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Scanning Electron Microscopy and Morpho-Physiological Features Imparting Differential Tolerance to Pre-harvest Sprouting (PHS) in Mungbean [*Vigna radiata* (L.) Wilczek]

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ABSTRACT

Background: In mungbean crop, the yield gap in many high yielding varieties is mainly due to lack of resistance to pre-harvest sprouting and wetting due to rains at physiological maturity or just before harvesting. Prevention of pre-harvest sprouting damage forms the most important challenge in mungbean since it has been identified as the crucial constraint for improving seed yield and quality seed. With the above perspective, the present study is aimed to identify morpho-physiological features responsible for pre-harvest sprouting and to reveal ultra structural architecture of pod and seeds of mungbean genotypes, imparting susceptibility or tolerance to pre-harvest sprouting.

Methods: Four mungbean genotypes *viz*. ML 267 and MGG 295 (susceptible) and LGG 450 and K 851 (tolerant) were used to study the details of morpho-physiological characters such as: layers and thickness of podwall, seed coat, their external surface and internal structures of seed using scanning electron microscopy. The quantity of water accumulated, rate of moisture absorption, speed, path and pattern of water movement across the podwall and seed coat including locular space, cotyledonary area and embryonic region were studied using I_2-KI treated water in time course studies on water pathway.

Result: The ultrastructural architecture of pod, morpho-physiological features endowed with differential biomolecular alignment together determine the trait of tolerance or susceptibility to pre-harvest sprouting in mungbean genotypes.

Key words: Biomolecules, FSD, I_2-KI water, Mungbean, PHS, SEM.

INTRODUCTION

India is the largest producer, consumer and exporter of pulses in the world with an annual production of 18.31 million tones representing 34.8% of world area and 25.08% of world's pulse production. However, pulse production was stagnated since last two decades, which resulted in reduced per capita pulse consumption per day from 60 (1950-51) to 32 gm/capita/day (2018) as against the recommendations of World Health Organization (WHO) of 80 kg/capita/day (Deol *et al.* 2018). In order to increase the productivity of pulses in India, there is dire need to find out the existing constraints in its production. Several factors responsible for the low productivity of pulses in India, among them pre-harvest sprouting (PHS) is one of the most important factors for the poor yields. Pre-harvest sprouting is the phenomenon of *in-situ* germination of physiologically mature grains in the ear or panicle or pod under wet conditions (>30% seed moisture) before harvest. The pre-harvest sprouting problem was high in areas of high rainfall region, resulting in great economic losses (Li Tai *et al.* 2021). This constraint is frequently seen in almost all the pulses particularly in mungbean due to its susceptibility to pre-harvest sprouting. It is cultivated over an area of 13.1 m ha, with production of 8.46 m.t of grain in India (Ministry of Agriculture 2020-21). The average productivity stakes at 389 kg/ha occupying an area of 14.68% of the total pulse grown, contributing 8.06% of total pulse production (1.3 M per annum). Mungbean seeds, despite being protected inside the pod, are susceptible to pre-harvest sprouting at physiological maturity stage under incidental rainfall due to lack of fresh seed dormancy (FSD), which deteriorates the quality of the seed/ grain. Therefore, development of mungbean genotypes with short (10-15 days) period of fresh seed dormancy has become vital to curtail losses incurred by pre-harvest sprouting. In this study, it is described a simple technique for tracing the route of water transport from the exterior pod surface across the podwall tissues, locular space, seed coat and embryonic axis since moisture at embryonic axis is the root cause of pre-harvest sprouting. Pre-harvest sprouting/ weather damage has been described as a range of adverse physical and chemical changes that occur in seed following its exposure to prolonged periods of rainfall or high humidity

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while intact on mother plant prior to its harvest (Andrews, 1982). Incessant rains occurring at the time of harvest not only delay the pulse crop harvesting, but also cause loss of grain yield up to 70% or more and deteriorates seed quality due to in-situ sprouting of seeds making the produce unfit for either consumption or to use as seed (Durga and Kumar, 1997). Much effort was not made either to harness already existing genetic variation among mungbean for preventing pre-harvest sprouting or to exploit the physiological and biochemical mechanisms for preventing viviparous germination. The information on morpho-physiological features of pod as well as seed which impart tolerance to pre-harvest sprouting in mungbean, even now, is meagre. In order to find out appropriate defensive mechanism factors as against pre-harvest sprouting, the study was taken up under simulated rainfall conditions to identify morpho-physiological features responsible for pre-harvest sprouting in the present elite genotypes of mungbean and to reveal ultra structural architecture of pod and seeds of mungbean genotypes, imparting susceptibility or tolerance to pre-harvest sprouting.

