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EXPLORATION OF THE METABOLITES AND STRUCTURAL ANATOMY OF SEED COAT DORMANCY IN BLACK GRAM VBN-8 USING GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS) AND SCANNING ELECTRON MICROSCOPY (SEM) ANALYSIS

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ABSTRACT

Hard seed is a physical dormancy due to hard seed coat particularly present in leguminous crop. This type of dormancy is caused by impermeability of seed coat which prevents water imbibition and thus inhibits timely and uniform germination. It is one of the undesirable physiological traits that makes the seed unfit for immediate crop production after harvest. Thus, investigating metabolites and structural anatomy is an integrative approach to understanding the mechanisms that control seed coat dormancy. Seed coat characteristics are critical for seed germination, vigour, and longevity, with hard seed coats often leading to dormancy due to impermeability. Understanding dormancy mechanisms can help crop production become more adaptable to changing climate conditions, ensuring food security and sustained agricultural output in the face of environmental problems. This study was conducted at NPRC, Vamban for VBN 8 raised in field conditions and the Department of Seed Science & Technology, TNAU, Coimbatore for laboratory work to investigate the biochemical metabolites and cuticle and palisade layer structure of black gram seed coats using GC–MS & SEM. Freshly harvested seeds were soaked in water for this study. After the imbibition process, the seeds were categorized into two types: hard and non-hard. GC-MS analysis identified key metabolites contributing to hardseededness, including fatty acids, phenolic compounds, and alcohols. Among the fatty acids, octadecanoic acid was found in higher concentrations (27.48) in hard seeds compared to non-hard seeds. The phenolic compound 3-tert-butyl-4-hydroxyanisole (10.76) and the alcohol hexadecanol (1.29) were also recorded at higher levels in hard seeds compared to non-hard seeds. SEM analysis revealed that hard seeds have a thicker cuticle layer, a denser palisade layer and a rougher seed surface compared to non-hard seeds, contributing to their greater impermeability and dormancy. In contrast, non-hard seeds have a thinner, more permeable seed coat with larger pores, which facilitates faster water absorption and germination. These structural and biochemical characteristics result in the hydrophobicity and dormancy associated with hard seeds. The finding can guide the appropriate breeding program and designing suitable agronomic strategy is aimed at reducing the dormancy under variable environmental conditions to distinguish hard and non-hard seeds in black gram. Subsequently ensures high seed rate replacement and crop productivity in black gram. This innovative approach offers substantial advancements in understanding and improving seed quality parameters.

Keywords: Seed coat-imposed dormancy; Hard seed; Non-hard seed; Key metabolites; Cuticle and palisade layer.

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INTRODUCTION

Pulses are an affordable source of high-quality protein for humans and play a vital role in sustainable agriculture. Black gram is widely cultivated crop in India and can be grown as an intercrop, catch crop, or sole crop due to its short growing season The black gram (*Vigna mungo* L.), is a short-lived crop from the Leguminosae family, highly valued for its high protein content (25–26%). The black gram is not only important for food security but also serves as a cover crop that enhances soil health (Marimuthu *et al*., 2024).

India is both the largest producer and consumer of Black gram (Urdbean) globally. The major Black gram-producing states include Andhra Pradesh, Madhya Pradesh, Rajasthan, Uttar Pradesh, Tamil Nadu and

Maharashtra. Urdbean contributes 11% to India's total pulse production, which was 25.46 million tonnes in 2020-21 (Agricultural Statistics Division, DES, MoAF&W, 2022). Although Urdbean is a warm-season crop with a lower national average productivity compared to cool-season pulses, its adaptability to diverse growing conditions makes it a popular crop.

In 2022, Black gram was cultivated across 4.63 million hectares in India, producing 2.78 million tons, with an average productivity of 987 kg/ha (Project Coordinator Report 2023). However, the Agricultural Statistics Division, DES, MoAF&W (2022) reported a lower national productivity of 600 kg/ha. In Tamil Nadu, 2.75 lakh hectares (6.79 lakh acres) were under Black gram cultivation in 2022-23, accounting for 35.50% of India's total Black gram area. In 2023-24, although the cultivated area in Tamil Nadu slightly decreased to 2.55 lakh hectares (6.30 lakh acres), its share of the national total increased to 36.62%. In terms of trade, India imported 6.12 lakh tons of Urdbean in 2021-22 and exported 55.18 thousand tons. During 2020-21, India exported 15.28 thousand tons of Urdbean, valued at Rs. 147 crores. (Project Coordinator's Report 2023).

In legume crops, testa (Seed coat) is a main factor of seed germination, vigour & longevity. Testa is the seed's outermost protective layer of the seed. Many legumes' seed performance has been linked to specific seed coat characteristics such as the hilum, strophiole, and micropyle (Souza and Marcos-Filho 2001). Legume seeds suffer from this problem, which is caused by an impermeable hard seed coat (Physical dormancy). Physical dormancy in seeds results from the presence of an impermeable seed coat layer (Wang *et al*., 2023; Wen *et al*., 2024). According to Finch-Savage and Leubner-Metzger (2006), Physical dormancy prevents water and gas from penetrating the seed, thereby delaying germination even under optimal environmental conditions. Furthermore, the seed coat protects the embryo from harsh external circumstances while also nourishing and caring for the developing embryo. Seed dormancy is a temporary phenomenon that stops germination even under optimal environmental conditions.

Although much is known about the mechanical properties of the seed coat and their role in physical dormancy, there remains a significant gap in our understanding of the biochemical composition of the seed coat, particularly the metabolites that contribute to dormancy. Studies in other legume species, such as fava bean (*Vicia faba*) and pea (*Pisum sativum*), have found that the seed coats of hard seeds contain significantly higher levels of polyphenols, flavonoids, lignin and lignans, all of which are linked to increased seed coat impermeability (Cavallaro *et al*., 2021, Hradilová *et al*., 2017; Kantar *et al*., 1994). However, similar studies in black gram are limited and the role of metabolites in black gram seed coat-imposed dormancy remains largely unexplored.

Seed coat dormancy is further influenced by the thickness of the cuticle layer and the deposition of hydrophobic compounds such as lipids, tannins and other metabolites. These substances, along with the structural properties of the cuticle, form natural barriers to water and oxygen, thus contributing to physical dormancy. The cuticle layer, composed primarily of hydrocarbons, wax esters, free aldehydes, fatty acids, alcohols and phenolic acid esters, plays a significant role in regulating seed coat permeability (Shao *et al*., 2007). Despite these findings, little attention has been given to the specific metabolites that influence these structural characteristics in black gram and how their presence correlates with dormancy or germination capacity. In terms of seed coat properties, hard seeds exhibited a thicker cuticle layer (6-10 µm) compared to non-dormant seeds (4-6 µm). The most notable differences, however, were found in the inner integument, where not only did size vary, but the structure of the palisade cell walls also showed substantial differences, as described by Orrù *et al*. (2023).

While prior studies have focused on the mechanical aspects of seed dormancy in legumes, the biochemical and structural mechanisms, particularly in black gram, remain underexplored. This study aims to fill that gap by utilizing GC-MS to analyze the seed coat metabolites and the cuticle layer via SEM to study the structural properties of the seed coat in black gram (*Vigna mungo*) VBN 8. By identifying key metabolites and structural features that contribute to seed coat-imposed dormancy, this research offers a novel approach to improving seed germination and vigour in black gram.

