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Research Article
Optimization of Slow-Drying Conditions for Improving Short-Term Storage of Cacao (*Theobroma cacao*) Seeds

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Abstract

Background and Objective: The specific condition with slow-drying mechanisms of cacao seeds may prove to be a possible break through in improving the short-term storage of this recalcitrant species. This study aimed to evaluate the effect of storage conditions on the changes of physiological, microstructural and soluble sugars associated with slow-drying mechanisms in cacao seeds. **Materials and Methods:** Seeds from ripened cacao pods (PBC 123) were demucilaged, placed in zip-lock polyethylene bags and stored at 14 and 16°C (40 and 80% RH), RT (25°C) and control (freshly extracted seeds). **Results:** In this study, seeds at RT and 16°C, 40% RH showed a similar germination percentage as the control. Thus, it shall be convenient for the storability up to 12 days. The SEM micrographs proved that the mild dehydration in seeds of both treatments caused the least cells' morphological changes, which leads to lesser cell damage. However, seeds at RT reduced their storability due to 8-10% of germination occurrence during storage. Along with the results, seeds of both treatments maintained much lesser soluble sugars. The higher soluble sugars at the first 4 days after storage for seeds at 16°C, 40% RH than RT, reflected the negative feedback through the altered metabolisms during storage. Lesser respiration rate with more efficiency in utilizing seed reserves further leads to higher seedling performances for both treatments. **Conclusion:** This study recommended 16°C, 40% RH as the alternative storage condition for cacao seeds in at least 12 days due to their storability, least cells' damages and altered metabolisms.

Key words: Recalcitrant seed, hydrated storage, microstructural changes, osmotic cell homeostasis, carbohydrate reserves

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In Malaysia, cacao (*Theobroma cacao*) is considered one of the most important commodities traded globally. Despite the falling cacao bean production in recent years (2011-2018), Malaysia is still reputably known as the second-largest processing centre in Asia and the seventh-largest grinder in the world[1]. However, relying on cacao beans imported from other countries might reduce the potential of cacao bean production in Malaysia. The relevance of producing cacao beans in Malaysia, however, does not capture much attention from the local farmers due to the uncertain quality of the resource materials. As cacao produces abundant fruits regularly thus, little attention is given to the storing of seeds. However, the differences in the germination time of recalcitrant seed in the storage after shedding have marked effects on the degree of dehydration they will tolerate, which contributes to unpredictable variability[2].

Cacao trees are propagated using seedlings. Therefore, the handling (e.g., storage method) of good quality seeds is vital to ensure a continuous and cost-effective supply of tree seeds. Categorized as recalcitrance, cacao seeds are vulnerable to drying. Thus, it is best stored at its shedding water content[2]. Those seeds, especially the tropical species, are often chilling-sensitive and cannot be stored at temperatures below 15°C, which makes their long-term conservation difficult[3]. Therefore, the storage is strict as a short-term measure, from a few weeks to some months of many tropical recalcitrant species.

Researchers revealed the comparisons between two types of available recalcitrant seed storage after shedding; slow-drying is more harmful than fast-drying because the seeds maintain an intermediate water level for a more extended period and are susceptible to damage[4]. Holding seeds at the higher hydration level may lead to rapid degradation[5], particularly at higher temperatures, since all harmful reactions are likely to occur (e.g., chemical, metabolic). This might be due to the insufficient water to allow defence mechanisms in a completely hydrated molecular condition[5]. Hence, seed quality implications at the greater wet range and fully-imbibed seeds are based more on functional repair processes than water content per se. The slow-drying mechanism on recalcitrant seed quality during storage is still poorly understood. Thus, the continuous related research might be significant in establishing the suitable conditions for the easy-handled short-term storage of locally produced cacao seeds.

Recalcitrant-seeded species generally directly pass from development to germination due to the lack of a dehydration stage during seed ripening[6]. Those seeds usually keep organelles fully functioning and maintain active metabolism[7]. The slow-drying mechanism occurred due to the embryonic axis is slowly dehydrated naturally inside the seeds. The variable dehydration tolerance between various plant species was suggested to be due to the internal seed matrix's physical structure, which appears to include interactions between sugar and protein complexes with salts, organic acids and amino acids[8]. The damage of membrane phospholipids could occur in dry conditions via the chemical processes. Still, the consequences of membrane leakage or loss of organellar compartmentation are significant in a hydrated cell only when liquid water is present[5].

The drying rate effect on dehydration tolerance is related to the dehydration process (mechanical aspects) and metabolism control[9]. Following morphogenesis, developing seeds enter a 'maturation' stage, which seems to be a more active stage in gene expression and metabolism than the seed drying itself. Also known as 'reserves accumulation' periods, it involves re-organization the metabolism and storage compound synthesis, including starch, storage proteins and oil[8]. Protein, lipid and carbohydrate enzymatic hydrolysis and metabolites' transport are primarily based on water availability[10]. Soluble sugars, which are the primary direct substance essential during germination[11], are of similar importance for developing germination of cacao seeds during storage. The loss of dehydration tolerance in recalcitrant seeds before seed shedding and the concurrent decline of soluble sugars could be a phenomenon that is unique to tropical seeds and is related to both initiation and mobilization of the reserves during the final stages of development. Thus, germinated seeds and seedlings seem to be the most vulnerable phases for soluble sugar fluctuations due to the necessity for osmotic cell homeostasis growth and maintenance[12].