MATERIALS AND METHODS

The experiment with two susceptible (ML 267 and MGG 295) and two tolerant (LGG 450 and K 851) varieties was conducted in the laboratory of Seed Research Technology Centre (National Seed Project) and scanning electron microscopy (SEM) studies were carried out at RUSKA Lab, College of Veterinary Sciences, ANGRAU Campus, Rajendranagar during 2017-18 and 2018-19 to study the details of layers and thickness of podwall, seed coat, their external surface and internal structures of seed. The fresh seed samples were transferred into glass vials and fixed in 3% gluteraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 h at 4°C. The treated samples were then post-fixed in 2% aqueous osmium tetroxide for 4 hrs and later in the same buffer for 2 hrs. The samples were then dehydrated in series of graded alcohol and dried to a critical point of drying, for Electron Microscopy. The dried samples were then mounted over the stubs with double sided carbon tape. Finally, a thin layer of platinum (palladium) coat was applied over the samples using an automated sputter coater (JEOL JFC-1600) for about 3 min. All samples were coated with gold and examined with a Hitachi S 570 scanning electron microscope at 15 kV. The samples were then scanned using scanning electron microscope (Model: JOEL- JSM 5600) at magnifications from x50 to x1, 400.

Based on the response of mungbean genotypes to simulated rainfall condition, the pods of two genotypes LGG 450 (tolerant) and ML 267 (Susceptible) were subjected to I_2-KI treated water. Migration of this water from podwall across various parts of seed enroute the embryo was traced following Iodine staining of the starch. The procedure used by Mc Donald *et al.* (1994) was adopted for tracing water path with minor modifications. Each treatment after 24 h of 2% I_2 solution spray given intermittently for facilitating absorption for 6, 12 and 24 h. At the end of each period, the pods were collected and cut across the podwall. The fine sections were selected to observe the movement of water as stained by Iodine through the podwall, seed coat via locular space, endosperm and embryo, detected by brown shadow. The micrographs were recorded by photography at magnifications from 1.5 to × 2.5 by using NIKON SMZ 800 (Model C-DS) research microscope.

RESULTS AND DISCUSSION

The results of present investigation were briefly discussed as the ultra-structure of the pod and seed coats of the susceptible varieties of ML 267 and MGG 295 as against pre-harvest sprouting, the pod wall exhibited deep cracks and witnessed a greater number of pores on its surface (Fig 1 A and B). The thickness of podwall was lesser than K 851 and LGG 450. The podwall thickness of MGG 295 was 423 µm. The fairly large, elongated and a greater number of functional and turgid trichomes with high density on podwall surface. The outer surface of pod wall got thin cuticular layer followed by one celled epidermis. These made easy access for movement of water when it was wet or subjected to simulated rainfall (Fig 2 A and B). Water droplets adhere to trichomes could easily absorb water. Thus, wetting of seed coat or absorption of water could also be enhanced due to the high density of trichomes on the outer surface of the podwall.