MATERIALS AND METHODS

Seeds of blackgram VBN 8 were obtained from NPRC, Vamban. The crop was grown at NPRC, Vamban in *kharif* 2023 via the recommended package of practices (CPG, 2020). Freshly harvested seed were taken as laboratory work. The laboratory work was conducted at Department of seed Science & Technology, Tamil Nadu Agricultural university, Coimbatore.

Separation of hard seeds: Harvested seeds were soaked in water for 1 h to separate hard seeds from non-hard seeds. Take 25 seeds in 25 ml of distilled water. After one hour of imbibition, the seeds that do not absorb moisture this seed are sorted as hard seeds. After the hard seeds were separated, the remaining imbibed seeds were dried in the shade and then exposed to sunshine until they reached their original moisture content, at which point they were termed non-hard seeds used in this experiment.

SEM analysis: A (SEM) was used to conduct a histological study of the seed of both hard and soft seeds. Cut the seeds in cross-section for both hard and non-hard

seeds. Dry seeds were mounted on stubs and directly inserted into a Tescan vega3 & ZEISS SEM at different kV with different magnifications ranging for viewing and photographing (Paul *et al*., 2019).

Gas chromatography–Mass Spectrometry (GCMS): To facilitate the separation process, the blackgram seeds were first plunged into liquid nitrogen until boiling subsided. They were then drained and dried overnight in a hot air oven 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 (Mullin and Xu (2001).

The metabolites of seed coats were profiled using GC-MS. Fifty milligrams of seed coats from hard and non-hard seeds were weighed and ground with a mortar and pestle. The extraction procedure, adapted from Lisec *et al*. (2006), began by weighing 50 mg of seeds, followed by thorough grinding. A cold methanol solution (−20 °C, 400 µL) containing 200 nmol of cis-inositol as an internal standard was added for primary metabolite extraction. The sample was shaken in a thermomixer at 70 °C and 950 rpm for 10 minutes. Then, 200 µL of chloroform was introduced, and the mixture was shaken again at 70 °C for 5 minutes. After adding 400 µL of ultra-pure water, the solution was vortexed for 20 seconds and centrifuged at 74,009 g for 10 minutes. For GC-MS analysis, 50 µL of the methanol/water supernatant was dried using a speed-vac concentrator. This approach ensures an efficient extraction and preparation of metabolites for subsequent GC-MS analysis. The produced sample was run on a GCMS instrument (Agilent GC 7890A/MS5975C, capillary column DB5MS, column length of 30 m, internal diameter of 0.25 mm and film thickness of 0.25 microns). The treatment details are T_1-hard seeds and T_2-non-hard seeds (Fig 1).

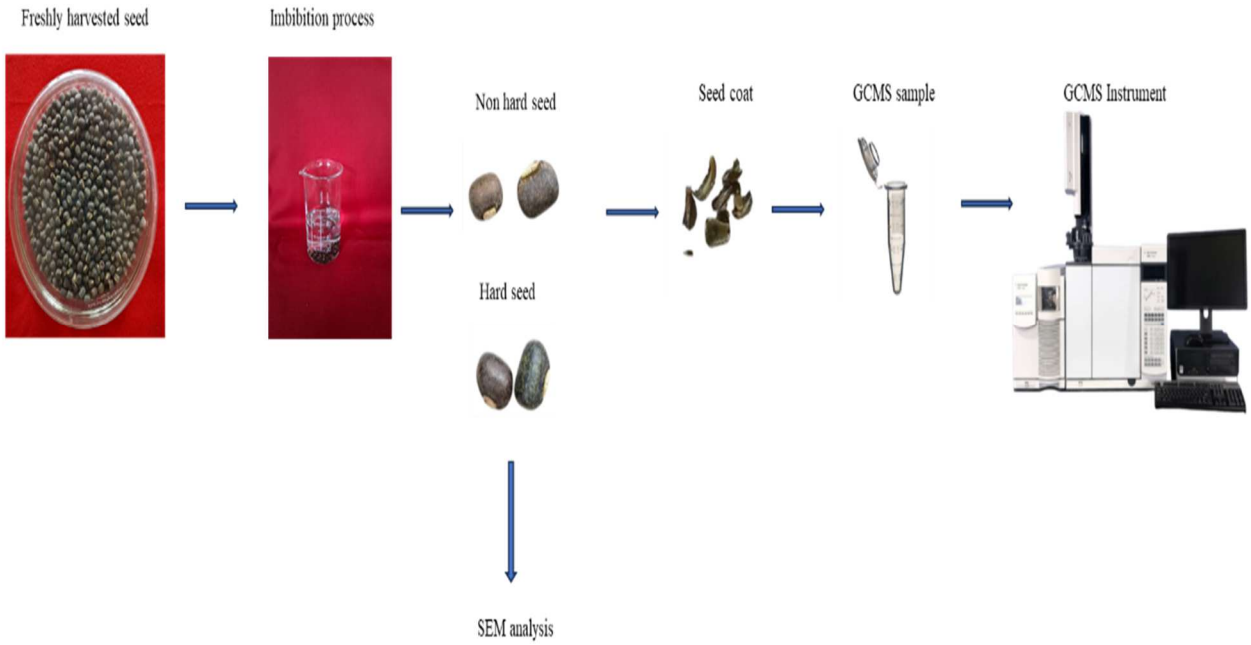

Fig 1. Procedure for sample preparing for GCMS and SEM analysis

Statistical analysis: The Mann-Whitney U test was used to assess the differences in GC-MS peak area percentages between hard and non-hard seed using Prism - GraphPad software. A p-value less than 0.05 will be taken as statistically significant difference between the seeds (Sundjaja *et al*., 2020).

RESULTS

Gas chromatography–Mass Spectrometry (GCMS) analysis: The results of the GC–MS analysis indicated the presence of 25 different components in hard seeds & 30 component in non-hard seed (Table 1, Fig 2 & 3). Fatty acid and phenolic compounds are likely associated with seed dormancy in *Symplocos paniculata* (Tang *et al*., 2024). Based on this aspect, phenolic component, fatty acid and alcohol from the metabolites of hard and non-hard seeds were taken and the present result were explicated.

The GC-MS analysis revealed significant differences in the composition of fatty acids, alcohols, and phenolic compounds between hard and non-hard seeds. Hard seeds exhibited higher levels of key fatty

acids such as hexadecanoic acid (3.30%), tetradecanoic acid (4.44%), 9,12-octadecadienoic acid (2.45%), octadecanoic acid (27.48%) and 10-methyl-undecanoic acid (2.20%) compared to non-hard seeds, which have lower concentrations of these compounds. In contrast, pentadecanoic acid is significantly higher in non-hard seeds (16.97%) than in hard seeds (2.45%). Additionally, hexadecanol (1.29%) is alcoholic compounds present only in hard seeds. Hard seeds also showed elevated levels of phenolic compounds like 3-tert-butyl-4-hydroxyanisole (10.76%), phenolic dibenzodioxin (3.85%) and 3-hydroxy-benzoic acid (1.42%), which contribute to seed dormancy by enhancing the toughness and impermeability of the seed coat.

