Allelopathic inhibitors in seeds kernel cause dormancy variation between hard and soft Sapindus mukorossi seeds

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Research Article

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Abstract

Seed dormancy helps seeds to survive in nature but often reduces uniform germination under cultivation. In *Sapindus mukorossi*, seed dormancy is influenced by both physical and chemical factors. Hard seeds of *S. mukorossi*, which remain firm even after prolonged soaking, germinate earlier, while soft seeds that soften after soaking exhibit delayed germination. To unravel the mechanisms underlying dormancy variation, we combined allelopathic bioassays, UPLC–MS/MS–based metabolite profiling, and SEM-guided anatomical characterization of the seed coat. Bioassay experiments revealed that soft-seed kernel extracts of *S. mukorossi* caused strong allelopathic inhibition in Chinese cabbage seeds germination. The 100% soft-seed kernel extract (100g/L) reduced germination percentage to only 6%. In contrast, the 100% hard-seed kernel extract showed much weaker allelopathic effects, with germination percentage reaching up to 100%. At 100% concentration, soft-seed kernel extracts completely inhibited root growth while soft-seed coat extracts allowed limited root elongation, with roots reaching to 2.9 cm. Metabolomic profiling identified 1,120 metabolites in soft and hard seed kernels, of which 129 differed significantly between the two seeds tissue. Notably, soft seed kernels were enriched in 33 unique flavonoids and 11 unique alkaloids, including Hesperetin-7-O-glucoside, (-)-epicatechin, phlorizin derivatives, strictamine, methyl dioxindole-3-acetate, hexadecanamide, and caffeine. These compounds act as allelochemicals and inhibited germination and seedling growth. SEM showed that soft seeds had a rough outer surface with palisade layers containing spaces, crystal-like formations, and a porous endotesta. In contrast, hard seeds exhibited a porous outer surface but a highly compact palisade layer and interconnected endotesta. Overall, our results indicate that delayed germination in *S. mukorossi* seeds is primarily caused by allelopathic inhibitors accumulated in the seed kernel rather than by physical restrictions of the seed coat.

Introduction

Seed dormancy is a vital adaptive strategy in plants, enabling seeds to delay germination until environmental conditions are favorable, thereby enhancing seedling survival [1]. However, prolonged dormancy can pose significant challenges for propagation and large-scale cultivation, particularly in economically and ecologically important species [2]. One such specie is *S. mukorossi* Gaertn., commonly known as soapberry, valued for its medicinal, industrial, and ecological applications. Despite its commercial significance, the species exhibits low germination rates, limiting its effective propagation and cultivation [3].

Seeds dormancy arises from a combination of physical, physiological, and chemical factors. Physical dormancy occurs when the seed coat is impermeable to water, preventing imbibition and delaying germination. This is commonly associated with hard-seeded varieties, where lignified, thick-walled cells act as a mechanical barrier to water and gas exchange [4, 5]. However, physical dormancy alone does not fully explain delayed germination, as some seeds that absorb water readily still fail to germinate, indicating the presence of additional physiological or chemical constraints. Chemical dormancy involves the presence of inhibitory compounds within the seed tissues that suppress germination by modulating metabolic and hormonal pathways [6]. Secondary metabolites, including flavonoids, phenolics, alkaloids, and tannins, have been shown to influence dormancy by altering hormone balance, enzyme activity, and oxidative stress in embryos [7]. For instance, these compounds may inhibit gibberellic acid biosynthesis critical for breaking dormancy or enhance abscisic acid accumulation, which maintains dormancy by suppressing growth-promoting signals [8].

Moreover, allelopathic compounds within seeds may further inhibit germination. High concentrations of phenolics and flavonoids can suppress germination of both conspecific and heterospecific seeds, potentially serving as a self-inhibitory mechanism to ensure seedling establishment under optimal conditions [9]. Despite increasing research

on *S. mukorossi* dormancy, the interplay between physical barriers and chemical inhibitors remains underexplored. However, the role of hard seed coats in water impermeability has been studied. [10]. This study aims to elucidate the dual role of physical and chemical barriers in *S. mukorossi* seed dormancy by combining detailed analyses of seed coat structure with allelopathic bioassays and metabolomic profiling of the seed kernel. By identifying the key structural and biochemical factors responsible for delayed germination, our work provides critical insights for overcoming dormancy and optimizing germination protocols for large-scale propagation.

Materials and methods

Collection of S. mukorossi seeds

The seeds were collected from the new variety of *S. mukorossi*, called 'Yuanhua' growing at Jianning County, Sanming City, Fujian Province in 2022 (26°49' N latitude, 116°52' E longitude, 300 m above sea level), and stored in the National Energy R&D Center at 4ºC in the seed cabinet. The seeds were collected by breaking the pericarp of the fruit. The seeds used in this study were cultivated by our research group in the Soapberry National Forest Germplasm Banks (affiliated to the National Forestry and Grassland Administration). The collection of seeds from Soapberry National Forest Germplasm Banks has been approved by the National Forestry and Grassland Administration. The representative herbarium of *Sapindus mukurossi* in this study is deposited in Chinese Virtual Herbarium, the voucher number is PE 01391570. The species was identified by Prof. Liming Jia, School of Forestry, Beijing Forestry University, China.

Rolled paper towel and sand storage imbibition method for the germination of S. mukorossi seeds

After seed collection, the seeds were washed with tap water, and 100 seeds were soaked in water for 14 days. Hot water (initial temperature 80°C) was used for the first 3 days, followed by 11 days in normal water with daily water changes. After soaking, 30 soft and 30 hard seeds were wrapped in double-layer sterilized damp muslin cloth, rolled, placed in zip-lock bags to maintain humidity, and kept in a germinator at 25°C. Water was sprinkled on the cloth to keep it moist, and germination was observed for 2 months.

For sand imbibition, a 3–4 cm layer of wet sand (9–11% moisture) was placed in a tray, followed by a layer of seeds and another layer of sand. The tray was kept in a refrigerator at 4°C for 15 days while maintaining moisture. Afterward, soft seeds (enlarged due to water absorption) were separated from the sand, and the remaining seeds were considered hard. Both treatments (30 soft and 30 hard seeds) were sown in pots containing sandy loam soil at a depth of 0.5–1.0 cm. Pots were kept in a greenhouse under partial sunlight, watered with a hand sprayer, and seedling emergence was recorded for 2 months.

Preparation of aqueous extract from soft and hard S. mukorossi seeds

After collection of soft and hard seeds, the seed coat and seed kernel were separated and freeze dried in lyophilization machine for 3 days at 45ºC temperature and 1pas pressure (Bhatta, Stevanovic Janezic, & Ratti, 2020). After freeze drying, the extract was prepared from both hard and soft seeds coat and seeds kernel separately. The freeze-dried samples were grounded using ball miller grinding machine (MM400; Retsch, Arzberg, Germany) to make the powder. Powder was sieved with 8.0 mm size wire mesh and large particles were removed. The 100g of seed coat and seed kernel powder was soaked separately in one liter of water in a beaker for 72hrs (Fig. 1). After this the extract was stirred with a magnetic stirrer for 24hrs, then filtered through Whatman filter paper using funnel and muslin cloth into conical flasks to obtain the final volume of 1litre. Aqueous extracts of 25, 50, 75, and 100% concentrations were prepared by diluting the original 100% extract with distilled water: 25% (25 mL

extract + 75 mL water), 50% (50 mL extract + 50 mL water), 75% (75 mL extract + 25 mL water), and 100% (undiluted extract).

Seed bioassay test of S. mukorossi extracts on cabbage seeds germination and seedling growth

To evaluate the allelopathic effects of extracts from soft and hard *S. mukorossi* seeds, the germination tests were conducted using Chinese cabbage (*Brassica oleracea*) seeds. A total of five treatments, including 0%, 25%, 50%, 75%, and 100% extract concentrations were used. For each treatment, 30 seeds of cabbage were used with three biological replicates. The seeds were surface-sterilized with 10:1 sodium hypochlorite solution for 2 min and washed thoroughly with distilled water [11]. Seeds were placed in each of the five Petri dishes (each with the surface area of 70.8 cm^2) with different concentrations of extracts. All petri dishes were placed in a lighted room at 24 ºC in germinator. Each petri dish was moistened after two days with 5ml of different concentrations of extract treatments. The Number of germinated seeds were recorded and counted for 14 days. Dates of seeds germination each day was recorded, and the lengths of roots and hypocotyl were measured with a digital caliper. The germination percentage (GP) and mean germination time (MGT) was calculated by the following equation,

GP (%) = Ni/N × 100

$$\mathrm{MGT} = \Sigma\left(\mathrm{d} \times \mathrm{n}\right)/\Sigma\,\mathrm{n}$$

where Ni is the number of germinated seeds in the 14th day, N is the total seed number, d is the number of seeds emerging on a given day and n is the time after setting the seeds for germination. The roots and hypocotyl length of the germinated seeds of cabbage were measured using vernier caliper. The roots and hypocotyl were excised from the seedlings 14 days after the start of the germination test and measured for their length in cm.