The movement of water could also be made easy due to thin cuticular layer and one - celled epidermis and kept moistened. Further, the mesocarp and endocarp seen in perforated tissues exhibited large longitudinal cracks in MGG 295 as well as in ML 267. This is immediately followed by a large locular space (0.48 mm) in MGG 295 and 1.06 mm in ML 267 formed a reservoir around the seed proper for incoming water. Several researchers (Dougherty and Boerma, 1984 in soybean; King and Richards, 1984 in wheat; Lush and Evans 1980 in mungbean) revealed that pod morphological characters are made congenial for wetting, rapid movement and water absorption of podwall and kept it moist condition which leads to pre-harvest sprouting under adverse climatic conditions due to thin podwall ultrastructural features like deep cracks with more number of pores, thin unicellular cuticular layer, high density, size and shape of the trichomes on the pod wall got thin cuticular layer followed by one celled epidermis. K 851 and LGG 450 have shown pre-harvest sprouting resistance due to attribution of pod morphological characters. LGG 450 and K 851 pod wall surface had no cracks with very few numbers of pores (Slight and slits). Trichomes sparsely with short, weiry twisted few in number along the margin seen as it dried and less turgid nature of trichomes and thick podwall. A very few short, flattened, less turgid, wiry and twisted trichomes (Fig 3 A and B) in these genotypes indicate their dysfunction for absorption of water. This further emphasizes less scope for trapping of water droplets when they are wet. These morphological characters shown resistance against pre-harvest sprouting. Similar findings were also reported by Singh *et al.* (2017), Sarfraz Ahmad *et al.* (2014), Satyanarayana *et al.* (1991) and Harris (1987).

The seed coat of ML 267 and MGG 295 (Fig 4A and B) are relatively thinner 77.3 µm (MGG 295) than that of K 851. The cotyledons of ML 267 had smaller starch granules embedded within protein bodies. The above described layers of pod wall, locular space and seed coat of ML 267 and MGG 295 clearly indicate an easy access to water movement across the seed layers. All the aforesaid structural features culminated for easy movement of imbibed water and might prone the seed of these genotypes to pre-mature sprouting, when they were subjected to simulated or unseasonal rainfall.

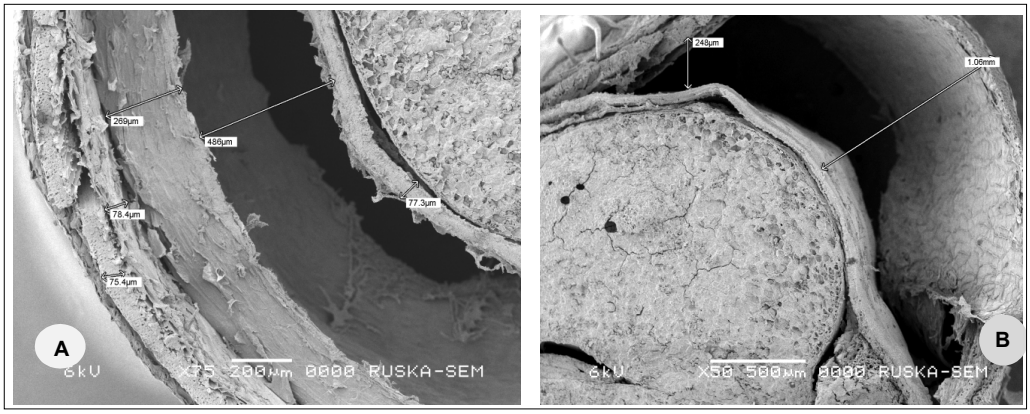

Fig 1: Scanning electron micrograph of ML 267 and MGG 295 mungbean genotypes showing a large locular space with epidermal pores and cracks. Podwall (pw), Seed coat (sc), Locule (lo) and Cotyledon (Co).

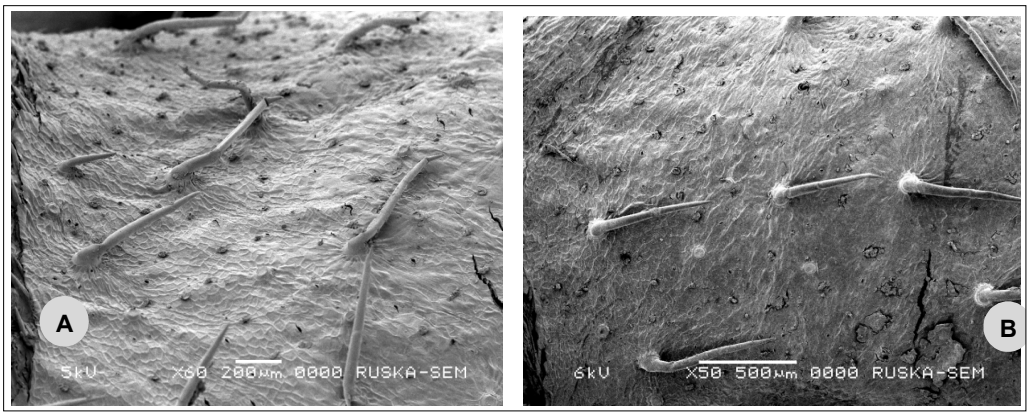

Fig 2: Scanning electron micrograph of mungbean genotypes (ML 267 and MGG 295) showing the turgid trichomes on outer surface of the podwall.