In nutshell, the GC-MS analysis revealed that a fatty acid, octadecanoic acid (27.48%), hexadecanol (1.29%) an alcohol and phenolic substance 3-tert-butyl-4-hydroxyanisole (10.76%) were found at significantly higher levels in hard seeds compared to non-hard seeds, which had lower concentrations of octadecanoic acid (6.00%) and 3-tert-butyl-4-hydroxyanisole (1.78%) with hexadecanol completely absent. These elevated levels of metabolites in hard seeds likely contribute to their greater impermeability and dormancy. The accumulation of fatty acids, phenolic compounds and alcohols on the seed coat plays a major role in inhibiting water absorption, reinforcing seed dormancy through increased impermeability and suggesting that these metabolites are key factors in hardseededness while non-hard seeds have more permeable seed coats that facilitate faster germination (Table 1).

Table 1. List of metabolic components profiled from seed coat of hard seed and non-hard seed through GC-MS in blackgram var VBN 8.

S. No	Metabolites	Peak area %	
		Hard seed	Non hard seed
	Fatty acids		
1.	Hexadecanoic acid	3.30	0.62
2.	Tetradecanoic acid	4.44	0.98
3.	9,12-octadecadienoic	2.45	1.20
4.	Octadecanoic acid	27.48	6.00
5.	10-Methyl-undecanoic acid	2.20	0.94
6.	Pentadecanoic acid	2.45	16.97
	Alcohols		
7.	Hexadecanol	1.29	-
	Phenolic compounds		
8.	3-tert-Butyl-4-hydroxyanisole	10.76	1.78
9.	Phenolic dibenzodioxin	3.85	1.97
10.	3-Hydroxy-benzoic acid	1.42	0.64

The Mann-Whitney test compares hard seed and non-hard seed, showing a statistically significant difference between the two. The exact P-value is 0.0272, which is less than the standard significance threshold of 0.05. This indicates that the difference between the treatments is statistically significant. Hard seed has a median value of 2.875 (n=10), while non-hard seed has a median value of 1.090 (n=10). The actual difference between these medians is -1.785, with a Hodges-Lehmann estimated difference of -1.54, indicating that non-hard seed has a lower median than hard seed. The sum of ranks for hard seed is 134 and for non-hard seed, it is 76, with a Mann-Whitney U value of 21. Since the test is two-tailed and the P-value is below 0.05, we can conclude that the difference in medians between the two treatments is statistically significant and not due to random variation (Table 2).

Table 2. Comparative analysis of metabolite concentrations in hard and non-hard seeds using Mann-whitney U test.

Type of seed	Median	Sum of rank	P value (Two tailed)	Hodges-Lehmann	Mann-Whitney U
Hard	2.875	134	0.0272*	-1.54	21
Non-hard	1.090	76			