UPLC–MS/MS-based qualitative phytochemical profiling of soft and hard seed kernels

Ultra-Performance Liquid Chromatography coupled with Tandem Mass Spectrometry (UPLC-MS/MS) was performed to identify metabolites responsible for allelopathy in *S. mukorossi* seed kernels. After separating the kernels from the seeds, the samples were freeze-dried using a vacuum lyophilizer (Scientz-100F). The dried samples were then ground into powder using a grinder (MM 400, Retsch) at 30 Hz for 1.5 min. Next, 50 mg of the powdered sample was weighed using an electronic balance (MS105DM) and extracted with 1,200 µL of pre-cooled 70% methanol containing an internal standard (kept at -20°C; extraction ratio: 1,200 µL per 50 mg sample). The mixture was vortexed for 30 s every 30 min, six times in total. After centrifugation at 12,000 rpm for 3 min, the supernatant was collected, filtered through a 0.22 µm microporous membrane, and stored in an injection vial for UPLC–MS/MS analysis. The sample extracts were analyzed using a UPLC–ESI–MS/MS system equipped with a triple quadrupole–linear ion trap (QTRAP) mass spectrometer. Chromatographic separation was achieved on an Agilent SB-C18 column (1.8 µm, 2.1 × 100 mm). The mobile phase consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The gradient program began at 95% A and 5% B, followed by a linear transition to 5% A and 95% B within 9 minutes, which was maintained for 1 minute. The conditions then returned to 95% A and 5% B within 1.1 minutes and were held for another 2.9 minutes. The flow rate was 0.35 mL/min, the column temperature was maintained at 40°C, and the injection volume was 2 µL. The UPLC effluent was introduced into the ESI source of the QTRAP MS for tandem mass spectrometric detection.

ESI-Q TRAP-MS/MS

The ESI source was operated at 500°C with an ion spray voltage of 5500 V (positive) and − 4500 V (negative). Ion Source Gas 1 (GS1), Ion Source Gas 2 (GS2), and Curtain Gas (CUR) were set to 50, 60, and 25 psi, respectively, and Collision-Activated Dissociation (CAD) was set to high. Data were acquired in multiple reaction monitoring (MRM) mode using nitrogen as the collision gas (medium). Declustering Potential (DP) and Collision Energy (CE) were optimized for each transition, and scheduled MRM was applied based on retention time windows.

Metabolite profiling and analysis

Unsupervised PCA (principal component analysis) was performed by statistics function prcomp within R (www.r-project.org). The data was unit variance scaled before unsupervised PCA. The HCA (hierarchical cluster analysis) results of samples and metabolites were presented as heatmaps with dendrograms. For HCA, normalized signal intensities of metabolites (unit variance scaling) are visualized as a color spectrum. For two-group analysis, differential metabolites were determined by absolute Log_2FC ($Log_2FC \geq 1.0$). Identified metabolites were annotated using KEGG Compound database (http://www.kegg.jp/kegg/compound/), annotated metabolites were then mapped to KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html).

SEM analysis of S. mukorossi seeds:

Scanning electron microscopy (SEM) was employed to examine the inner and outer surfaces of the seed coat in both soft and hard *S. mukorossi* seeds to assess potential physical barriers to germination. Seed selection for soft and hard categories followed the same criteria as described in the seed bioassay test. After collection of soft and hard seeds, the seeds were dried using freeze drying machine for 3 days at 45ºC temperature and 1pas pressure [12]. From hard and soft seeds,5 samples were selected. The removed seed coat was cut into 2 mm pieces to observe the outer seed coat surface, middle testa and the hilum area. Samples were then mounted on table with double-sided tape and placed on the revolving discs of Joel fine coat ion sputter (Joel, JFC 1100). Afterwards, the seeds were gold-coated with a gold sputter coat instrument (HITACHI E-1010) [13]. Specimens' stubs were then fixed to the specimen holder of Scanning Electron Microscope (Joel JSM 350) maintained at accelerating potential voltage of 15 KV. Images were processed and analyzed under SEM.

Statistical analyses:

Germination and seedling growth bioassays were conducted in a complete randomized design (CRD) with three replications. The data was subjected to analysis of variance (ANOVA). The significant differences between treatments means were separated using Tukey's test ($P < 0.05$).

Results

Effect of imbibition treatment on S. mukorossi seeds germination

A range of standardized methods are available for evaluating seed health and germination behavior. In our study, after imbibition in water and wet sand, rolled paper towel and soil pot methods were used to compare germination timing of soft and hard *S. mukorossi* seeds respectively. However, despite faster imbibition in both methods, soft

seeds showed significantly delayed germination. The average time of germination of soft seeds was approximately 55 days, whereas hard seeds germinated within an average of 32 days (Fig. 2).

These findings indicated that rapid water uptake does not necessarily enhance germination; instead, excessive or uncontrolled imbibition may inhibit the initiation of germination and subsequent seedling emergence. Based on these findings, soft and hard seeds extracts were used for seed bioassay tests.

Effects of soft and hard seed extracts on GP and MGT of cabbage seeds:

The soft-seed extracts significantly influenced the GP and MGT of cabbage seeds. By the 3rd day, most seeds treated with soft seed coat extract were germinated, however treated with soft seed kernel extract were unable to germinate. Even by the 14th day, seeds treated with the soft-seed kernel extract remained ungerminated, demonstrating a strong and persistent inhibitory effect (Fig. 3A). The hard-seed coat and kernel extracts showed only mild inhibitory effects, with most seeds germinating by the 3rd day but with seeds kernel effect failed to grow further (Fig. 3B). Cabbage seeds, at 100% extract concentration, the soft seed–coat extract resulted in 90% GP, whereas the soft seed–kernel extract reduced GP to just 6% (Fig. 3C).

In comparison, *S. mukorossi* hard seed coat and kernel extracts affected cabbage seeds germination but hard seeds coat and kernel extract was less significant. Both the hard seed–coat and kernel extracts maintained high GP at all concentrations, showing no difference between the two and remaining comparable to the control (Fig. 3D). MGT increased with concentration, remaining low with the soft seed–coat extract (1.53–2.0 days) but rising markedly with the soft seed–kernel extract (2.6–3.3 days), indicating stronger inhibition (Fig. 3E). In comparison with hard seeds extract showed lower MGT overall, with the seed–coat extract increasing MGT slightly (1.6–1.8 days) and the hard seed–kernel extract raising it from 1.7 to 2.1 days (Fig. 3F).

Impact of soft and hard seed extracts on cabbage seedlings:

Morphological changes in seedlings highlighted the strong contrast in allelopathic activity between soft and hard seed coat and kernel extracts (Fig. 4). In the soft seed coat treatments (Fig. 4A), cabbage seedlings exhibited reduced hypocotyl and root growth at the 100% concentration. In contrast, soft seed kernel extracts (Fig. 4B) caused severe suppression and seeds were failed to germinate, demonstrating that the soft seed kernel contains the strongest allelopathic compounds. Hard-seed extracts produced comparatively weaker effects. Under hard-seed coat extract, cabbage seedlings showed shorter root and hypocotyl length than control seedling (Fig. 4C). Hard seed kernel extract (Fig. 4D) caused more inhibition than the hard seed coat extract, at 50% kernel extract concentration allowed only cotyledon expansion without hypocotyl and root development, while at 75% and 100% concentrations the seeds merely sprouted but failed to grow.

Cabbage root length (RL) under the 100% soft seed coat extract was 2.9 cm, markedly shorter than the 6.3 cm observed in the control while with soft seed kernel extract caused a sharp reduction and nearly complete inhibition from 25% to 100% extract concentrations (Fig. 4E). Cabbage RL treated with hard seed coat extract was less affected compared to hard seed kernel extract. Hard seed kernel extract caused a significant effect on RL, decreasing from 6.2 cm at 0% extract to 0.01 cm at all concentration of extract (Fig. 4F).