A recent study demonstrated that cacao seeds could be stored at 16°C, 40% RH in sealed polyethylene bags for at least 144 hrs and still produced high germinability, with no dead seedlings recorded[13]. A further experiment to extend the storage durations might expand the scope to elucidate the changes of mechanical and metabolism aspects within seeds which may yield useful information regarding the loss of seed quality during storage. Therefore, the objective of this study was to evaluate the effects of different storage conditions on the changes of physiological, microstructural and soluble sugar content associated with slow-drying mechanisms of locally produced cacao seeds.

MATERIALS AND METHODS

Study area: This study was carried out from August, 2019 to March, 2020. It started with cacao pods arrival, the immediate seeds extraction in the laboratory and the seedling growth evaluations (for 8 weeks planting duration) grown under a rain shelter with 70% shade.

Plant materials: Ripened cacao pods of PBC123 clone were provided by the Malaysian Cocoa Board, Tawau, Sabah, Malaysia. The cacao plants were planted at Pusat Penyelidikandan Pembangunan Koko Madai, Kunak, Sabah (4.7965000487779506, 117.9670069587197). The harvested pods were transferred soonest possible to the laboratory at Universiti Malaysia Sabah, Sandakan, Sabah. Upon arrival, seeds were extracted from pods, demucilaged using sawdust and cleaned using a soft sponge.

Storage conditions: One hundred and sixty cacao seeds were packed into each zip-lock polyethylene (PE) bags (23×15 cm) and stored at (i) air-conditioned room temperature, RT (25±2°C, 55±5% RH), (ii) 16°C, 80% RH, (iii) 16°C, 40% RH, (iv) 14°C, 80% RH, (v) 14°C, 40% RH and (vi) control (seeds freshly extracted from pods). Each treatment carried 18 PE bags. A microprocessor-controlled console-style germinator (Seed buro MPG-3000, USA) was used for seed hydration. All PE bags were opened daily for 1-2 min and the seeds were stirred gently to allow the aerated condition within the PE bag. Seeds were evaluated every 48 hrs for 12 days for germination characteristics, seedling performances, microstructural changes through Scanning Electron Microscopy (SEM) observation and soluble sugars changes.

Measurement of seed Moisture Content (MC): Ten seeds per replication for each treatment were drawn and dried at 103±2°C for 16±1 hrs[14]. The following formula was used to determine seed MC:

$$\text{MC (\%)} = \frac{\text{Fresh weight-Dry weight}}{\text{Fresh weight}}$$

Measurement of Leachate Conductivity (LC): LC was determined by the method of Bonner[15] with modification. Five seeds per replication for each treatment were drawn, weighed and soaked in 25 mL ultra pure water. After 24 hrs, the leachate was decanted, measured using the Eutech PC2700 Conductivity Meter and expressed in µS g^{-1} FW.

Estimation of respiration rate (carbon dioxide CO_2 evolved): Respiration rate was measured by a modification of the CO_2 measurement system procedure of Raudiene *et al.*[16]. Twenty freshly after-stored seeds were weighed and put into the hermetically closed chamber (250 mL), with the probe attachment on its top occupied with the infrared detector and CO_2 gas sensor (CO_2-BTA Vernier, USA). The sensor measured CO_2 concentration by a digital interface connected straight to the data collection software and recorded every minute. Respiration rates were expressed as mL CO_2 produced per g of cacao seeds per hour and calculated as follows:

$$\text{Respiration rate} = \frac{\text{Change of } CO_2 \text{ concentration} \times \text{Chamber volume}}{\text{Sample weight} \times \text{Sampling period}}$$

The measurement range of the CO_2 sensors was 1000-10,000 ppm in the extended measurement range (±10% of reading). The response time of the sensors was 95% full-scale reading in 120 sec. The normal operating temperature and humidity range of the sensor was 25±5°C and 5-95% RH, respectively.

Measurement of Germination Percentage and Germination Rate Index (GRI): Germination was conducted in trays and maintained at the laboratory (25±2°C). Fifty seeds per replication were kept between moist cloth towels and observed for 14 days. Seeds were considered germinated when 2 mm protrusion of the radicles were visible. GRI reflects the speed ofgermination[17] and calculated as follows:

$$\text{GRI (\% per day)} = G1/1 + G2/2 + ... + Gx/x$$

where, G1 is germination percentage×100 on the first day after sowing.

Measurement of seedling dry biomass and evaluation of normal, abnormal and dead seedlings: Thirty germinated seeds were sown in a mixture of 20% chicken manure and 80% topsoil in the black PE bags (22×10 cm). Seedlings were grown under a rain shelter with 70% shade. Seedlings were harvested at the end of the 8th week, dried in an oven at 80°C for 24 hrs and the weight was recorded[18]. The percentage of normal (morphologically complete), abnormal and dead seedlings were recorded at the end of the test period.

Scanning Electron Microscopy (SEM) observation: SEM was used to evaluate the damages from the slow-drying and chilling conditions exposure, following the method proposed

by Ignatz *et al.*[19]. Seeds were cut by transverse-section using a razor blade; the radical cells of the meristematic axis and the cotyledon mesophyll were visualized. Then, samples were sputter-coated with a gold layer using a Quorum Q150R Thin-Film Coater and observed under SEM (Hitachi S-3400N, Japan).

