethical standards

Conflict of interest: Authors declare no conflict of interest

Ethical issues: None

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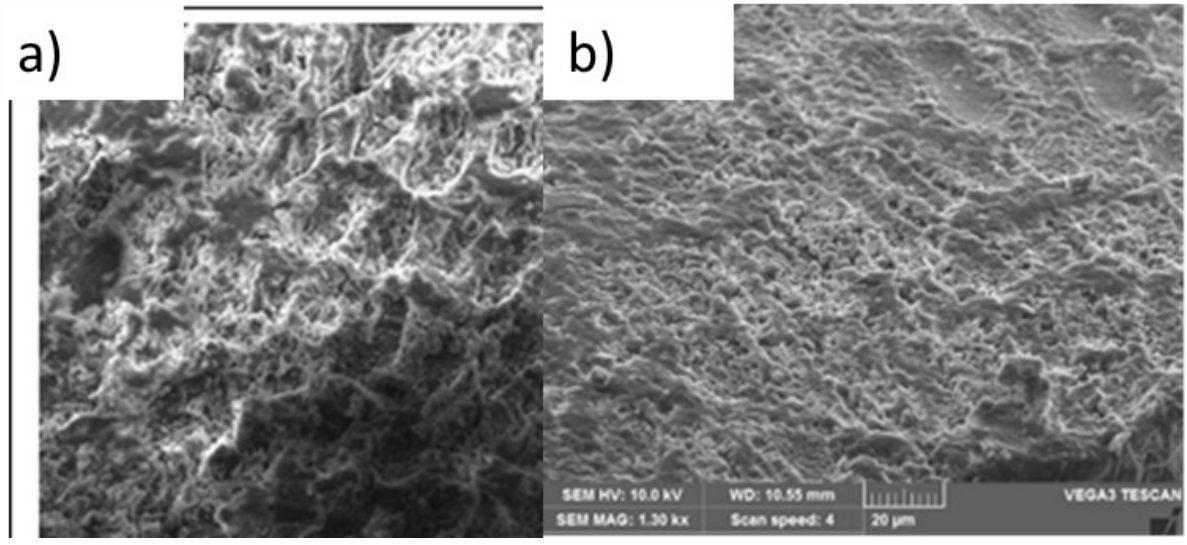

a) Hard seed

b) Non-hard seed

Fig 7. SEM image of seed coat surface of Blackgram a) Hard seeds and b) Non-hard seed

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