The aqueous extracts of *S. mukorossi* soft seed kernel extract exhibited significant effects on hypocotyl length (HL) of cabbage seeds. Soft seed kernel extract was more allelopathic than soft seed coat extract and severely inhibited

cabbage seeds, with germination completely suppressed at concentrations from 25% to 100% (Fig. 4G). Hard seed coat extract caused a gradual decrease in HL from 3.3 cm at 0% extract to 1.8 cm at 100% extract. However, hard seed kernel extract drastically reduced HL from 3.4 cm at 0% extract to 0.01 cm at 25 and 50% extract concentrations (Fig. 4H).

Qualitative phytochemical analysis by UPLC MS/MS

The soft seed kernel extract caused stronger germination and growth inhibition in cabbage seeds than the hard seed kernel extract, whereas both seed coat extracts had only limited effects. Based on these biological responses, we focused on the seed kernels to investigate the chemical basis of their allelopathic differences. UPLC–MS/MS qualitative metabolomics was conducted to characterize the key phytochemicals present in soft and hard seed kernels. Total ion current chromatograms demonstrated stable signal detection across time points for each sample, with smooth baseline peaks (Fig. 5A, B). Principal component analysis (PCA) was performed to accurately distinguish the two groups of *S. mukorossi* seeds based on seed kernel composition. PCA analysis showed clear separation between soft and hard seed kernels, with both groups clustering distinctly apart, indicating pronounced differences in their metabolite composition. Cumulative contribution rate of PC1 and PC2 is 61.44%, with 36.28% going to PC1 and 25.16% to PC2 (Fig. 5C). A total of 1,120 metabolites were identified from them 129 metabolites were significant including 40 upregulated and 89 were downregulated in soft seeds kernel compared to the hard. Among the identified metabolites, alkaloids (13.23%) and flavonoids (11.44%) represented the most abundant classes, indicating their dominant contribution to the overall seed kernel metabolome (Fig. 5D). To visualize the overall metabolite distribution, a heatmap was constructed using all identified metabolites. The heatmap revealed distinct metabolic profiles between the soft and hard seed kernels, with the majority of metabolite classes exhibiting higher accumulation in the soft seed kernel (Fig. 5E, F). Moreover, 33 identified flavonoids and 11 alkaloids (Tables 3 and 4) were found to be abundant and uniquely enriched in the soft seed kernels, showing markedly higher intensities than in hard seeds kernel. The fold change of metabolites of two seeds kernel was compared and analyzed, Bar graphs were plotted using the data after the multiplicity of variance Log_2 treatment; we found that the top 20 metabolites with the highest multiplicity of variance were Sazp004372 (Bursehernin), pma6298 (3-Hydroxypyridine), Wmhp000055 (2,5-Dimethoxybenzoquinone) and others (Fig. 5G). All 129 significant metabolites were classified into two distinct subclasses based on their accumulation patterns. Subclass 1 included metabolites that were highly accumulated in soft seed kernels, whereas Subclass 2 contained metabolites showing higher levels in hard seed kernels (5H). A broader list of differentially a up and down regulated metabolites provided in Table 1.

Table 1. Top up and down regulated metabolites in soft seeds kernel compared with hard seed kernel.

Index	Compound	Type	Seed kernel
Lmcp002302	N6-(2-Hydroxyethyl)adenosine*	down	hard
Hmyp002656	Methyl dioxindole-3-acetate	down	hard
Lhmp110108	1-((Pentanoyl)phloroglucinyl]-β-D-glucopyranoside	up	soft
Lasp002993	Isobavachalcone glucoside	up	hard
YC512118	Oleamide (9-Octadecenamide)	down	soft
pme1474	5'-Methylthioadenosine	down	hard
Hamp011640	Hexadecanamide	down	soft
MWSHY0203	(+)-Catechin*	up	hard
Zbkp003359	Fisetinidol-4β-ol*	up	hard
MWSslk148	Rutin Trihydrate	down	hard

Enrichment Analysis of Differentially Expressed Metabolites.

According to KEGG functional classification, the majority of the identified metabolites were categorized under metabolism category predominantly enriched in pathways related to the biosynthesis of secondary metabolites to the criteria of fold change of values ≥ 2 or ≤ 0.5 and VIP values ≥1. Only a small fraction of metabolites was associated with MetMap and Environmental Information Processing categories (Fig. 6A). The KEGG enrichment plot highlights the top 20 significantly enriched pathways in soft and hard seeds kernel. The KEGG enrichment plot showed that metabolites were mainly concentrated in secondary metabolite biosynthesis pathways, with the highest rich factors observed in diterpenoid, flavonoid, caffein metabolism and flavone and flavonol biosynthesis. These results highlight that secondary metabolism, especially flavonoid-related pathways, was the most active and significantly enriched category among the identified metabolites (Fig. 6B). Given the pronounced accumulation of flavonoid- and alkaloid-related metabolites revealed by metabolomic profiling, a KEGG pathway heatmap was constructed to enable a direct comparative analysis of these biosynthetic pathways between the soft and hard seed kernels. The analysis showed that metabolites associated with both flavonoids and alkaloids biosynthesis were markedly enriched in soft seeds kernel. To investigate the metabolic differences between soft and hard seeds kernel, we constructed a heatmap of flavonoids metabolites. The analysis revealed higher accumulation of flavonoids metabolites in soft seeds kernel compared to hard seed kernel (Table 2). In particular, MWSHY0174 ((-)-Epicatechin), Lmzp002365 (Hesperetin-7-O-glucoside) and mws2118 (Phlorizin) were highly enriched flavonoid in soft seeds kernel (Fig. 6C). In addition to flavonoids, the metabolomics data revealed that several alkaloids biosynthesis metabolites accumulated to markedly higher levels in soft seeds kernel compared to hard seeds (Table 3). Notably, Wdgp004715 (strictamine) Hmyp00265 (Methyl dioxindole-3-acetate), mws2218 (Caffeine), Zahp012577 (Octadecadienamide), Wccp011838 (Octadec-8-enamide*) Hamp011640 (Hexadecanamide) and others were among the most enriched alkaloids in soft seeds kernel (Fig. 6D). In addition to these significant metabolites, several other differential metabolites belong to other class of metabolites were also accumulated in

higher quantity in soft seeds kernel named as Cmmn013378 (Levopimaric acid), Lmyjn03753 (Tropate), Zmln002252(Sideretin) and Cmmn012997 (Abietate) (Fig. 6E).

Table 2
Detail of unique flavonoids compounds in soft seeds kernel.

#	Q1 (Da)	Q3 (Da)	Molecular weight (Da)	Ionization model	Compounds
1	611.16	303.06	664.1851	[M-3H2O + H]+	Rutin Trihydrate
2	465.1023	303.0582	464.0955	[M + H]+	Hyperin
3	611.1607	303.0519	610.1534	[M + H]+	Quercetin-3-O-glucoside-7-O-rhamnoside
4	611.1607	303.0494	610.1534	[M + H]+	Quercetin-3-O-neohesperidoside
5	465.1391	303.09	464.1319	[M + H]+	Hesperetin-7-O-glucoside
6	465.1019	303.0613	464.0955	[M + H]+	Quercetin-3-O-alloside; Isohyperoside
7	465.1	303.05	464.0955	[M + H]+	Quercetin-5-O-β-D-glucoside
8	291.0863	139.0397	290.079	[M + H]+	(-)-Epicatechin
9	773.21	303.05	772.2062	[M + H]+	Quercetin 3-rutinoside-7-glucoside
10	741.23	433.12	740.2164	[M + H]+	Luteolin 7-rutinoside-4'-O-Rhamnoside
11	741.2237	433.1122	740.2164	[M + H]+	Robinin
12	435.0922	303.0505	434.0849	[M + H]+	Avicularin
13	465.1028	303.051	464.0955	[M + H]+	Isoquercitrin
14	465.1028	303.05	464.0955	[M + H]+	6-Hydroxykaempferol-7-O-glucoside
15	449.1078	303.0471	448.1006	[M + H]+	Quercitrin
16	435.0922	303.0522	434.0849	[M + H]+	Morin-3-O-xyloside
17	435.0922	303.0606	434.0849	[M + H]+	Quercetin-3-O-xyloside (Reynoutrin)
18	595.16	271.06	594.1585	[M + H]+	Apigenin 7,4'-diglucoside
19	611.16	303.05	610.1534	[M + H]+	Multinoside A
20	579.1709	285.0758	578.163	[M + H]+	Acacetin-7-O-glucosyl(1→4)xyloside
21	617.1137	303.0513	616.1064	[M + H]+	Quercetin-3-O-(6"-O-galloyl)galactoside
22	773.21	303.05	772.2062	[M + H]+	Quercetin-7-O-rutinoside-4'-O-glucoside
23	431.0984	285.0388	432.1056	[M-H]-	Kaempferol-3-O-rhamnoside (Afzelin) (Kaempferin)
24	463.0882	301.0363	464.0955	[M-H]-	Quercetin-4'-O-glucoside (Spiraeoside)*
25	463.1246	301.0707	464.1319	[M-H]-	Hesperetin-5-O-glucoside
26	463.0889	301.03	464.0955	[M-H]-	6-Hydroxyluteolin 5-glucoside*
27	451.13	289.07	452.1319	[M-H]-	Epicatechin-3'-O-β-D-glucopyranoside*
28	451.12	289.07	452.1319	[M-H]-	Catechin 4'-O-beta-D-glucopyranoside*