Measurement of total soluble sugar: This method was carried out according to Nath *et al.*[20]. A 100 mg of seeds were homogenized with 5 mL of 80% ethanol and centrifuged at 3000 rpm for 10 min. Supernatants from 3 times extraction (with 80% ethanol) were collected into 25 mL volumetric flasks. The extract (0.3 mL) was pipetted in separate tubes from each treatment and placed in a boiling water bath for 3 min to evaporate ethanol. Each test tube was added with 1 mL pure water and 4 mL of (0.2%) anthrone reagent (200 mg in 100 mL H_2SO_4) and placed in ice-cold water. The blank reagent was prepared with 1 mL of distilled water and 4 mL of anthrone reagent. The intensity of colour was measured using a spectrophotometer (DeNovix DS-11, USA) at 600 nm. A standard curve was prepared using 10 mg glucose per 100 mL distilled water with the equation was $y = 1.45x+0.32$ ($R^2 = 0.84$).

Measurement of glucose, sucrose and raffinose-family oligosaccharides (RFOs): Methods to quantify glucose, sucrose and RFOs were based on a diagnostic kit (Product K-RAFGL, Megazyme Int. Ireland Ltd., Bray, Ireland). Aliquots at 200 µL of ethanol 80% (v/v) sugar extracts were added to three test tubes labelled A, B and C. Tube A was directly assayed for glucose. Sucrose was hydrolyzed to glucose and fructose in Tube B through digestion with 200 µL of invertase in 200 µL of 50 mmol L^{-1} NaOAc buffer (pH 4.5). RFOs were hydrolyzed into galactose, glucose and fructose in Tube C through digestion with 200 µL α-galactosidase and invertase in 200 µL of 50 mmol L^{-1} NaOAc buffer. Three millilitres of GOPOD reagent was added to each tube and all solutions were incubated at 50°C for 20 min. Glucose concentrations were determined using glucose (standard) and spectrophotometer absorbance was read at 510 nm. Tube A absorbance was used to calculate glucose concentration. The absorbance difference between Tubes A and B was to calculate sucrose concentration since sucrose was hydrolyzed (by invertase) to glucose+fructose. The absorbance difference between Tubes B and C was to calculate RFOs concentration since sucrose and RFOs were converted (by invertase and α-galactosidase) to equivalent moles of glucose.

Experimental design and statistical analysis: The experiment was arranged in a split-plot randomized complete block design with three replications. Data were subjected to analysis of variance with SAS version 9.4 and the significant means were separated by the Least Significant Difference (LSD) test at $p<0.05$.

RESULTS

Seed Moisture Content (MC), Leachate Conductivity (LC) and respiration rate: In this study, only seeds at RT and 16°C, 40% RH showed a significant reducing pattern of MC (8-17%) than the control, at 4 and 10 Days After Storage (DAS), respectively in Fig. 1a. Seeds at RT further maintained the lowest MC until 12 DAS. Seeds at the higher saturated RH (80%) were found to remain as stable MC as the control before showed some diversion towards the end of storage. Among them, seeds at 14°C, 80% RH showed a significant increase (11%) of MC at 10 DAS to record the highest MC. Moreover, only seeds at 14°C, 40% RH showed a significantly lower (8-14%) MC during early storage(2-4 DAS), before maintained the same level as the control until 12 DAS.

More significant results showed on the pattern of LC measured as the indicator of membrane damage. In parallel with MC's reducing pattern, seeds at RT showed a significantly higher (48%) LC than the control, at 2 DAS in Fig. 1b and later declined back to the same level as the control until 10 DAS. Meanwhile, both seeds at 16°C recorded a significantly higher (29-31%) LC, only at 6 DAS. The contrast results showed for seeds at 14°C. Seeds at 14°C, 40% RH showed a significant increase of LC; from 82.1 µS g^{-1} FW (control) to 131.2 and 120.8 µS g^{-1} FW during early storage (2 and 6 DAS, respectively). Meanwhile, seeds at 14°C, 80% RH showed a significant increase of LC (45-52%) towards the end of storage (6-12 DAS), to exhibit the highest LC.

On the other hand, during storage at the higher temperature condition (RT), there was a highly continuous significant lower respiration rate than the control (50-74%) along 12 DAS in Fig. 1c. The slower declining pattern of respiration rate showed for both seeds at 16°C; with 35-50% of the declining rate at 4-6 DAS. Later, only seeds at 16°C, 40% RH showed a sharp, significant increase of respiration rate (from 36.6-62.4 µS g^{-1} FW) at 10 DAS. The rise of respiration rate reflected that there might be more compensation processes to accommodate the disrupted metabolisms. Seeds at 14°C, 80% RH showed a significant increase in respiration rate (from 36.6 µS g^{-1} FW to within 47.1-68.3 µS g^{-1} FW) at 2-10 DAS and recorded the highest at most storage durations.