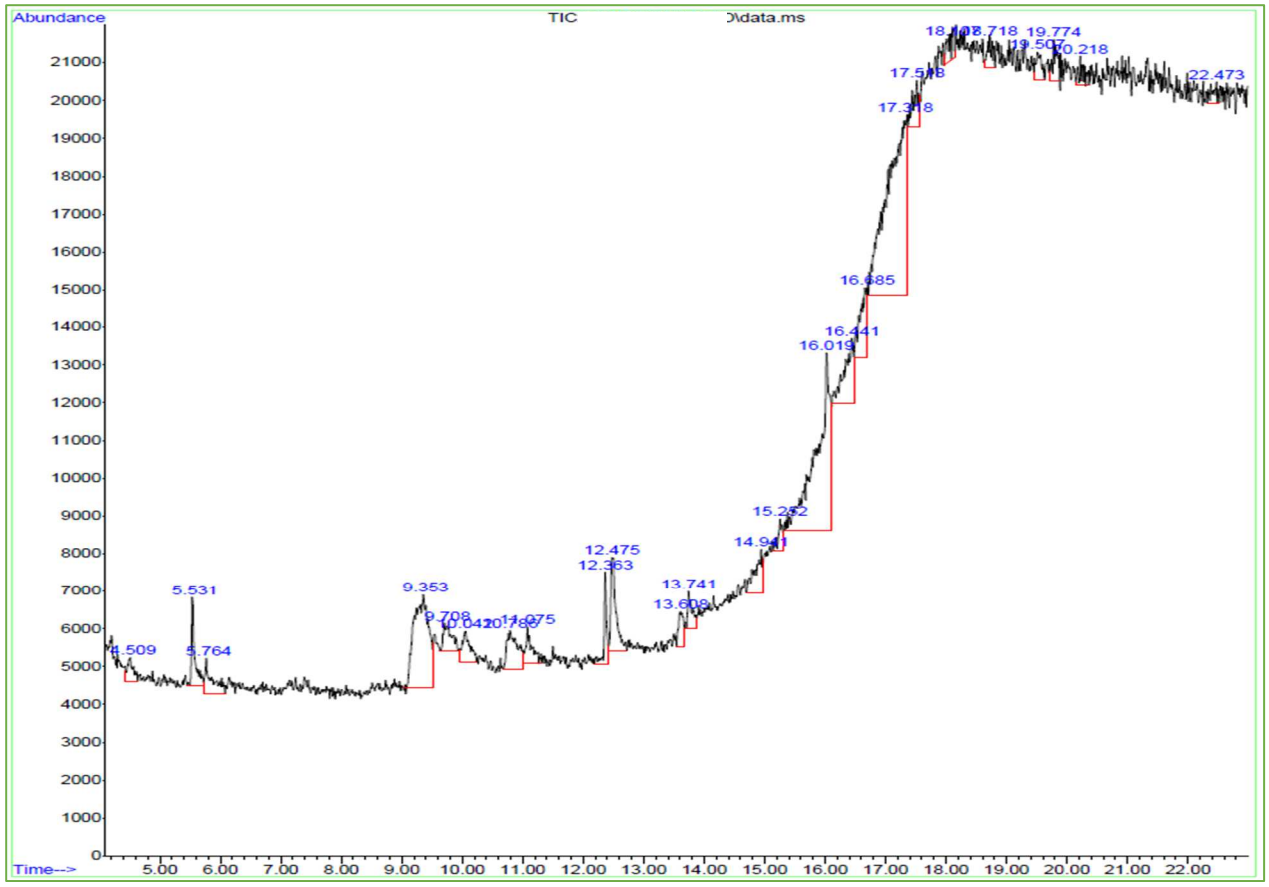

Fig 2. GC–MS spectra of phytochemicals in the hard seed coat of black grams

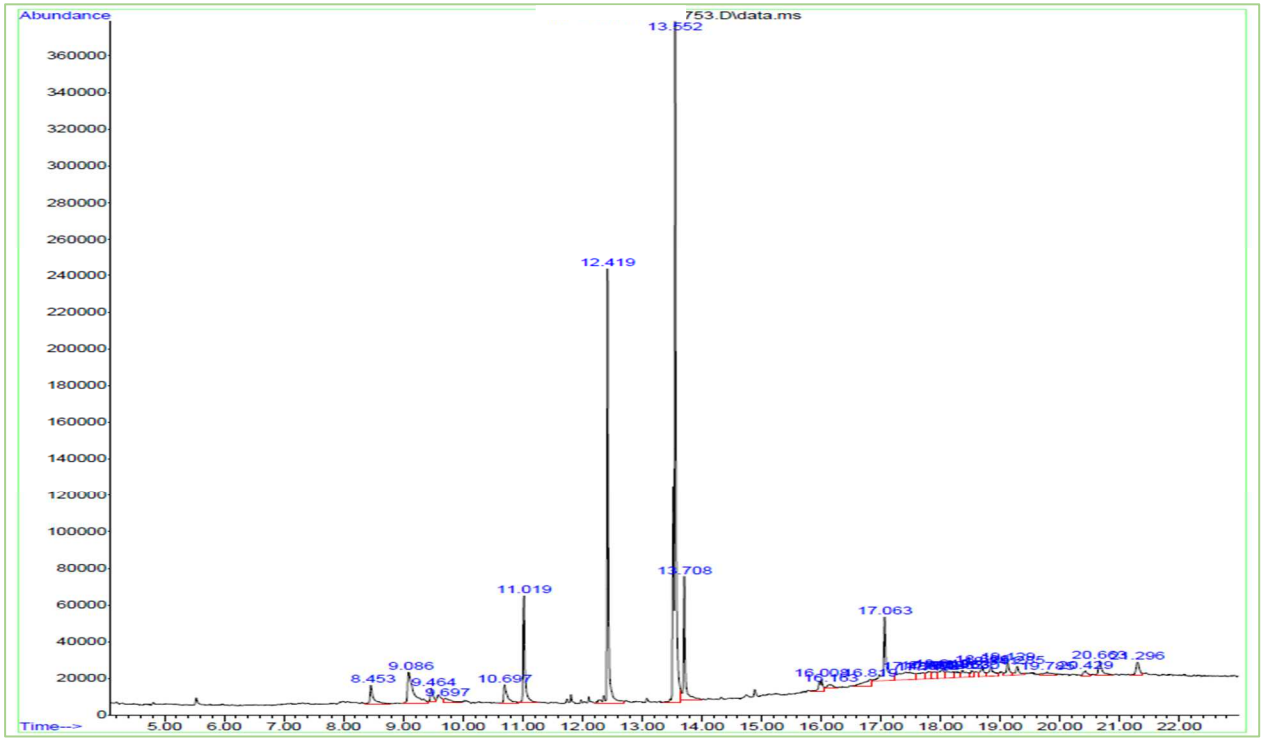

Fig 3. GC–MS spectra of phytochemicals in non-hard seeds coats of black grams

Scanning Electron Microscope (SEM) analysis: SEM analysis in hard seeds is generally spherical, similar to non-hard seeds, but there are notable differences in surface texture. The hilum is apparent and more visible in hard seeds than in non-hard seeds. Hard seeds exhibit more pronounced marks or ridges on their surfaces, implying a denser, more orderly outer layer (Fig.4a). These seeds have a thicker, more impermeable seed coat composed of macrosclereid (Malpighian) cells, which contribute to their hardness and water resistance, unlike non-hard seeds. SEM images reveal that hard seeds have more distinct layers within the seed coat, indicating a thicker cuticle and stronger underlying cell layers, such as osteosclereids and parenchyma cells.

Obviously, the non-hard seed has a smooth surface, lacks the thicker and impermeable layers present that distinguish hard seeds. The hilum, or scar where the seed adhered to the ovary wall, is clearly evident (Fig.4b). This most likely reflects the absence of highly thickened galactomannan or mannan polymers. The surface of the seed coat has no substantial flaws, which aids in protecting the seed from quick moisture penetration and microbial invasion, although it is not as strong as a hard seed coat. These traits indicate that this is a non-hard seed, which often has a more permeable seed coat than hard seeds do.

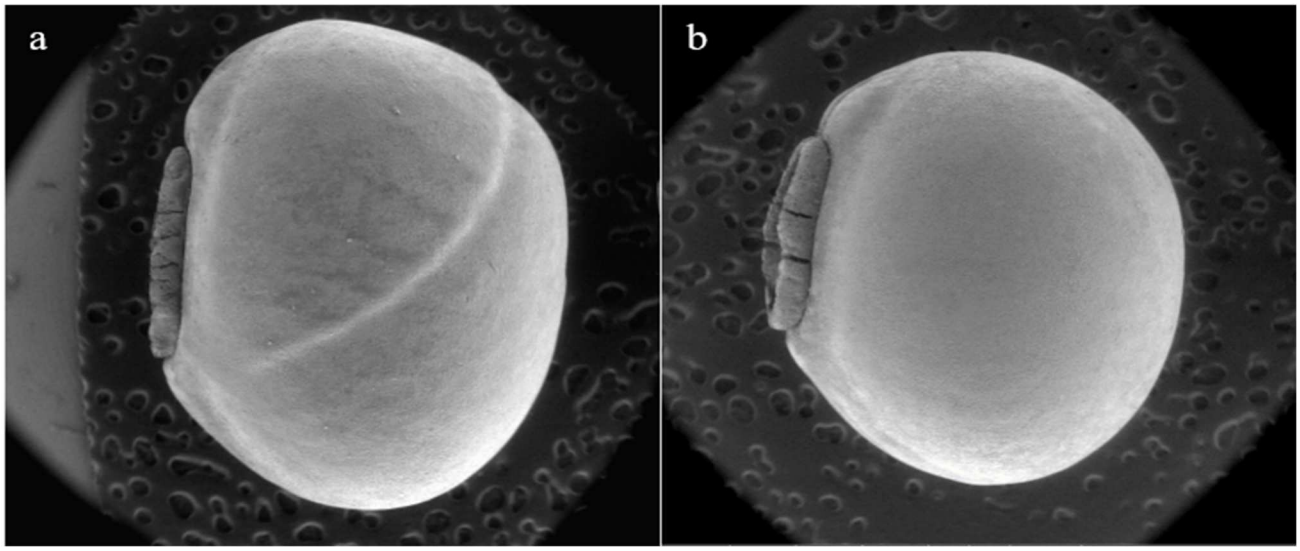

Fig 4. Full view of hard seed: a) Deposition of higher metabolites and cuticle layer on surface of seed coat. b) Full view of Non-hard seed: Seed size round in shape and regular, Smooth on seed surface.

The cross section through SEM shown (Fig.5a) that the cuticle layer marked as C of a hard seed is thicker and denser than that of a non-hard seed, acting as a waxy barrier that prevents water and gas exchange, thus contributing to the overall impermeability of the seed coat which is crucial in maintaining dormancy in hard seeds. Generally, cuticle layer is a outermost layer responsible for the waterproofing and protection of the seed. The cuticle layer appears as a smooth, thin layer on the surface. The palisade layer (Pl), located just below the cuticle (Fig.5b) consists of tightly packed, elongated cells that provide mechanical protection to the seed. This layer is highly lignified, which contributes to both the hardness of the seed and its impermeability to water. The dense arrangement of cells in the palisade layer effectively prevents water absorption, thus playing a crucial role in enforcing seed dormancy. The osteosclereids, also known as hourglass cells, are often found beneath the palisade layer (Fig.5b). These specialized cells have thickened walls that contribute to the structural rigidity of the seed coat. Their role is significant in both water movement and seed hardness, as they provide an additional layer of protection, enhancing the seed's impermeability and reinforcing its overall hardness. The SEM image (Fig.5b) provides a more detailed view of the palisade layer (Pa) with a focus on thickness measurement. The image shows precise dimensional data, indicating the thickness of the palisade layer as approximately 46.08µm. The thick palisade layer, along with the tightly arranged cells, forms a robust barrier against water uptake.: The measurement Pa R1 (Fig.5b) refers to the orientation or thickness of a subregion within the palisade layer, possibly emphasizing its structural integrity.