#	Q1 (Da)	Q3 (Da)	Molecular weight (Da)	Ionization model	Compounds
29	435.1297	167.0351	436.1369	[M-H]-	Phlorizin
30	463.0882	301.0357	464.0955	[M-H]-	Quercetin-7-O-glucoside
31	609.15	300.03	610.1534	[M-H]-	Quercetin-3-O-robinobioside
32	739.21	431.1	740.2164	[M-H]-	Vitexin-7-O-rutinoside
33	579.1744	433.1188	578.1636	[M + H]+	Isovitexin-2"-O-rhamnoside(2"-O-alpha-L-Rhamnopyranosyl-isovitexin)

Table 3
Detail of unique alkaloids compounds in soft seeds kernel.

Id	Q1 (Da)	Q3 (Da)	Molecular weight (Da)	Ionization model	Compounds
1	222.0754	130.064	221.0688	[M + H]+	Methyl dioxindole-3-acetate
2	256.26	102.09	255.2562	[M + H]+	Hexadecanamide
3	340.103	178.0495	339.0954	[M + H]+	6-beta-d-glucopyranosyloxyindole-3-carboxylic acid
4	181.12	107.09	180.115	[M + H]+	Octadec-8-enamide
5	280.26	81.07	279.2562	[M + H]+	Octadecadienamide
6	284.3	88.08	283.2875	[M + H]+	N-(12-methyltetradecyl)propionamide*
7	284.29	88.08	283.2875	[M + H]+	Octadecanamide*
8	282.2783	69.0703	281.2719	[M + H]+	Octadec-2-enamide*
9	195.0877	83.0604	194.0804	[M + H]+	Caffeine
10	323.1756	291.1495	322.1681	[M + H]+	Strictamine
11	443.15	161.06	442.14099999999996	[M + H]+	N5-(1-Carboxy-2-((3-(4-hydroxy-3,5-dimethoxyphenyl)allyl)thio)ethyl)glutamin

Structural Analysis of S. mukorossi Seed Coat Using SEM

Despite their smooth external appearance, *S. mukorossi* seeds possess a seed coat consisting of three layers: the outer testa, the palisade (macrosclereid) layer, and the inner parenchyma layer. SEM analysis revealed clear structural differences between soft and hard seeds. In soft seeds, the outer surface of seed coat was rough and uneven, with loosely arranged epidermal cells (Fig. 7A left). Their palisade layer appeared disorganized, consisting of elongated, rod-like macro-sclereids separated by visible air spaces. The cracks in soft seed coat were much wider than those of the hard seeds. The wider cracks in seed coat might be related to the relatively easy water permeability in soft seeds. (Fig. 7B left). Such gaps reduce mechanical continuity and increase permeability to water. The endotesta of soft seed exhibited a highly interconnected, net-like framework formed by hourglass cells (Fig. 7C left), further facilitating internal water movement. The hilum region of soft seed contained a linear opening surrounded by spongy tissue and a cone-shaped ring of lignified cells (Fig. 7D left), providing an additional entry

point for moisture. These structural features of soft seeds promote rapid imbibition, which may enhance the mobilization and activation of allelochemicals from the kernel. In contrast, the hard seed coat contained compact, densely packed cells and a uniform outer surface. The surface was covered by a thick cuticular layer, contributing to its low permeability. (Fig. 7A right). Their palisade layer was highly organized, with tightly aligned, elongated rod-like cells forming a continuous, non-porous barrier between the cuticle and the inner testa. So, the water absorption between the cells is inferior (Fig. 7B right). This uninterrupted palisade layer provides a strong mechanical shield and limits water penetration. The endotesta displayed a densely packed interlocking structure with smaller and more restricted pits (Fig. 7C right), contributing to a thicker and more rigid internal barrier. Although the hilum in hard seeds appeared rough, non-linear and not fully opened, its architecture did not offset the overall impermeability imposed by the palisade and endotesta layers (Fig. 7D right).

Figure 7 Comparative seed coat ultrastructure of soft and hard *Sapindus mukorossi* seeds under SEM. (A) Seed coat outer surface of soft and hard *S. mukorossi* seeds. (B) Palisade layer of soft and hard *S. mukorossi* seeds. (C) Endotsta hourglass cells of soft and hard *S. mukorossi* seeds. (D) Hilum structure of soft and hard *S. mukorossi* seeds.

These anatomical features indicate that hard seeds restrict water entry more effectively, thereby maintaining physical dormancy and promoting more controlled and timely germination when conditions become favorable.

Discussion

Seed germination is often regulated by dormancy mechanisms that ensure germination occur only under favorable conditions. Seeds may exhibit physical dormancy, caused by a hard, water-impermeable seed coat, or physiological dormancy, controlled by internal biochemical inhibitors or hormonal imbalance [4]. Building on this understanding, our study aimed to elucidate the mechanisms underlying both physical and physiological dormancy in *S. mukorossi* seeds. The stronger inhibitory effects of soft seed coat and kernel extracts on cabbage GP, MGT and seedling growth indicate that these tissues contain higher levels of allelochemicals than hard seed tissues. The elevated allelochemical content in soft seeds likely disrupts germination processes, as reflected by the sharp decline in cabbage GP to only 6% at the highest extract concentration. This concentration-dependent inhibition is consistent with previous reports showing that aqueous *Sesame* seed extracts can delay germination and suppress seedling growth of *Phyllostachys edulis* due to the presence of water-soluble allelochemicals (J Zhao, Z Yang, J Zou and Q Li [14]. The stronger inhibitory activity of soft seeds can be explained by their higher permeability and fast water absorption, which allow allelochemicals to dissolve and diffuse more readily into the surrounding environment [15]. As a result, active compounds such as saponins, phenolics, flavonoids, and alkaloids are released more quickly, leading to stronger suppression of germination and early seedling growth [16, 17]. In *D. ferox*, soaking seeds in water restricts gas exchange from and to embryo through the hilum, preventing oxidation of endogenous inhibitors and thereby inhibiting germination, whereas dry seeds do not show this effect [18]. Similarly, soft seeds of *S. mukorossi* imbibe water rapidly, which may limit oxygen availability and prevent the oxidation of allelochemicals, increasing their inhibitory effects on germination. Hard seeds, with their rigid and less permeable coat, absorb water slowly and release fewer metabolites, which greatly reduces their allelopathic impact [15]. Hard seeds often undergo a prolonged maturation process, during which some allelochemicals may degrade or become less bioavailable. This difference in chemical composition can result in reduced allelopathic activity in hard seeds compared to soft seeds [19, 20].

Roots and hypocotyl length of cabbage was more sensitive to inhibition when growing in the extract environment of soft and hard seed kernels compared with seed coat extracts. During imbibition, embryos release more solutes than

seed coats, creating a stronger allelopathic environment that suppresses root and hypocotyl growth. [21]. This aligns with previous findings that roots are particularly vulnerable to allelopathic effects due to direct and prolonged exposure to inhibitory compounds in the growth medium [22]. The stronger inhibition of roots caused by soft seeds kernel extracts can be attributed to higher concentrations of bioactive compounds which interfere with cell division, enzymatic processes, and hormonal balance, thereby suppressing root elongation [23].