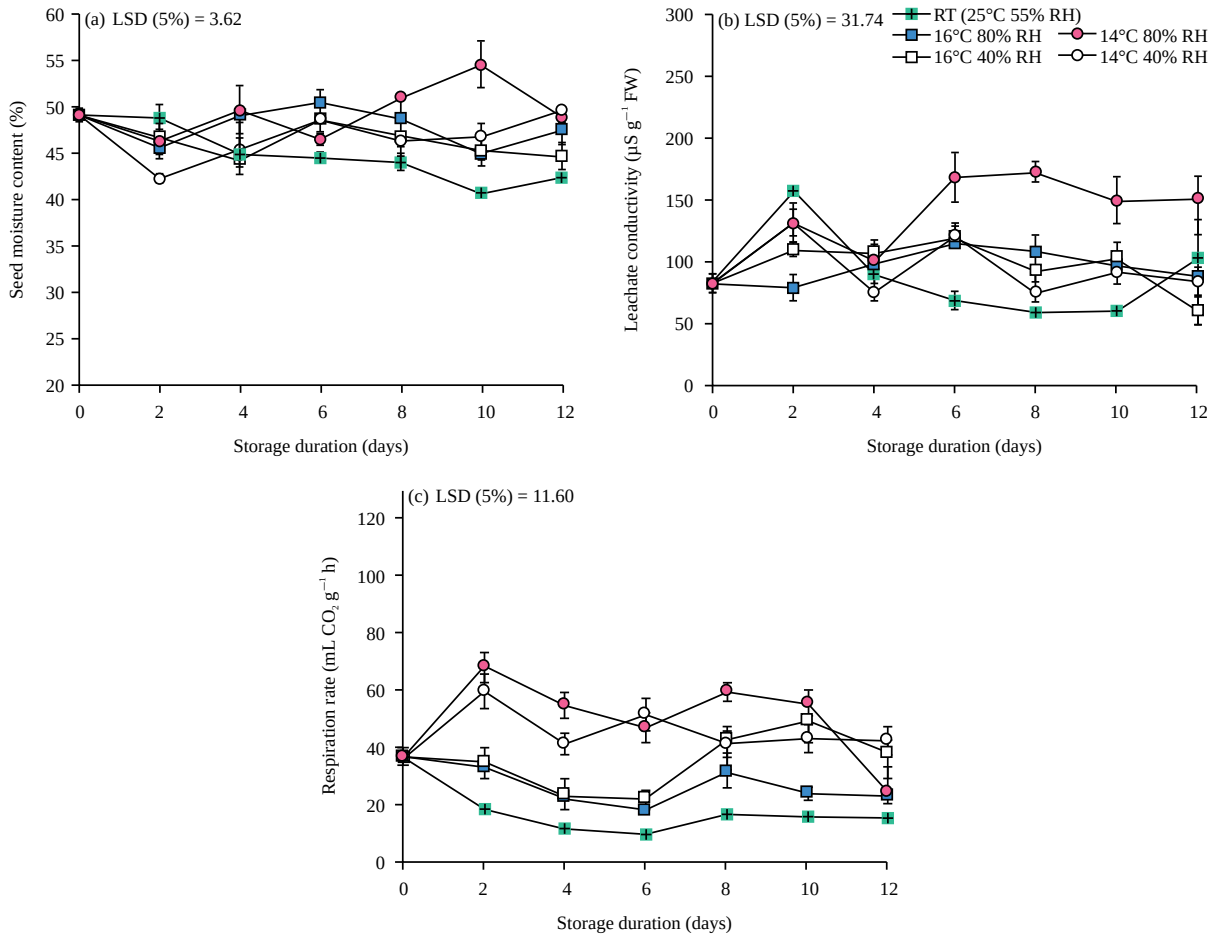

Fig. 1(a-c): (a) Seed moisture content, (b) Leachate conductivity and (c) Respiration rate of cacao seeds stored under different conditions for 12 days
Data are Mean±standard error

Final Germination Percentage (FGP), Germination Rate Index (GRI)and seedling growth performance: Seeds at RT demonstrated FGP to reach the same levels as the control along 12 DAS in Fig. 2a and showed a gradual and significant higher GRI in Fig. 2b. However, seeds at RT also recorded about 8-10% of germination occurrence during storage, compared to none germination occurrence by the other treatments. More hydrated seeds at 16°C, 40% RH (higher MC), displayed the equality of producing the insignificantly higher FGP than seeds at RT. Seeds at 16°C, 80% RH, which seem to retain more water within cells, displayed the gradually decreasing (13-23%) trend of FGP, at 10 to 12 DAS. Seeds at 14°C, 40 and 80% RH produced a significantly lower (10%) FGP than the control, at 2 DAS. Seeds at 14°C, 40% RH, later declined (13-18%) significantly at 8-12 DAS. Meanwhile, seeds at 14°C, 80% RH, showed a significant decline (17-23%) of FGP at 4-12 DAS and produced among the lowest FGP.

On the other hand, seeds at RT led the positive trend of seedling performance by recorded 100% of normal seedlings along 12 DAS in Fig. 2c. This followed by seeds at 16°C, 40% RH, with a higher normal:abnormal ratio (until 10 DAS), than seeds at 16°C, 80% RH. Stunted and tip dry seedlings were the common abnormalities observed in this study. Meanwhile, seeds at 14°C continue to exhibit low vigour status, with the lowest normal seedlings recorded along 12 DAS.

Moreover, seeds at RT and 16°C produced higher seedling dry biomass (MASS), indicating higher vigour status. Seeds at RT led the positive trend to exhibit the most increased MASS with 33-44% higher than the control, at 6-12 DAS in Fig. 2d. Seeds at 16°C, 40% RH followed to exhibit the significantly higher MASS, from 1.4-1.9 g plant^{-1}, at 2-4 DAS and later reduced to the same level as the control, until 12 DAS. Meanwhile, seeds at 16°C, 80% RH showed a similar trend to exhibit the significantly higher (30%) MASS, at 2 DAS, before