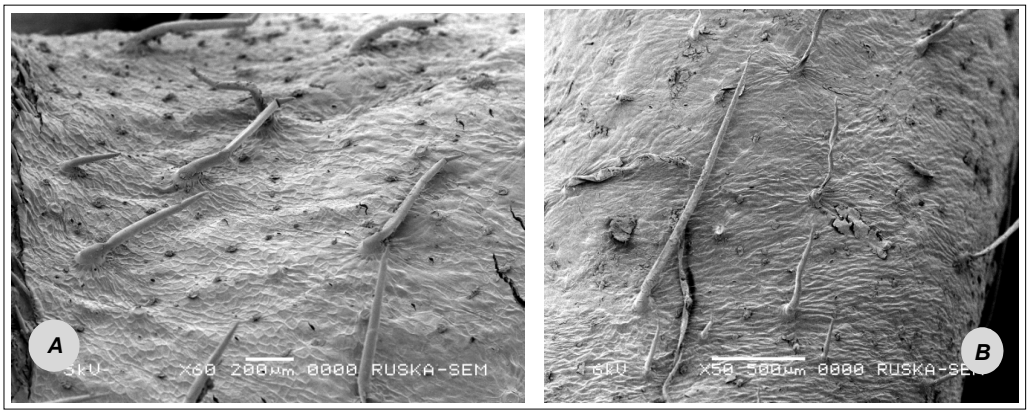

Fig 3: Scanning electron micrograph of mungbean genotypes (LGG 450 and K 851) showing withered, damaged trichomes on outer surface of the podwall.

The thickness of the mesocarp and endocarp of LGG 450 together measures 195 µm (Fig 5A and B). The locular space was reduced conspicuously in LGG 450 while it was very small compared to that of K 851. Both these genotypes had three times lesser locular space compared to that of ML 267 (Fig 5A). The locular space at placental region or at other places ranges from 34-39 µm in LGG 450. The seed coat of LGG 450 and K 851 is thicker (Fig 6 A and B) compared to ML 267 and MGG 295 (77.3 µm). The cotyledons contain few but larger starch granules, partially covered with protein deposits. The embryo was seen interlocked within cotyledons measuring 2 × 3 and 558 × 275 µm. The embryonic space around the embryo was relatively more at mid width. This indicates that the embryo is unready to take up imbibitional growth for sprouting unlike that of ML 267 (Fig 7A). The longitudinal embryo (Fig 7B) was seen surrounded by a large space; embryonal space also indicates a space for accumulation of water helped at the time of imbibition.

The thicker mesocarp almost tight with endocarp together with narrow locular space (no gap in case of LGG 450) forms a barrier for impeding free movement of water or with less scope for accumulating water around the seed coat. The cotyledons contain almost naked starch granules with splashed protein bodies indicate less prone for absorption of water; also, the median width of the embryo indicates its unreadiness for sprouting. These observations in the testing genotypes are in accordance with the findings of Sarfraz Ahmad *et al.* (2014) and Harris (1987).

The matured seed germination on pod itself under high rainfall conditions triggered with absorption of water from the podwall layers across the seed coat into embryo. The movement of water from podwall across its layers via locular space and seed coat into embryo depends on the rate, pace and pattern of movement of water. The quantity of water with time that could reach the embryo trigger imbibition, hydration of embryo, hydrolysis of reserves and biochemical process of imbibed seed which decides the seed whether sprout, delay or not to sprout at all. Thereby, the path of water movement across the tissues from seed coat to embryo was traced using iodine water, as per the technique developed by Mc Donald (1994). The study conducted with