Flavonoids and alkaloids act as key allelochemicals, suppressing germination by disrupting enzyme activity, water uptake, and hormonal regulation [24, 25]. In soft seed kernels of *S. mukorossi*, flavonoids that accumulated at high levels, including (-)-epicatechin, which strongly inhibits root growth, and rutin trihydrate, which predominantly affects shoot elongation [26–30]. Other highly accumulated flavonoids metabolites in soft seeds such as quercetin-3-rutinoside-7-glucoside and kaempferol-3-O-xylosyl(1,2)glucoside inhibit root and seedling growth in *Lactuca sativa* L Nogales, JC Alías, J Blanco-Salas, I Montero-Fernández and N Chaves [31] and *Arabidopsis thaliana* [32] by interfering with auxin transport and reactive oxygen species signaling. Similarly, soft seed kernel enriched luteolin-7-O-rutinoside flavonoid, identified as an allelochemical from *Castanea henryi* leaf extract, significantly reduced seed germination, seedling growth, chlorophyll content, and antioxidant enzyme activities in *Brassica pekinensis* and *Zea mays* at high concentrations [33]. Among the alkaloids, strictamine, methyl dioxindole-3-acetate, hexadecanamide, and caffeine were found at high levels in the soft seed kernels and are known for their allelopathic effects on DNA synthesis, respiration, and electron transport. Strictamine, a well-characterized compound from *Rhazya stricta* (Alstonia spp.), is biologically active and has been frequently reported to exhibit phytotoxic effect [34]. Hexadecanamide, often present in root exudates, is known to interfere with cell division and elongation, disrupting essential metabolic processes that are consistent with delayed germination [35].

In addition to the biochemical inhibition caused by high allelochemical content in soft seed kernels, SEM analysis revealed that soft seeds also exhibit distinct physical characteristics that may facilitate water uptake and influence germination. Soft seeds possess a rough, irregular outer surface with a disorganized palisade layer, intercellular spaces, crystal-like structures, and a porous endotesta. These features resemble those observed in *Vicia sativa*, where irregular palisade layers contribute to seed dormancy [29, 36]. The hilum in soft seeds is permeable and open, serving as the primary water entry point. An open hilum and cracked palisade layer were seen in the permeable seeds of Lima beans (*Phaseolus lunatus L.*) compared to nonpermeable seeds [37]. In contrast, hard seeds have a much more compact and rigid seed coat. The thicker outer surface and cuticle of hard seeds increase mechanical resistance and act as a barrier to water uptake, effectively limiting hydration and reducing the release of allelochemicals [38]. Although soft seeds of *S. mukorossi* absorb water rapidly due to their porous structure, germination remains inhibited, indicating that dormancy is not solely caused by physical barriers. Similar observations have been reported in *Cinnamomum migao*, where seeds are water-permeable but fail to germinate because their embryos contain inhibitory compounds that suppress germination in cabbage and rye grass seeds, even when physical dormancy is removed [39]. These findings suggest that physical traits of soft seeds enable fast water imbibition, which can prevent the oxidation of endogenous inhibitors and sustain their allelopathic effect, leading to delayed germination compared with hard seeds.

Conclusion

This study examined the physical and chemical barriers to *S. mukorossi* seed germination through bioassay tests, UPLC, and SEM analyses. The dormancy of soft seeds reflects a dual mechanism, combining potent chemical inhibitors with unique seed coat structures that distinguish them from hard seeds. The soft seed kernel contains high concentrations of flavonoids and alkaloids that contribute to the prolonged dormancy period. Although soft

seeds rapidly imbibe water and lack strong mechanical barriers, their germination is delayed by these chemical compounds, highlighting the dominant role of physiological dormancy. These results demonstrate that seed dormancy is largely governed by internal chemical factors, with seed coat impermeability playing only a secondary role. Understanding the function of endogenous inhibitors provides valuable insights for overcoming dormancy and improving germination efficiency in agroforestry and reforestation practices. In conclusion, alkaloid and flavonoid compounds within the seed kernel are identified as the primary contributors to dormancy in *S. mukorossi* seeds. Moreover, the strong inhibitory effects of soft seed kernel extracts on cabbage germination and seedling growth further confirm the presence of potent allelochemicals, highlighting the need for targeted biochemical approaches to enhance seed germination and seedling establishment.

Declarations

Ethics approval and consent to participate

All plant-related experiments were conducted in accordance with applicable institutional, national, and international regulations and guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests

Fundings

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Author Contribution

Areeba Bano and Muhammad Tahir contributed equally to this work. Areeba Bano performed the experiments, conducted data analysis, and wrote the initial draft of the manuscript. Muhammad Tahir assisted in experimental design, data interpretation, and manuscript revision. Jing Zhong supervised the experimental work. Mah Noor supported data analysis. Liming Jia conceived and supervised the study, provided funding and resources, and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

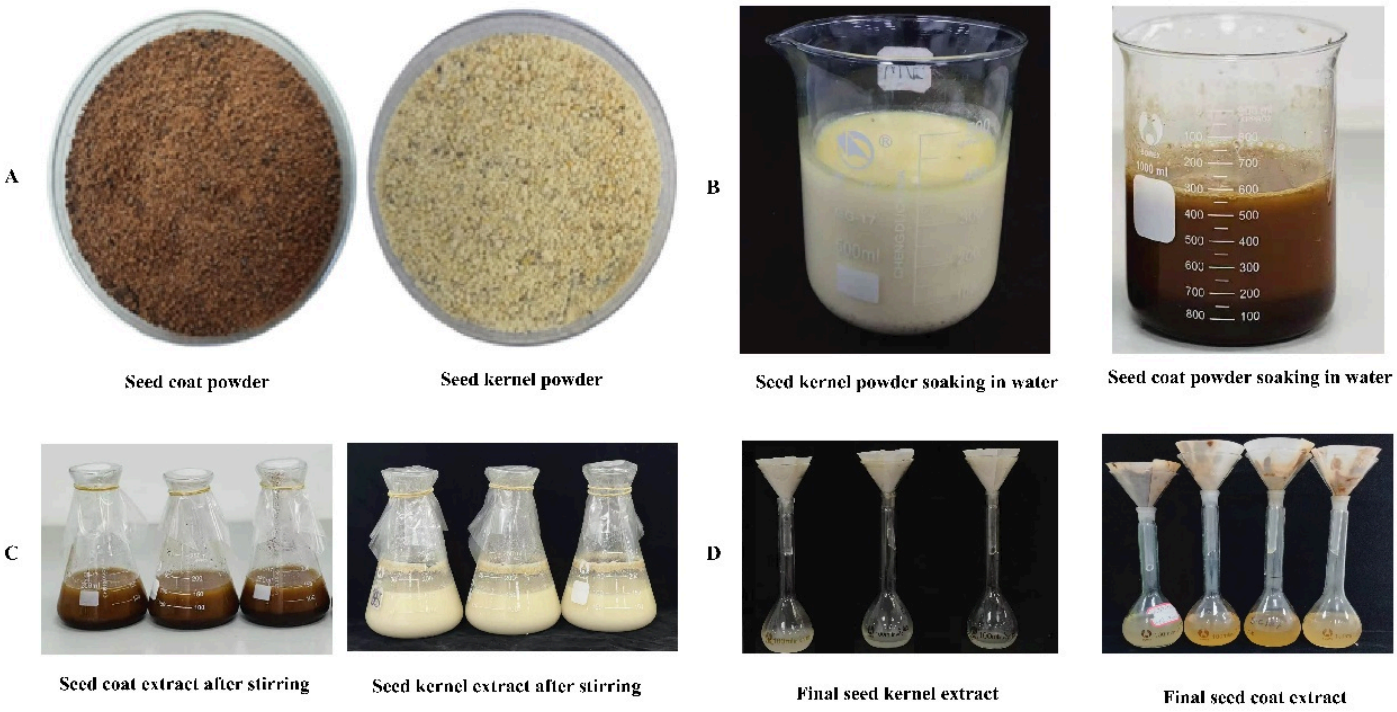

Figure 1

Grinding and preparation of extract from *S. mukorossi* soft and hard seed coat and seed kernel