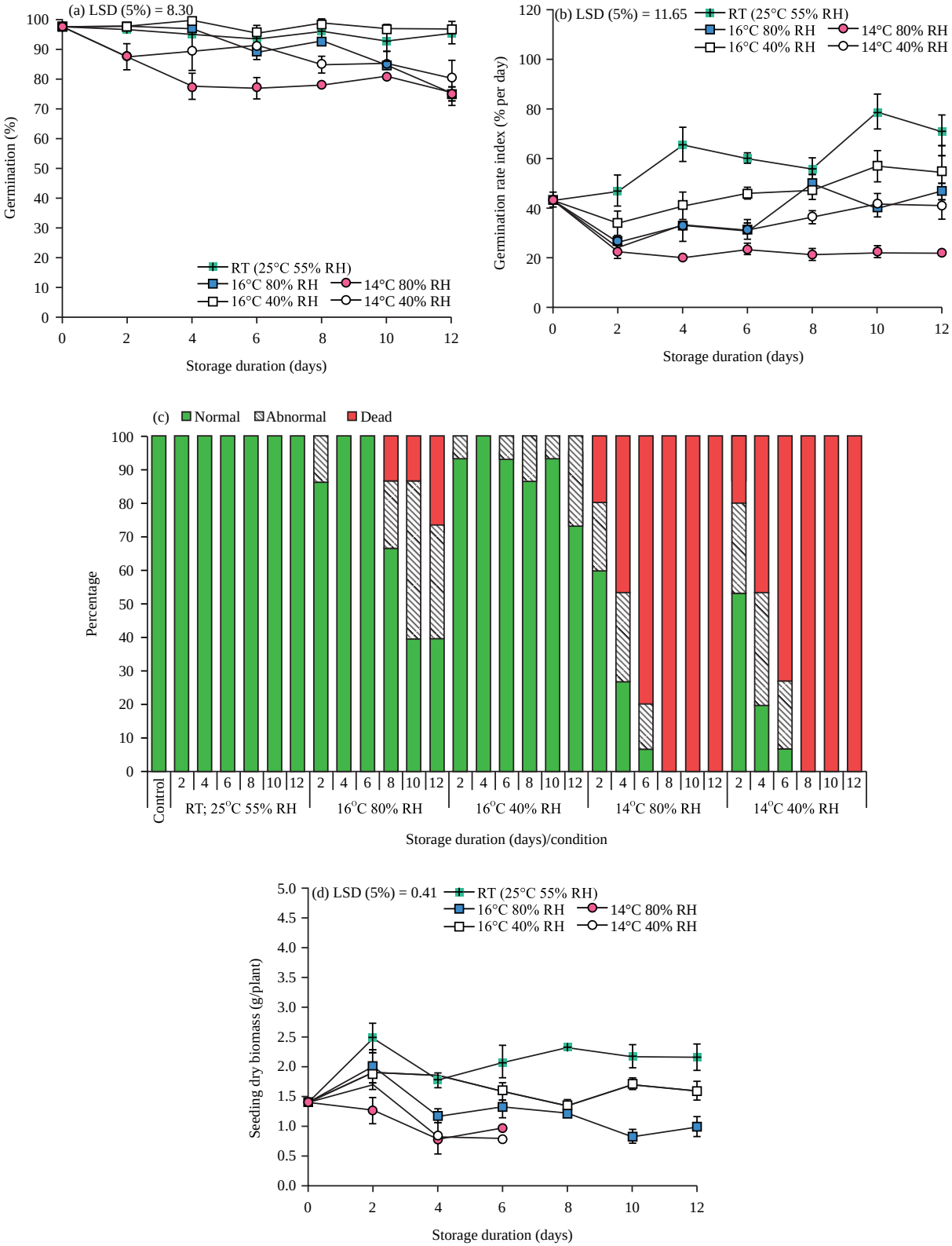

Fig. 2(a-d): (a) Final germination percentage, (b) Germination rate index, (c) Seedling performance and (d) Seedling dry biomass of cacao seeds stored under different conditions for12 days

Data are Mean±standard error

declined significantly (29%) to record among the lowest MASS, until 12 DAS. Seeds at 14°C, 40 and 80% RH showed a significant decline (32-44%) of MASS, at 4-6 DAS. The data collections stopped until 6 DAS due to 100% of dead seedlings recorded at 8-12 DAS, for seeds at 14°C.

SEM observation on seed micro structural changes: The SEM micrographs on the pattern of the structural change of cacao seed samples after being exposed to different storage conditions along 12 DAS are presented in Fig. 3. Freshly extracted cacao seed tissue has a well-organized structure consisting of cells, intercellular spaces and regular cells shape at the meristematic axis of the radicle (MAR) in Fig.3a(i-ii). Rare damages were also visualized at their cotyledon mesophyll cells (CMC) in Fig. 3a(iii-iv). On the other hand, turgid cells of the MAR showed for seed at RT and 16°C, 40% RH. A folding and thickening of the cell wallsand more pores/intercellular spaces, with irregular cells shape, were observed for seed at RT in Fig. 3b(i-iii). Those visible structures made cell walls more resistant and stable. On the contrary, lesser pores and starch granules were found on the surface of the folded, homogenous and tight cells, while they still retained their original shape structure of seed at 16°C, 40% RH in Fig. 3c(i-iii). It is interesting to note that more distinct changes occurred at the CMC in those two conditions. Very minimal damages with the amorphous deposits appeared at the CMC for seed at RT in Fig. 3b(iv-vi) and vice versa with the ones at 16°C, 40% RH in Fig.3c(iv-vi). On the other hand, seed at 16°C, 80% RH showed many significant structural changes as they produced the wrinkled, tight and compact structure of cell walls at the MAR in Fig. 3d (i-ii). The adverse effects of retaining much water within cells appeared with more lesions and breakdown of cell walls detected towards the end of storage in Fig. 3d(iii). The same reason might cause the loosen cell-to-cell structure and some plasmolysis events appeared at their CMC in Fig. 3d(iv-vi).