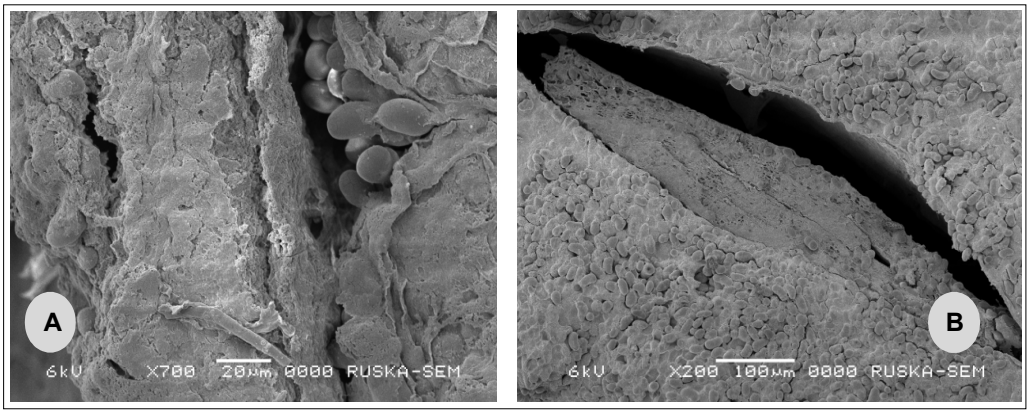

Fig 4: Scanning electron micrograph of (A) LGG 450 seed coat sub hilum showing compact podwall(pw) and seed coat(sc) with clean starch granules(sg) of endosperm without protein deposition seen in the locular cavity; (B) ML 267 showing starchy embryo (se) with embryonic cavitations indicates provision for storage of water around the embryo.

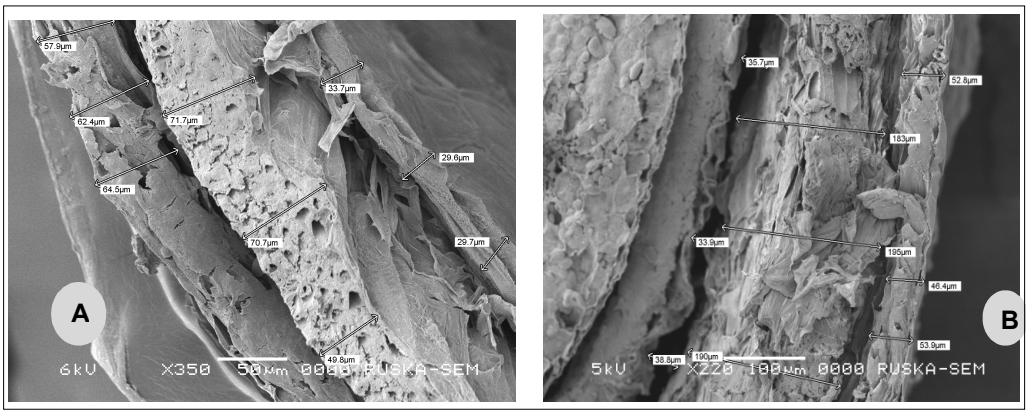

Fig 5: Scanning electron micrograph of podwall and seed coat (A) ML 267 showing thinner multicellular mesocarpellary tissue to large locular space. (B) LGG 450 showing packed podwall (pw) layer with cavitative cells (cc) and very narrow locular space (ls) of multicellular, thicker mesophyll tissue and cotyledon (co).