The photograph clearly illustrates multiple layers of the seed coat in a cross-section of a non-hard seed. The uppermost layer looks to be a relatively smooth cuticle. The value "41.10 µm" reveals the thickness of the parenchyma layer, which is significantly thinner than the dense layers observed in hard seeds. The labels "Pa R1" and "Pa1" refer to parenchyma cells, which form the

deepest layer of the seed coat. These cells are typically responsible for nutrient storage and transport. The outermost layer looks to be less dense and more permeable, as is typical with non-hard seed. This layer may be more vulnerable to water penetration, resulting in quicker germination. This cross-sectional image demonstrates the structural variations between non-hard and hard seeds, namely, the content and thickness of the seed coat layers. The non-hard seeds coat is thinner, less dense and more permeable, which allows for faster germination but also makes it more susceptible to environmental conditions (Fig 5 c & d).

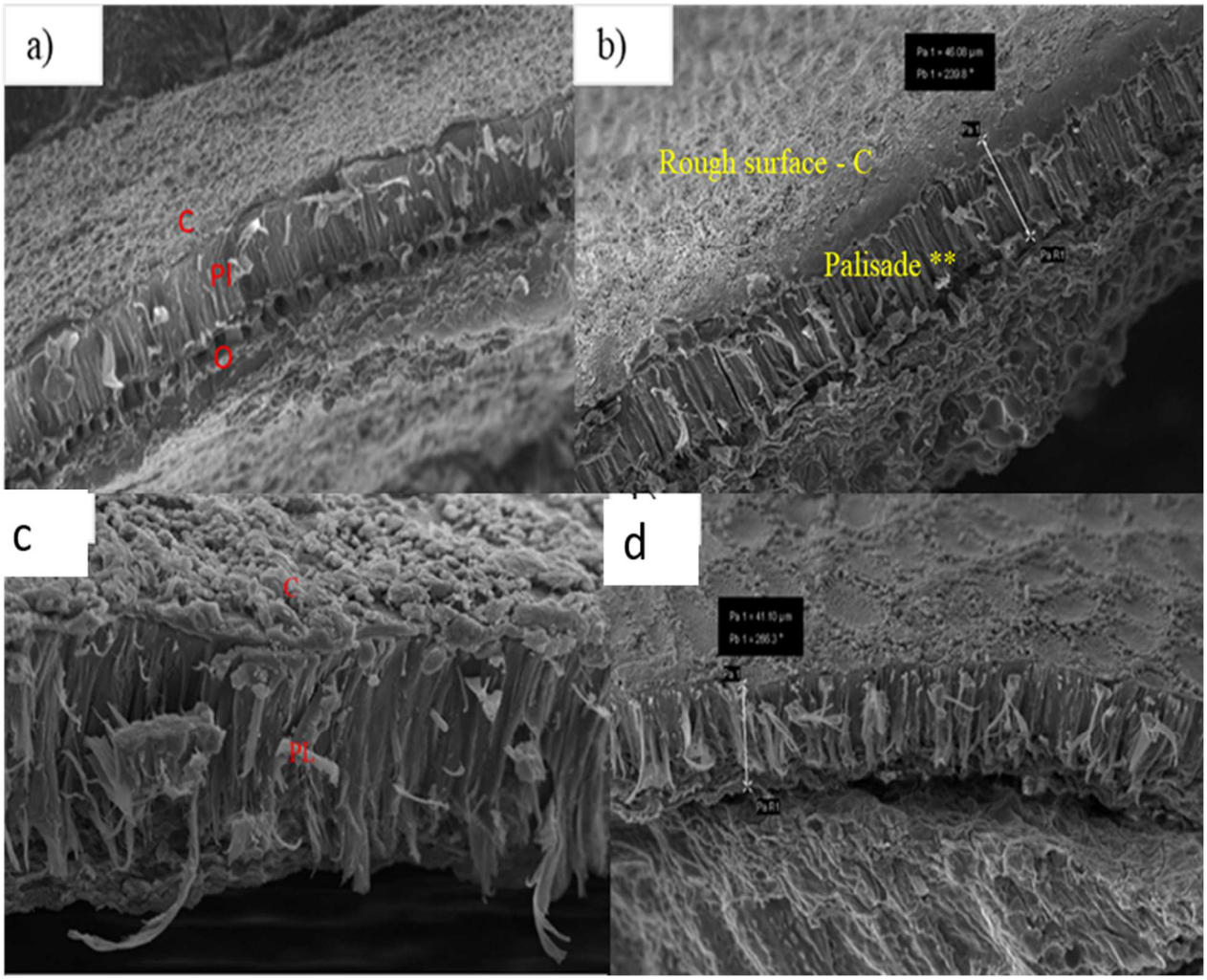

Fig 5. Cross section view of hard seed coat. a) SEM analysis represent the First layer is cuticle (C) layer is rough, b) Represent the measurement of palisade layer represent thickness of palisade layer, Palisade layer (PL) - presence of tightly arranged cell, Ostoscelriod layer (O) & absence of intercellular spaces. Cross section view of non-hard seed coat c) presence of intercellular spaces and d) the measurement of palisade layer represents thinness of palisade layer.

The provided SEM images (Fig.6) illustrate key surface characteristics of seed coat of hard seeds, highlighting features that contribute to seed dormancy and impermeability. In image (Fig.6a) a waxy layer is visible, with circled regions showing areas of wax deposition that create a hydrophobic barrier, preventing water penetration. Image (Fig.6b) displays higher deposition, where concentrated substances further enhance the seed's impermeability by forming a dense protective layer. In image (Fig.6c), the lack of pores is emphasized, with circled areas indicating tightly sealed surfaces that block water and gas exchange, a crucial feature in maintaining dormancy. Finally, image (Fig.6d) reveals a rough surface, where the irregular texture provides additional mechanical protection and reinforces the seed coat's barrier against water absorption. Together, this structural trait is waxy coating, high deposition, pore absence and surface roughness are essential for the seed coat's ability to resist moisture and preserve dormancy, particularly in hard seeds.