Different types of damage are displayed when cacao seed is exposed to the chilling conditions, seen from Fig. 3e and f. Seed at 14°C, 40% RH showed the signs of cell wall disruption, cell deformation, the collapse of cell structure and even lesions at the MAR in Fig. 3e(i-iii). Their CMC showed the signs of imbalance osmotic gradient conditions through the more plasmolysis events in Fig. 3e(iv-vi). On the other hand, the final display for seed at 14°C, 80% RH, is characterized by the dense and wrinkled cell structure, implying a harder texture in Fig. 3f(iii). The small, irregular, but frequently visible pores may further be increased their water retain within seed cells. That evidence can be seen through the regularly appeared membrane cell lesions at their MAR in Fig. 3f(i-iii). Furthermore, quite major damages of CMC also displayed for seed at 14°C, 80% RH in Fig. 3f(iv-vi).

Total soluble sugar (TSS), glucose (GC), sucrose (SC) and RFOs content: Seeds at RT show the lowest TSS (2-12 DAS) with an insignificant gradually increasing trend in Fig. 4a. Meanwhile, seeds at 16°C, 40 and 80% RH showed a significantly lower TSS than the control, until 12 DAS. Seeds at 14°C exhibited much higher TSS with no significant values than the control until 4 DAS. Seeds at 14°C, 40 and 80% RH later showed much lower TSS than the control; from 42.4 mg glu eq g^{-1} FW (control) to 21.9-29.2 and 15.5-24.4 mg glu eq g^{-1} FW, respectively.

In this study, almost all the storage conditions showed higher soluble sugars (glucose, sucrose and RFOs), than the control (mostly until 6 DAS). Except for seeds at RT, all the storage conditions show a significant increase of GC than the control; from 17.7 μmoles g^{-1} FW (control) to 25.7-30.7 μmoles g^{-1} FW, at 2 DAS in Fig. 4b. Seeds at 16°C, 40 and 80% RH later showed a sharp decreasing (39 and 58%, respectively) GC at6 DAS. Those seeds further displayed the significant decreasing trend of GC, until 12 DAS. Seeds at RT showed a significant decrease of GC; from 17.7 μmoles g^{-1} FW (control) to 9.8-10.7 μmoles g^{-1} FW, at 8-12 DAS. Moreover, seeds at 14°C demonstrated the rapid decreasing trend of GC at 6 DAS. Seeds at 14°C, 40% RH showed significantly lower (51-93%) GC than the control and later maintained the lowest (6-12 DAS).

Further, except for seeds at RT and 16°C, 80% RH, all the storage conditions showed a significant increase (19-32%) of SC than the control, at 2 DAS in Fig. 4c. Seeds at 16°C, 40% RH showed the SC reverted to the same level as the control until 12 DAS. Seeds at RT and 16°C, 80% RH showed a significant decrease of SC; from 35.1 μmoles g^{-1} FW (control) to 25.5-28.9 μmoles g^{-1} FW, at 8-10 DAS. Only seeds at 14°C continued to accumulate the significantly higher (31-38%) SC at 4-6 DAS.

On the other hand, only seeds at 14°C, 80% RH showed a significant increase (51-57%) of RFOs than the control at 2-4 DAS in Fig. 4d. Meanwhile, seeds at RT and 16°C, 40% RH showed a significant increase of RFOs; from 6.3 μmoles g^{-1} FW (control) to 10.8-11.5 μmoles g^{-1} FW, at 2 DAS. Seeds at 16°C, 80% RH and 14°C, 40% RH displayed no significant differences on RFOs, along with 12 DAS.