selective genotypes (ML 267, MGG 295, LGG 450 and K 851) based on their response of sprouting to simulated rainfall, revealed that the genotype ML 267 sprouted early while LGG 450 failed to sprout. Under conditions of wetting / simulated rain, the water movement took its path across podwall, locular space, podwall, space around cotyledons and embryo as clearly seen in ML 267 (Plate 1a). The time-course movement of water in the seed was traced by using I_2-KI treated water showing dark areas (Plate 1a). This path had been found dissipating slowly first into the locular space within 4 h slightly around cotyledon *i.e.,* in the podwall space and by 12 h, prominent accumulation of water occurred in locular space indicating intense accumulation of water all through the circumference of podwall (Plate 1b), whereas in LGG 450 which exhibited relative tolerance to wetting, showed a slow and irregular diffusion of water through the podwall and locular space by 6 h (Plate 1b) but a prominent dark colour could be seen by 6 h, 12 h in the locular space and around cotyledons, (partially) in Plate 1c. By 24 h, much accumulation of water was seen only in the locular space with a little diffusion into the cotyledonary area. A white embryo scar indicates no reach of water to the embryo (Plate 1d) even by 24 h. The restricted water movement may also be attributed to very low sprouting compared to the variety ML 267. Water imbibition (%) by pod showed highly significant and positive correlation with seed germination percentage. Higher amount of water absorbed by the pod makes sufficient moisture available for the seeds present inside to initiate process of germination. Similar role of pre-harvest with rate of water imbibitions through pod wall has been reported by Uwins *et al.* (1996), Sarfraz Ahmad (2014), Renata Anna *et al.* (2016) and Singh *et al.* (2017).

The genotype ML 267 showed dark colouration in the podwall layers indicates the presence of water between the podwall and seed coat moved carrying I_2 ions. The colour intensity was still more in the locular space, which represents the presence of more water accumulated around the seed coat. The intensity of colour was lighter in the seed coat wall but darker in the space between seed coat and cotyledons. The cotyledonary area showed a little shade, indicating the presence of relatively less water. The embryonal space also showed dark colour clearly suggesting the presence of water around the embryo (Fig 7A and B). It was concluded that water accumulation inevitably occurs in

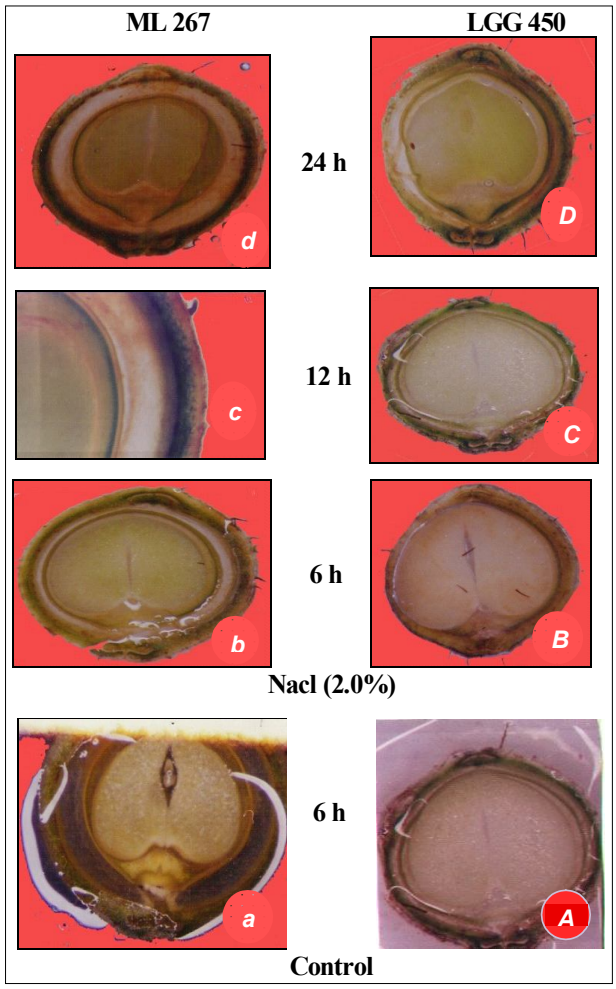

Plate 1: Time course studies on water pathway in NaCl treated susceptible (ML 267) and tolerant (LGG 450) mungbean genotypes to pre-harvest sprouting.