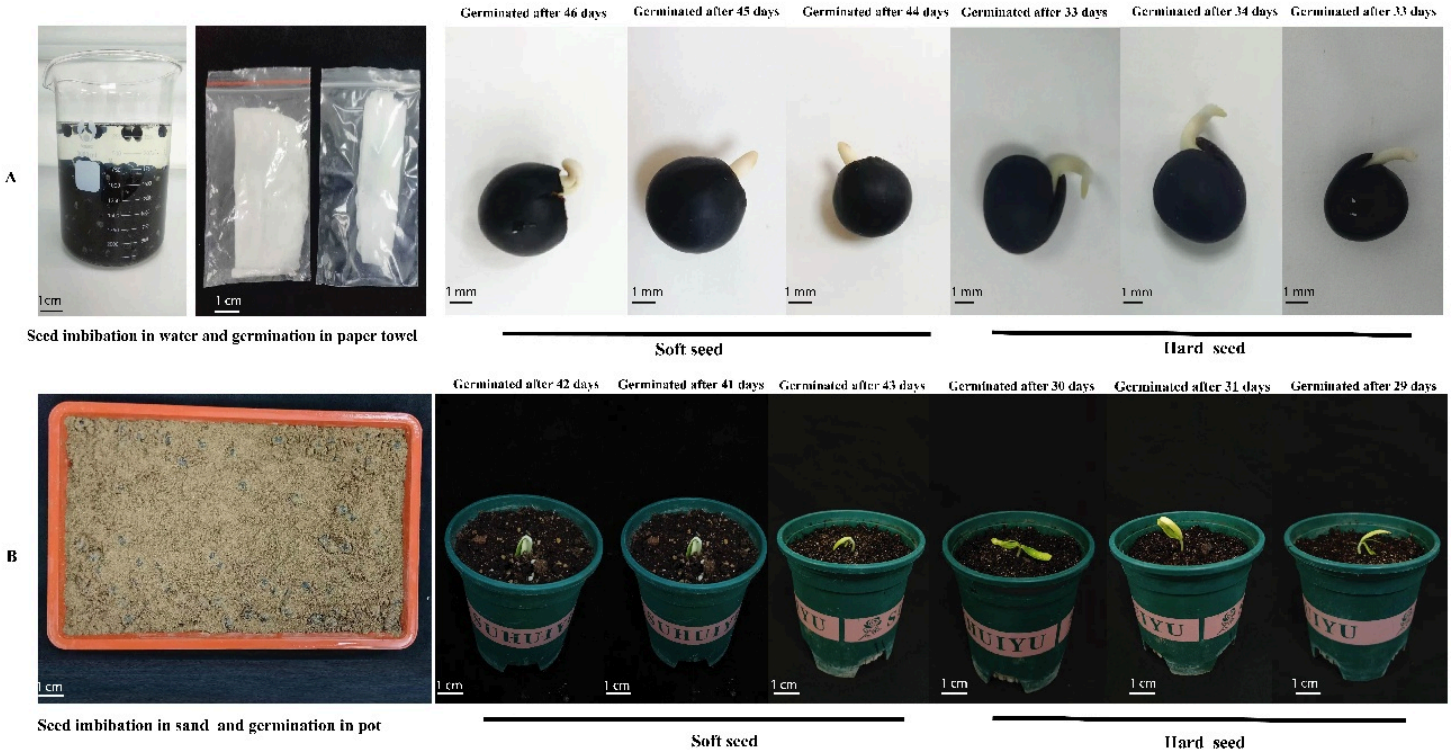

Figure 2

Water imbibition and germination assessment of *Sapindus mukorossi* seeds. (A) Seeds were soaked in water to separated soft and hard seeds and then the seeds were placed in rolled paper towel for germination test. (B) Seeds were placed in wet sand then soft and hard seeds were picked from sand and germination were checked out in pot soil.

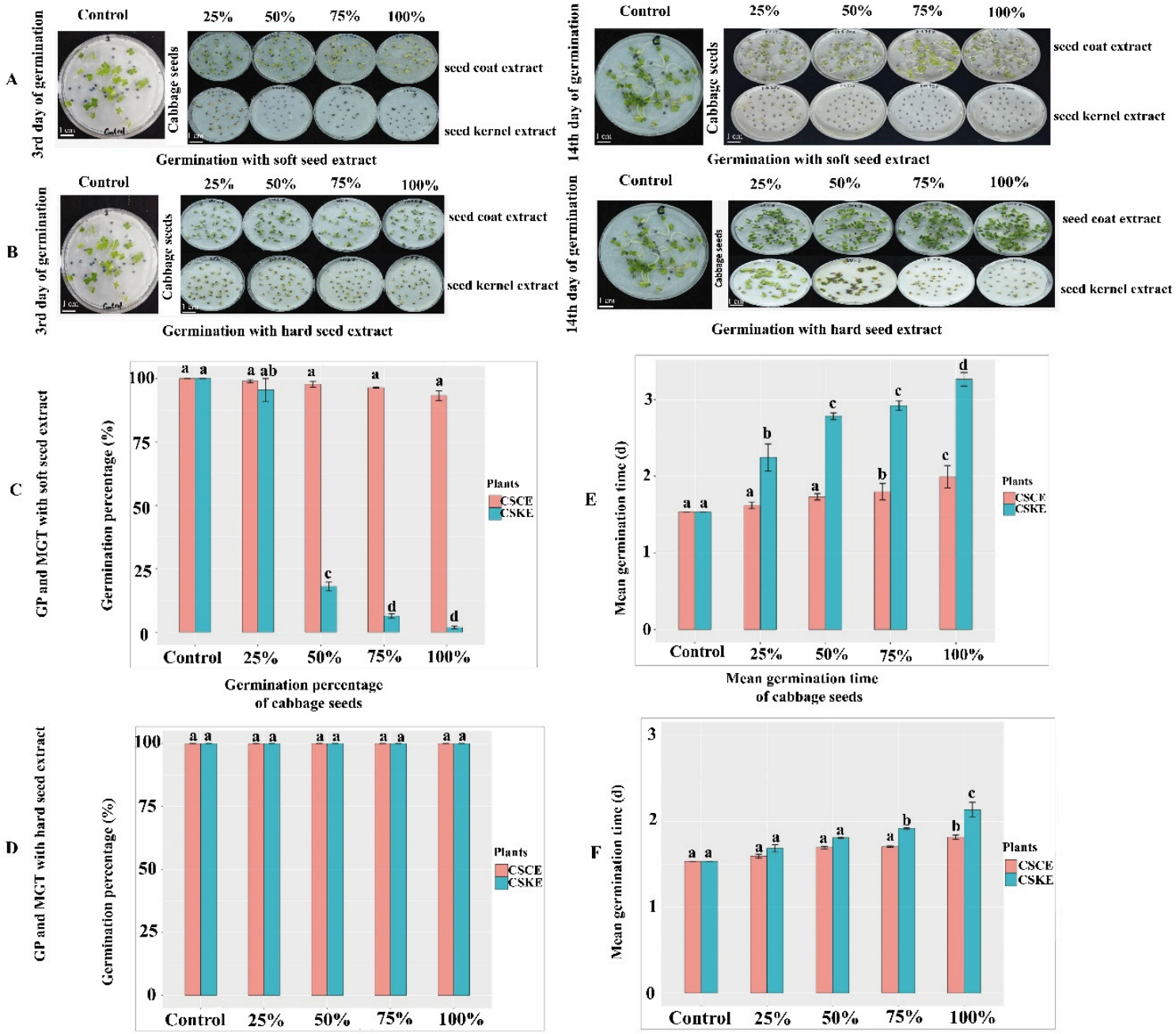

Figure 3

GP and MGT of cabbage seeds treated with *S.mukorossi* soft and hard seed coat and seed kernel extract. CSCE (effect of seed coat extract on cabbage), CSKE (effect of seed kernel extract on cabbage), (A) cabbage seeds germination at 3rd and 14th day treated with *S. mukorossi* soft seeds extract. (B) cabbage seeds germination at 3rd and 14th day treated with *S. mukorossi* hard seeds extract. (C) GP of cabbage seeds treated with *S. mukorossi* soft seeds extract. (D) GP of cabbage seeds treated with *S. mukorossi* hard seeds extract.