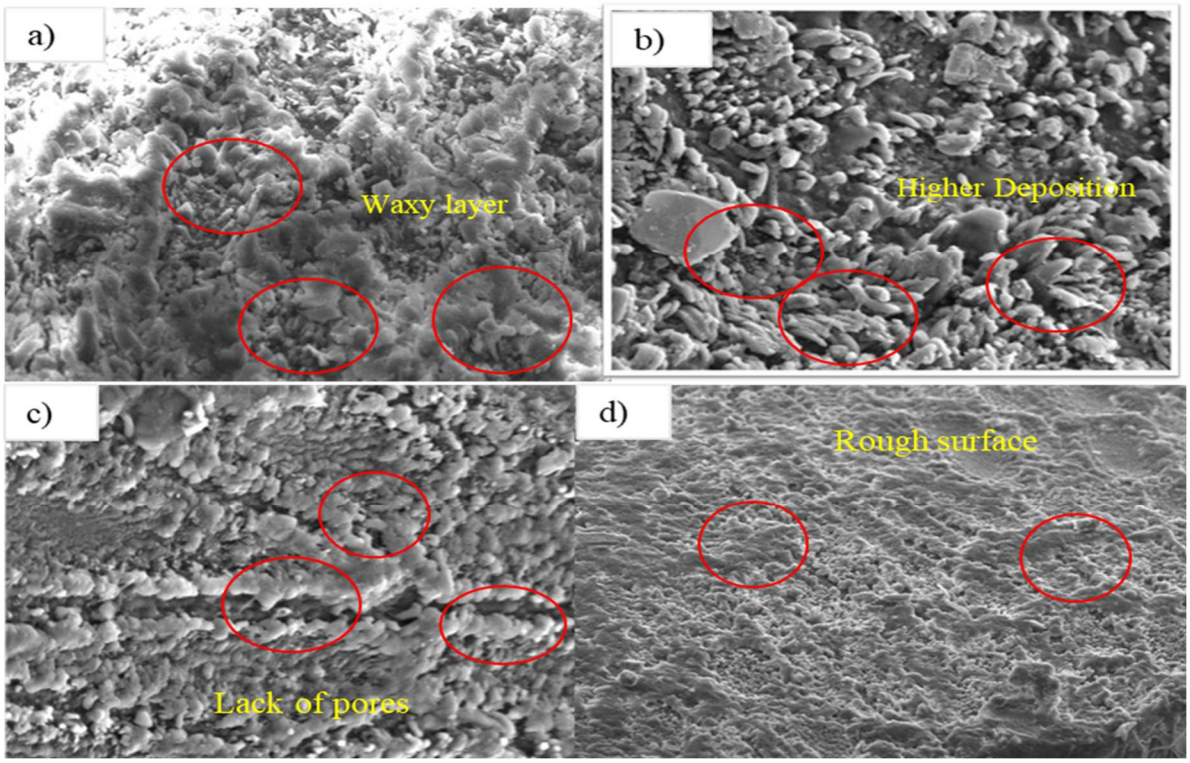

Fig 6. Seed coat surface of hard seeds. (A & B) Deposition of waxy substance and secondary metabolites as seed from the Testa surface, C) Represent lack of pores due to higher deposition, D) Seed coat surface contains stronger & more Impermeable layer.

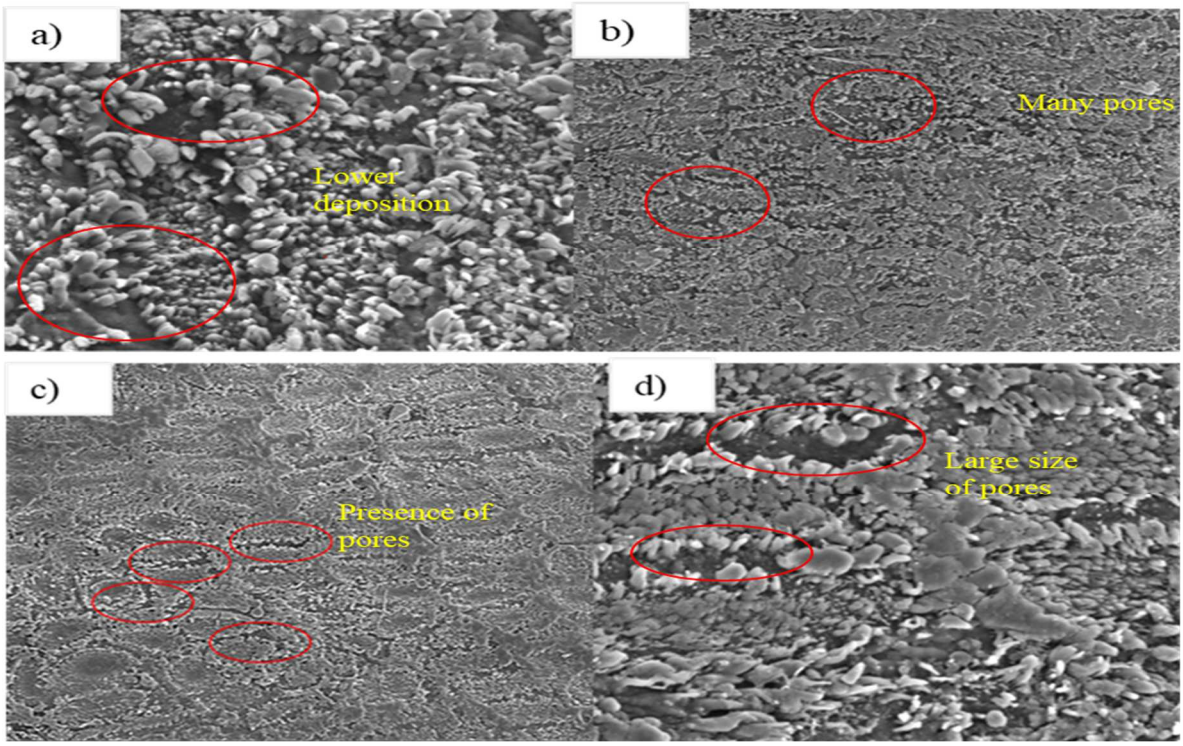

Fig 7. Seed coat surface of non-hard seeds a) red ring represent the deposition of low waxy substances, b & c) red ring represent the presence of many pores, d) Higher Magnification of c it shows large size of pores occur (red ring indicates presence of large size pores).

The provided SEM images (Fig.7) illustrate varying surface characteristics of seed coat of non-hard seeds, focusing on deposition levels and pore distribution, which play significant role in seed permeability and dormancy. Image (Fig.7a) highlights areas of lower deposition, suggesting a thinner protective barrier that potentially increases seed coat permeability, facilitating faster germination by allowing more water and gas exchange. Image (Fig.7b) showcases numerous pores, with circled regions demonstrating how these openings can enhance permeability, allowing for easy penetration of water and gases that can stimulate germination. Image (Fig.7c) also displays the presence of pores, pointing out scattered openings which regulate water uptake and influence the seed's germination capabilities under favourable conditions. Image (Fig.7d) depicts large pores, indicating that these larger openings allow for quicker water absorption, potentially leading to a faster breaking of dormancy and accelerated germination. These images collectively demonstrate how structural variations, such as deposition thickness and pore size, can significantly affect the seed coat's properties, impacting water absorption rates and the overall germination process.