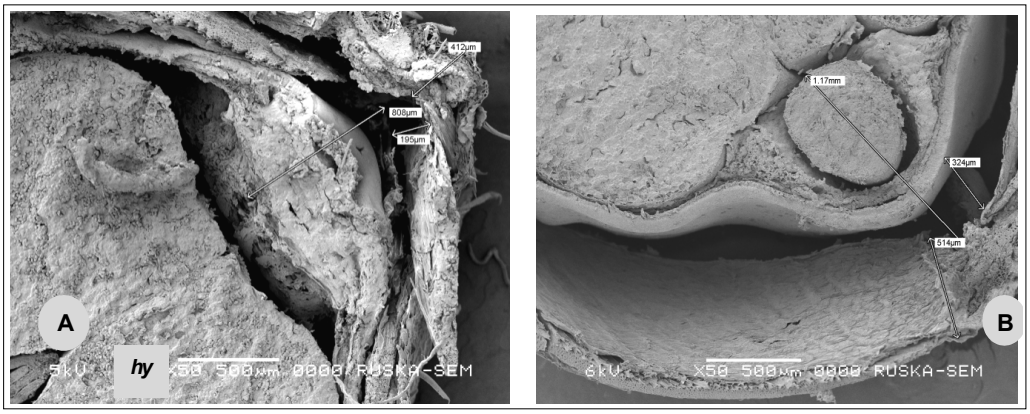

Fig 6: Scanning electron micrograph of LGG 450 and K 851 mungbean genotypes showing the thickness of cuticle and pod wall. Micropylem (m), Vascular bundle (vb), Placenta (pl) and Hypocotyl (hy).

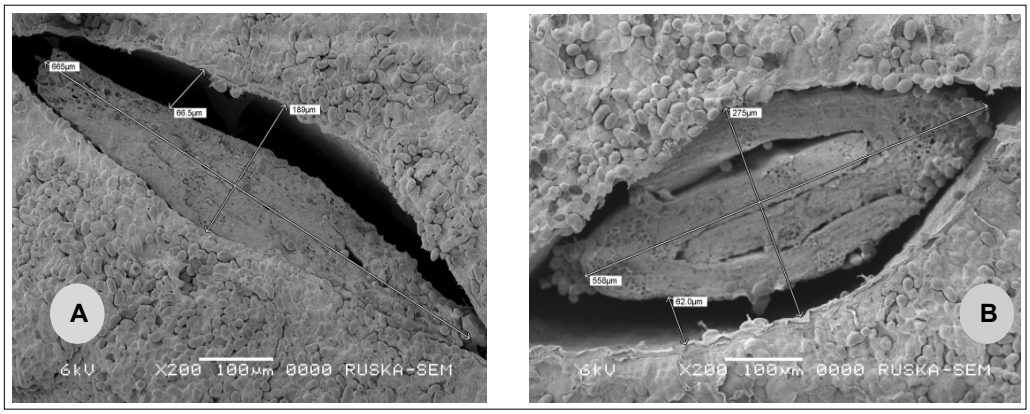

Fig 7: Comparison of scanning electron micrograph of figure (A) ML 267 showing longitudinal and elongated embryo and large space around it showing linear growth getting ready for germination (B) LGG 450 also a relatively starchy endosperm with 558 x 215 µm embryo and large space around the embryo showing the status of embryo unready for germination: Sg : starch granule; se : starchy embryo.

locular space around the seed and embryo which was readily prone the seed for sprouting condition. The genotype LGG 450 showed dark colouration in the podwall layers or seed coat, even though they were faint to dark colouration in very tight locular space. It was suggested that I_2 water movement in this seed was very slow and low even after 6 h of treatment with I_2 water. The cotyledons also showed very faint colouration at the distal end.