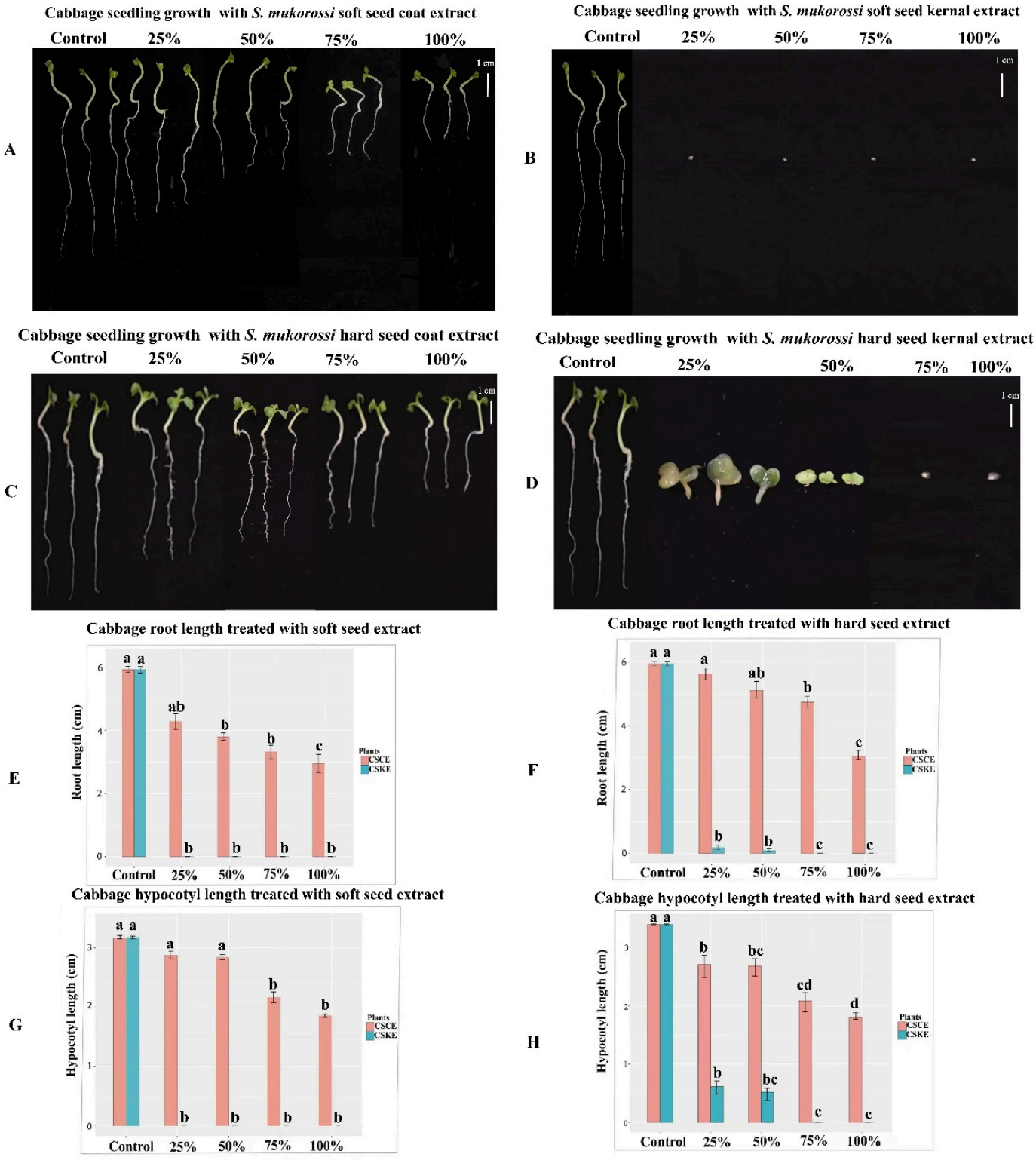

Figure 4

Roots and hypocotyl length of Cabbage seeds treated with S. mukorossisoft and hard seed coat and seed kernel extract. CSCE and CSKE indicates effect of seed coat extract and seed kernel extract on cabbage plants

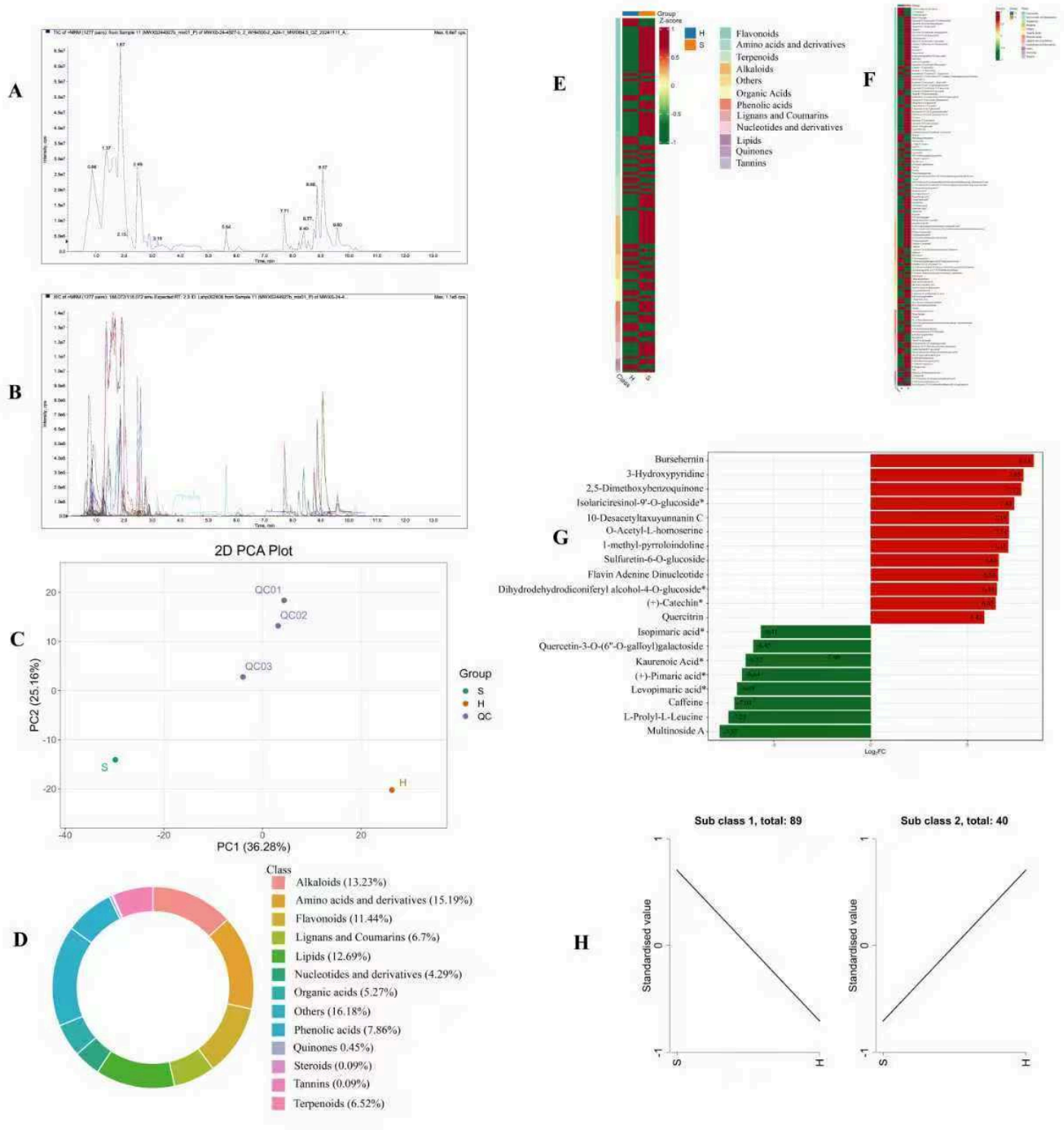

Figure 5

Metabolomics profile of hard and soft seed kernel. (A, B) Mass spectrometry of total ion current overlap plot (C) PCA analysis of S. mukorossi soft and hard seeds kernel (D) Class count ring of differential metabolites (E, F) Heat map showing the metabolite profiles of soft (S) and hard (H) seed kernels of S. mukorossi, with Z-scores indicating the relative abundance of metabolites across different classes. (G) Histogram of fold change of the top 20 metabolites after log2 treatment (H) Subclass of up and down regulated metabolites.