DISCUSSION

GCMS Analysis: GC-MS analysis revealed that hard seeds have significantly higher levels of several fatty acids, including hexadecanoic acid (palmitic acid), tetradecanoic acid (myristic acid), octadecanoic acid (stearic acid) and 9,12-octadecadienoic acid (linoleic acid). These saturated fatty acids are known to contribute to seed coat impermeability by forming a waxy layer that restricts water uptake. Hexadecanoic acid, is a saturated fatty acid with the molecular formula $C_{16}H_{32}O_2$. (Eastwood., 2013; Kumar *et al*., 2023 and Muhammad *et al*., 2023) potentially linked to the impermeability of the seed coat, may influence the seed coat's lipid composition and its role in seed coat function. Octadecanoic acid ($C_{18}H_{36}O_2$) is a saturated fatty acid. Fatty acids serve a variety of functions in metabolism, such as roles in energy transport and storage, cell membrane structure, signalling molecules and gene regulation. (Dowhan and mikhail., 2002). GC-MS analysis and validation trials revealed that fatty acids and phenolic compounds, including hexadecanoic acid, oxadecanoic acid and m-cresol, were the primary endogenous inhibitors found in the endosperm. These findings suggest that seed dormancy in *S. paniculata* is caused by both mechanical restriction from the endocarp and the presence of physiological inhibitors, as reported by Tang *et al*. (2024).

Fatty acids can generate extremely long chain fatty acids, which are employed as direct precursors for wax production and derivatives such as aldehydes, alcohols, alkanes, ketones and esters (Yeats and Rose 2013). These acids add to the hydrophobicity of wax and can improve its protective characteristics by establishing a stronger barrier against water loss and environmental damage. The long hydrophobic carbon chain of hexadecanoic acid is important for lipid bilayer formation in cell membranes, contributing to both the structure and function of biological membranes. Long chain fatty acids (LCFAs), particularly very long chain fatty acids (VLCFAs), are indeed related to physical dormancy in seeds. They contribute to the structural integrity and impermeability of the seed coat, which plays a crucial role in preventing water absorption and radicle emergence. This hardness and impermeability are key characteristics of physically dormant seeds, as they help maintain dormancy by protecting the seed from environmental factors until conditions are favourable for germination (chai *et al*., 2021). A primary role of lipids in cellular function is in the formation of the permeability barrier of cells and subcellular organelles in the form of a lipid bilayer and presence of hydrophobic properties (Dowhan and mikhail., 2002).

Tetradecanoic acid (myristic acid) is a saturated fatty acid present in the seed coat with the molecular formula $C_{14}H_{28}O_2$. Its structure comprises a straight chain of 14 carbon atoms with a carboxyl group (-COOH) at one end. Vijayan *et al*. (2023) reported that tetradecanoic acid was relatively higher in Bhatt seeds. Studies, such as Doria *et al*. (2019), indicate that a higher concentration of fatty acids in the seed coat reduces water absorption, which may explain why hard seeds exhibit elevated levels of fatty acids. Pentadecanoic acid (pentadecylic acid), a fatty acid with a 15-carbon aliphatic chain and a carboxylic acid group (-COOH) at one end (Venn-Watson and Schork 2023) which was particularly high in non-hard seeds, while its specific function is unclear.

GC-MS analysis also revealed that hard seeds exhibit higher levels of phenolic dibenzodioxin ($C_{12}H_8O_2$) which consists of two benzene rings connected by two oxygen atoms, forming a dioxin ring structure. Phenolic compounds are secondary metabolites known for their antioxidative properties, which can have significant benefits for human health (Mirali *et al*., 2017). Marathe *et al*. (2011) found a strong positive correlation ($r^2 > 0.95$) between antioxidant activity and phenolic content in Black gram. Additionally, Eswaran *et al*. (2022) identified lignin subunits, including aryglycerol-β-ether dimers, biphenyl/phenolic dibenzodioxin and pino/resinol, suggesting that dibenzodioxin molecules contribute to the structural stability and impermeability of the seed coat. El-Tabey Shehata. (1992) linked hard texture formation in stored legumes to the lignification of cell walls. The variation in dibenzodioxin levels between hard and non-hard seeds implies that these compounds may influence seed coat impermeability and permeability. The aromatic properties and strong intermolecular interactions of dibenzodioxin likely play a

crucial role in forming a robust barrier against water and environmental factors (Eswaran *et al*., 2022). Different environmental factors such as precipitation, radiation, temperature and soil characteristics affect the production of phenolic compounds in plants reported by Cohen *et al*. (2010).

3-tert-Butyl-4-hydroxyanisole (BHA), a phenolic compound, is found in higher levels in hard seeds. Although its exact role in seed dormancy is not fully understood, BHA may contribute to the seeds' antioxidant properties or affect the permeability of the seed coat. The permeability of the seed coat is influenced by the concentration of phenols and fatty acids. Phenolic compounds play a major role on hard seed reported by Sun *et al*. (2018). These compounds protect seed lipids from oxidation, which is crucial for maintaining seed viability and longevity during storage and dormancy (Shi *et al*., 2020). Slattery *et al.* (1982) showed that *Trifolium* seeds with high phenol content are impermeable, indicating that phenolic compounds are important for seed coat dormancy. According to Chandra *et al*. (2020), phenolics can affect seed coat hardness and permeability in soybean and wild *text tuna*. Cavallaro *et al*. (2021) suggest that dormancy in this species is not solely related to seed coat hardness, as traditionally thought, but also involves the release of polyphenols. Polyphenol analysis of dormant and the few non-dormant seeds from various genotypes supports this hypothesis, as non-dormant seeds had nearly half the total polyphenol content (an average of 17.0 mg g^{-1} seed FW) compared to dormant seeds (34.8 mg g-1 seed FW). Additionally, the findings enhance the understanding of dormancy processes, which are positively influenced by hot water treatments.

Hexadecanol (cetyl alcohol) is a long-chain primary fatty alcohol with the molecular formula $C_{16}H_{34}$ which was present exclusively in hard seed. Its structure comprises a straight 16-carbon alkane chain with a hydroxyl group (-OH) attached to the terminal carbon atom. The long hydrocarbon chain is hydrophobic, whereas the hydroxyl group imparts hydrophilic properties, making hexadecanol amphiphilic. Most of alcohols are made from unsaturated fatty acids, which are needed for the formation of long-chain esters. Alcohols have antibacterial activity. However, high concentrations of alcohols can impede germination (Beisson *et al*., 2007). Sahai and Pal (1995) reported that the seed coat is impermeable to water due to the presence of hydrophobic substances, including cutin, lignin, quinones, pectin's, suberin and wax. In Arabidopsis, the presence of various alcohol components influences suberin formation Suberin biosynthesis results in the production of fatty acids, primary alcohols and other components. These primary alcohols are important components of the polyaliphatic domain of suberin, which contributes to its hydrophobicity and barrier characteristics (Gou *et al*., 2017).

Fatty alcohols play a significant role in the suberin of the *Arabidopsis* seed coat, enhancing its barrier properties (Vishwanath *et al*., 2013). Suberin mutants in *Arabidopsis* are critical for seed dormancy, as they impede the movement of water and oxygen, thus affecting seed viability (Fedi *et al*., 2017).