The variation in anatomical/ultra-structural features of pods and seeds of mungbean varieties of ML 267, MGG 295, LGG 450 and K 851 examined under the scanning electron microscopy revealed that more number of pores with few deep cracks on podwall surface, large elongated and a greater number of turgid and functional trichomes, thin unicellular cuticular layer with one-celled epidermis, a large locular space and protein bodies embedded between many smaller starch granules in cotyledonary area. ML 267 and MGG 295 have shown the above traits which favors for susceptibility to pre-harvest sprouting. In a time course study, the quantity of water accumulated, rate of moisture absorption, speed, path and pattern of water movement across the podwall and seed coat including locular space, cotyledonary area and embryo shown that ML 267 and MGG 295 accumulated more water in turn sprouted early. Such accumulation of water, absorption and movement of water across the podwall and seed coat were less in LGG 450 and K 851 due to their morphological features. Thereby, it was either delayed or no sprouting in LGG 450 and K 851 repectively. Determination of susceptibility/tolerance as against pre-harvest sprouting in mungbean genotypes (ML 267, MGG 295, LGG 450 and K 851), based on the traits of topography, architectural built up, nature of podwall surface and thickness, type of cells of podwall and seed coat including locular space. The biochemical nature and stimulating enzymes of cotyledons were led to sprouting. The variation in above characters would be useful for crop improvement programme and development of tolerance/ resistance in mungbean varieties against pre-harvest sprouting. Ultra-structural studies in understanding and determining the factors for pre-harvest sprouting of seeds in various crops are available. However, majority of the scanning electronic microscopy studies are in the lines of understanding the physiological biochemistry of pre-harvest sprouting. In a study by Cai and Chen (2008), it was reported that the activities of α-amylase, IAA, GA1+3 and ZR were higher in easy-germinated rice than in uneasy-germinated rice. However, ABA content was lower in easy-germinated rice than in uneasy-germinated ones. The research findings further indicate that pre-harvest sprouting in rice is related to α-amylase, endogenous hormone contents and glume structure. However, research on ultra-structural studies using Scanning Electronic Microscopy in determining factors for pre-harvest sprouting in mungbean is scanty.

In a study using SEM in wheat, it was observed that starch granules in sprouted seed samples were partially hydrolyzed. Further, the high-performance size exclusion chromatography (HPSEC) profiles indicated that the starch of sprouted samples had relatively lower molecular weight than that of non-sprouted samples. Overall, the results indicated that α-amylase activity caused changes to the physicochemical properties of the pre-harvest sprouting damaged wheat (Simsek *et al.* 2014).

Fresh seed dormancy is a desirable trait in mungbean to overcome the problem of pre-harvest sprouting. Earlier research on fresh seed dormancy in mungbean indicated developing cultivars with a fresh seed dormancy of 10 to 15 days could curtail the losses associated with pre-harvest sprouting (Lamichaney *et al.* 2017). The biochemical activities that determine the fresh seed germination (FSG) and high pre-harvest sprouting in mungbean genotypes also are to be investigated for reducing the yield losses. It is established that high activity of α-amylase is responsible for high fresh seed germination and pre-harvest sprouting. For rapid identification of genotypes with varied levels of fresh seed dormancy and pre-harvest sprouting, α-amylase can be an effective biochemical marker (Lamichaney *et al.* 2017).

Despite identification of mungbean genotypes with high degree resistance to pre-harvest sprouting based on

desirable plant, seed, pod traits, ultra-structural studies and biochemical activities such as low α-amylase activity, it is necessitated to thoroughly screen the genotypes at field level. Several researchers have evaluated the mungbean germplasm at field level for establishing the pre-harvest sprouting tolerance (Singh *et al.* 2017). For field resistance to pre-harvest sprouting, availability of suitable donor germplasm lines in breeding programmes is mandatory.

Recent research findings in other crops also suggest the application of genetic tools in imparting pre-harvest sprouting resistance. For example, in wheat, genetic loci that determine pre-harvest sprouting resistance were identified that facilitate breeding for sprout-resistant wheat cultivars. These results on molecular approaches not only provide genetic resources for pre-harvest sprouting resistance, but also the important breeding tools for marker-assisted selection based on genome-wide linkage mapping for pre-harvest sprouting using 15K SNP arrays (Lingli li *et al.* 2021).

CONCLUSION

Overall, our results suggest the multi-faceted approach in establishing the resistance/ tolerance of mungbean genotypes with respect to pre-harvest sprouting. Since the scanning electron microscopy studies have established ultra-structural features that determine the resistance to pre-harvest sprouting of mungbean, development of future resistant lines should be identified for these ultrastructural features by adapting field level screening of existing mungbean genotypes. Further, wherever genotypic variation for resistance to pre-harvest sprouting is not available, spraying of Nacl@ 2.0%, Coumarin @ 10 ppm and Mo @200ppm may be advocated for inhibiting pre-harvest sprouting in susceptible mungbean genotypes since these chemicals promisingly inhibited pre-harvest sprouting in our studies. Further research may be conducted in these lines to find out appropriate ecofriendly chemical substances either of organic or inorganic that effectively inhibits the pre-harvest sprouting in any crop genotypes.

Conflict of interest: None.

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