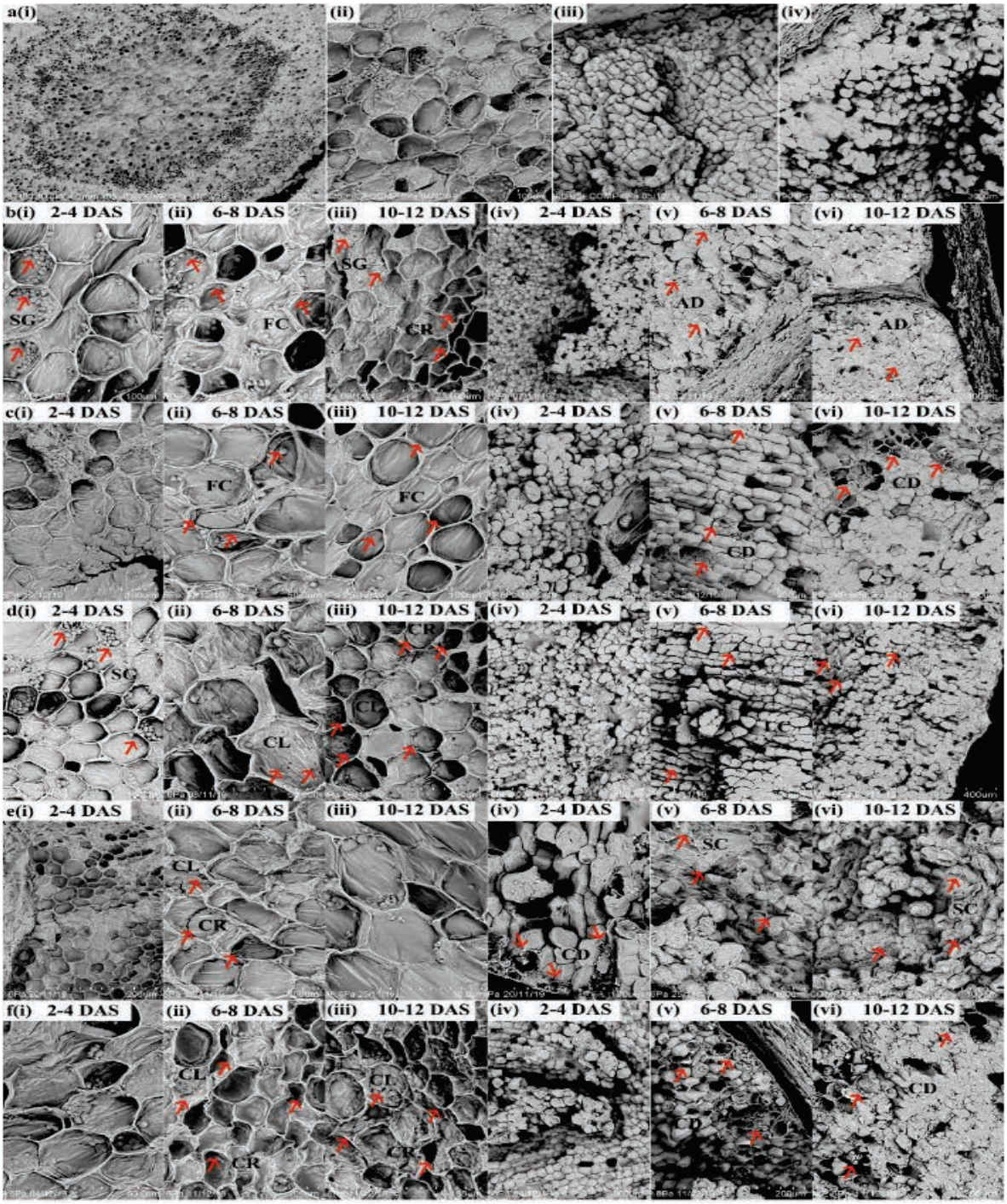

Fig. 3(a-f): Effects of storage conditions on microstructural changes of *T. cacao* seeds (from 2 until 12 DAS) viewed under SEM

(a) Rarely defected with regular cells shape of the freshly extracted seed (control) at the MAR(i-ii) and CMC (iii-iv). Bars (a: i-iv) = 100-500 µm. (b) Folded cell wall with irregular cells shape for seed at RT at the MAR (i-iii) and more amorphous deposits at the CMC (iv-vi). (c) Folded cell wall with regular cells shape for seed at 16°C, 40% RH at the MAR (i-iii) and cells damages mostly at the edge of inner cells at the CMC (iv-vi). (d) Wrinkled cell structures and membrane cell lesions for seed at 16°C, 80% RH at the MAR (i-iii), with the loosen cell-to-cell structure and plasmolysis events at the CMC (iv-vi). (e) Cell wall disruption and cell deformation for seed at 14°C, 40% RH at the MAR(i-iii) and more plasmolysis events at the CMC (iv-vi) and (f) Dense, wrinkled cell structures and more membrane cell lesions for seed at 14°C, 80% RH at the MAR (i-iii), with the major cells damage sat the CMC (iv-vi). Bars (b-f); (i-iii) = 50-200 µm, (iv) = 100-500 µm, (v) 100-300 µm, (vi) 100-400 µm. FC: Folded cell wall, CL: Membrane cell lesion, SC: Shrunken cell, CD: Cell damage, CR: Cell wall rupture, LC: Loosen cell-cell, AD: Amorphous deposits, SG: Starch granule