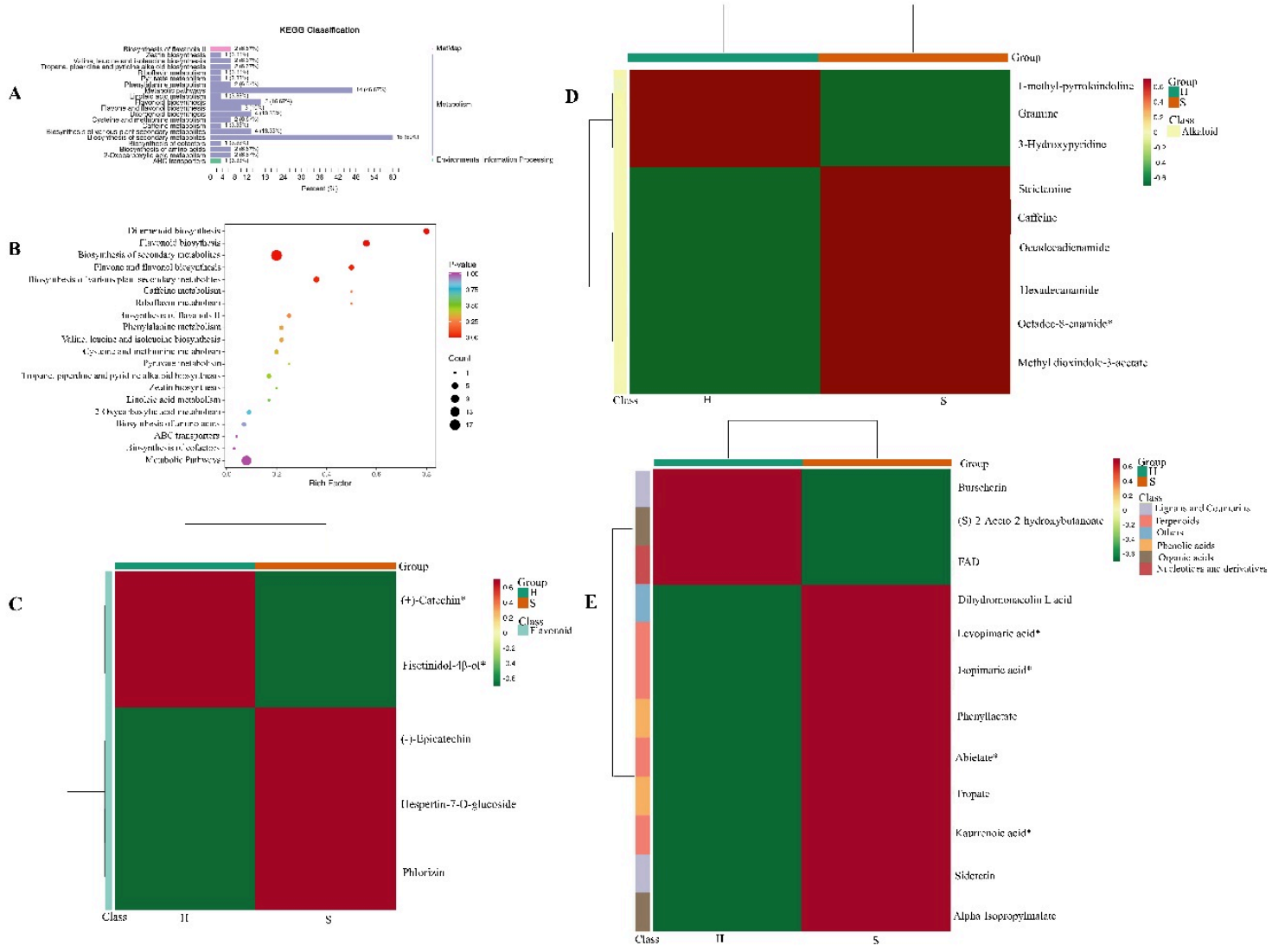

Figure 6

Differentially accumulated metabolites in soft and hard seed kernels. (A) KEGG classification of differential metabolites pathways (B) KEGG enrichment statistics of the differential metabolites in the soft and hard seeds kernel (C) Heatmap clustering of differential metabolites for flavonoids biosynthesis pathway (D) Heatmap clustering of differential metabolites for alkaloids biosynthesis pathway (E) Heatmap clustering of others differential metabolites biosynthesis pathways.

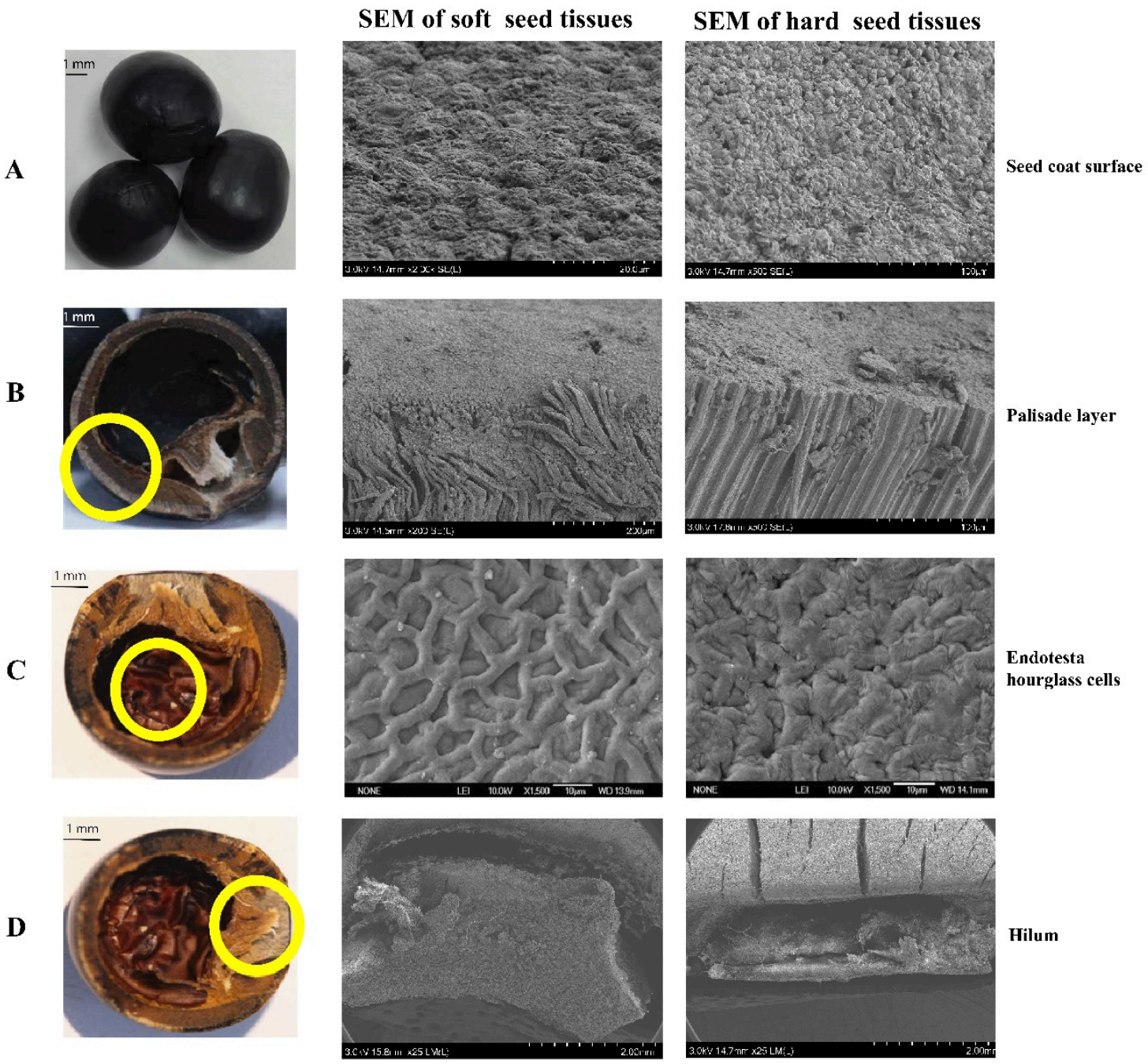

Figure 7

Comparative seed coat ultrastructure of soft and hard *Sapindus mukorossi* seeds under SEM. (A) Seed coat outer surface of soft and hard *S. mukorossi* seeds. (B) Palisade layer of soft and hard *S. mukorossi* seeds. (C) Endotsta hourglass cells of soft and hard *S. mukorossi* seeds. (D) Hilum structure of soft and hard *S. mukorossi* seeds