Overall, the GC-MS analysis suggests that hard seed coats in black gram are characterized by an abundance of fatty acids, phenolics and alcohol. These components likely contribute to the impermeability of the seed coat, hindering water uptake and causing dormancy. Conversely, non-hard seeds have a distinct composition with a higher concentration of pentadecanoic acid, possibly associated with a thinner and more permeable seed coat that allows for faster germination. Further research is needed to elucidate the specific mechanisms by which these metabolites influence seed coat properties and dormancy in black gram. These findings highlight potential strategies for reducing seed dormancy by targeting specific biochemical components like phenolic compounds and fatty acids, which influence seed coat impermeability. By understanding how these compounds contribute to dormancy, breeders can explore genetic or biochemical interventions to soften seed coats and improve germination rates. This offers practical implications for enhancing seed performance in agricultural settings, particularly in crops like black gram where dormancy poses challenges. Compared to earlier studies that primarily focused on mechanical or environmental methods to break dormancy, such as scarification or temperature treatments, this research provides a novel biochemical approach. Previous studies in crops like soybean linked dormancy to phenolic content, but this study further identifies the role of lipid-based compounds, especially fatty acids, which were less explored in prior research. This adds a new perspective to seed dormancy mechanisms across different legume species.

SEM Analysis: The morphological studies consistently highlight a strong connection between seed hardness and the structure of the palisade and epidermal layers. The SEM images revealed that the seed coat consists of three layers: the epidermis, palisade and sclereid, as reported by Wang *et al*. (2019). Chai *et al*. (2023) reported that the outermost cuticle of the seed coat forms a layer that is impermeable to water. According to Sedláková *et al*. (2021), the epidermis of the outer integument forms a layer of tightly packed, elongated palisade cells, which are sclerenchyma cells or sclereids. Moreover, the cuticle, a hydrophobic extracellular layer, plays a key role in seed coat impermeability by acting as a strong barrier to water uptake (Yeats and Rose, 2013). Vu *et al*. (2014) demonstrated that seed hardness is closely related to the palisade layer, particularly in seeds exhibiting physical dormancy. Similarly, Chai *et al*. (2016) confirmed that

seeds with physical dormancy have a hardened palisade layer, contributing to their impermeability. In lupin bean seeds, hard seeds possess a thicker palisade layer compared to non-hard seeds, which enhances their water impermeability (Ammirato, 1978).

According to Vijayan *et al*. (2023), phenols are the key compounds responsible for the impermeability of the seed coat, accumulating in the palisade cells of Bhatt seeds and contributing to both the impermeability and strength of the soyabean seed coat. Wen *et al*. (2024) reported that the thickness and arrangement of palisade layers can differ between species, influencing seed hardness. Corner's (1951) historical research emphasized the significance of leguminous seed structures, particularly the presence of an outer palisade layer and hourglass cells. Othman *et al*., (2023) reported that chemical dormancy was attributed to elevated levels of total polyphenol compounds, which inhibited seed germination and seedling growth, while physical dormancy was linked to the hardness of the seed coat, creating mechanical resistance to radicle emergence. Werker *et al*. (1979) further identified that the palisade layer, together with phenolic compounds, contributes to the rigidity and water resistance of the seed coat, which is crucial for pea seed impermeability.

Seed coat permeability is essential for successful germination, as it allows for water absorption (Gao *et al*., 2022). SEM analysis of licorice seeds revealed structural differences between hard and soft seeds, where hard seeds exhibited fewer, shallower and narrower cracks compared to soft seeds, creating a more intact surface that resists damage. Additionally, the hilar fissure in hard seeds was much narrower, reducing water permeability and contributing to dormancy. On the other hand, soft seeds had deeper and wider cracks, a broader hilar fissure, and a thinner endosperm, making them more permeable and less resistant to environmental stresses (Sun *et al*., 2018). In *Vicia sativa*, soft-seeded varieties have macrosclereid cells that average 39.24 µm in length and 5.75 µm in width, with a thin cuticle, as noted by Büyükkartal *et al*. (2013). Similar patterns were observed in non-hard seeds of Black gram, where a thinner seed coat and reduced cuticle thickness were associated with increased permeability and reduced dormancy. Zang *et al*. (2023) reported that scanning electron microscopy revealed lighter-colored seeds with a thin palisade layer and minimal surface deposits, whereas darker-colored seeds exhibited a thicker palisade layer. Chai *et al*. (2021) identified KNOX4, a class II KNOTTED-like homeobox transcription factor, as crucial for controlling hardseededness in *Medicago truncatula*. They also found that β-ketoacyl-CoA synthase (KCS12) plays a key role, with its expression downregulated in the knox4 mutant. KCS12 is primarily expressed in the seed coat, and its disruption leads to a loss of physical dormancy, allowing water absorption without scarification. KNOX4 directly regulates KCS12 expression, providing insights into the molecular mechanisms of seed dormancy, though lacking comparisons with similar studies in other crops.

Based on SEM analysis and supporting literature, it is evident that seed hardness is strongly tied to the structural characteristics of the seed coat, particularly the palisade and epidermal layers. Hard seeds consistently show thicker, denser palisade layers, fewer and narrower cracks and a more intact cuticle, all of which contribute to reduced permeability and increased dormancy. In contrast, non-hard seeds exhibit thinner palisade layers, wider cracks and a less robust cuticle, leading to increased permeability and reduced dormancy. These structural differences underscore the critical role of the seed coat in regulating water uptake and dormancy, providing valuable insights into the mechanisms of physical seed dormancy in legumes. Understanding these morphological traits lays the foundation for strategies to improve seed germination by modifying seed coat characteristics, with broader implications for enhancing agricultural productivity, especially in crops like Black gram.

In a nutshell, these findings provide valuable insights into the biochemical and structural mechanisms governing seed dormancy, particularly with respect to seed coat impermeability. By identifying key compounds such as fatty acids, phenolic compounds and alcohol, this study offers significant potential applications in crop breeding and agricultural practices. Understanding the role of these compounds could help breeders develop varieties with reduced dormancy traits in legumes like black gram, thereby enhancing germination rates and improving yield consistency. Furthermore, insights from this research could inform the development of dormancy management techniques, such as chemical scarification or targeted genetic modification, to break dormancy in seeds with impermeable coats, ensuring timely germination even in challenging climates. Additionally, the antioxidant properties of phenolic compounds and fatty acids could be utilized in breeding programs to extend seed viability and longevity during storage, benefiting seed banks and enhancing agricultural productivity. In conclusion, by manipulating the biochemical and morphological traits responsible for seed dormancy, these findings have the potential to improve germination practices, boost crop productivity and contribute to global food security.

Conclusion: This study, the first to report on hard and non-hard seeds in *Vigna mungo* VBN 8, identifies phenolic compounds, fatty acids and alcohols as key contributors to hard seed characteristics. The comprehensive characterization of these metabolites in both hard and non-hard seeds provides valuable insights into seed dormancy management. SEM analysis supports these findings by confirming that hard seeds possess

thicker and denser seed coats, reinforcing the biochemical evidence. These results underscore the critical influence of specific metabolites in regulating seed dormancy and offer promising strategies for improving germination in black gram and other leguminous crops. Reducing seed dormancy in crops like Black gram has significant implications for agriculture. Enhanced seed germination and vigour can improve crop establishment, increase yields and lead to more reliable production, especially in regions with suboptimal germination conditions. A deeper understanding of the biochemical mechanisms behind seed coat dormancy will not only support Black gram breeding programs but also provide strategies for reducing dormancy in other leguminous crops.

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