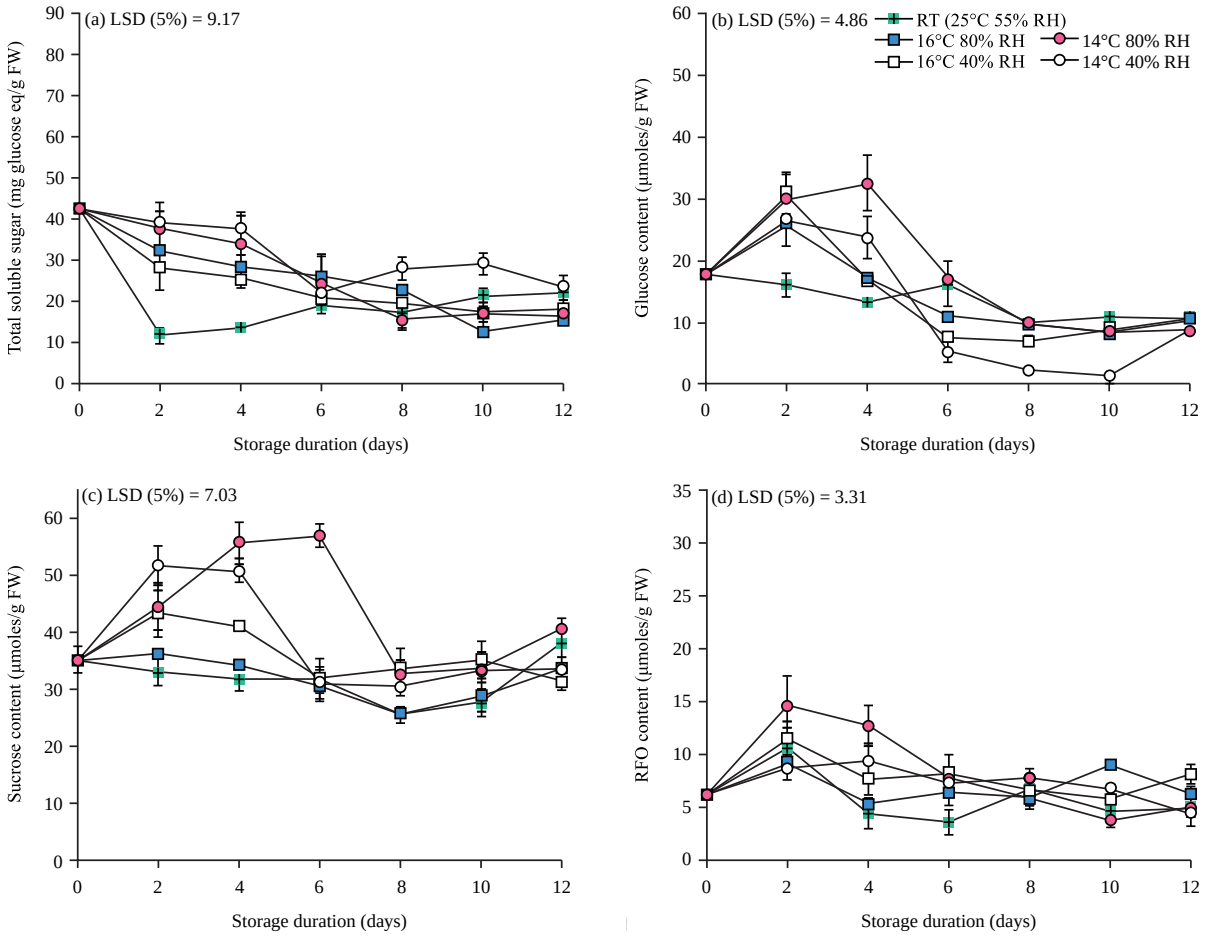

Fig. 4(a-d): (a) Total soluble sugar, (b) Glucose content, (c) Sucrose content and (d) RFOs content of cacao seeds stored under different conditions for 12 days

Data are Mean±standard error

Table 1: Pearson's correlation data

Correlations	MC	LC	RR	TSS	GC	SC	RFOs
FGP	ns	-0.38**	-0.31*	0.29*	ns	-0.25*	ns
GRI	-0.38**	-0.50**	-0.54**	-0.19*	-0.28*	-0.43**	-0.30*
MASS	-0.44**	-0.26*	-0.46**	ns	0.30*	ns	0.31*
NORM	ns	-0.33**	-0.50**	ns	0.20*	-0.24*	ns
ABN	ns	ns	ns	ns	0.21*	0.22*	0.36**
DEAD	0.25*	0.31*	0.44**	ns	-0.29*	ns	ns

**Significant at p≤0.001, *Significant at p≤0.05. MC: Moisture content, LC: Leachate conductivity, RR: Respiration rate, TSS: Total soluble sugar, GC: Glucose content, SC: Sucrose content, RFOs: Raffinose-family oligosaccharides content, FGP: Final germination percentage, GRI: Germination rate index, MASS: Seedling dry biomass, NORM: Normal seedling, ABN: Abnormal seedling, DEAD: Dead seedling

Correlations between seed/seedling vigour and physio-chemical status: Table 1 showed the Pearson's correlations between seed/seedling vigor (FGP, GRI, MASS, NORM, ABN and DEAD) and physio-chemical status (MC, LC, RR, TSS, GC, SC and RFOs) parameters. Among those parameters, LC showed the significant negative correlations with almost all seed/seedling vigor parameters measured; FGP (r = -0.38**), GRI (r = -0.50**), MASS (r = -0.26*) and normal seedling (r = -0.33**), as well as the significant positive correlation with dead seedling (r = 0.31*). On the other hand, only GRI parameter showed the significant correlations with all the physio-chemical parameters measured; MC (r = -0.38**), RR (r = -0.54**), TSS (r = -0.19*), GC (r = -0.28*), SC (r = -0.43**) and RFOs (r = -0.30*).

DISCUSSION

In the present study, the gradual decrease of seed MC (mild dehydration) might cause a reduction of cell metabolism[21] for seeds at RT. Moreover, the low LC indicated fewer membrane disruptions and thus it might induce more efficient deposition of insoluble storage reserves in seeds. The decreasing trend of LC for seeds at RT after 2 DAS suggesting the sufficient water content of the cytoplasm for metabolic (repairing) activities to be maintained or activated and to have remained in close equilibrium with the associated conditions. A decreasing trend of MC from 4 DAS onwards was evidenced by the presence of the amorphous deposits (at the cotyledon cells) [(Fig. 3a(v-vi)] which might be due to the desiccation of salts and cytosolic constituents[22]. As the capillary water forces reduced with the reductions of MC, the stretched appearance of the hydrated cells (at the meristematic cells) further changed to more folded and collapsed cell walls against the cellular content (4-12 DAS) [Fig. 3a(ii)]. During dehydration, the cells cracked or burst because of the combination temperature and moisture gradients exceeding the cells' binding force[23]. This could be observed by the abundant presence or leaked of the protein matrix and starch granules at the meristematic cells of seeds at RT [Fig. 3a(iii)].

On the other hand, the meristematic cells of seeds at 16°C, 40% RH showed compact structures at 2 DAS. Nevertheless, it became loosen with the stretched and folded cell walls against the cellular content until 12 DAS [Fig. 3b(i-iii)]. Different from seeds at RT, a decreasing trend after increased (at 6 DAS) of MC may cause the spatially scattered pattern of damages (at the cotyledon cells) at 8 DAS onwards, which located peripherally near seed coat or at the edge of the inner cells, but rarely for cells interior the tissues [Fig. 3b(v-vi)]. These conditions suggested the slowly loosen capillary water forces and later developed hydrophobic water-binding state [Fig. 3b(v)]. Whereas the enhanced permeability of cell membranes (high LC) disrupted the processes such as hydrolysis, macromolecules biosynthesis and respiration[10]. The present study found some discernible indicators through the negative correlations of LC with the FGP, GRI, MASS and NORM, as well as the positive correlation with the dead seedling. The higher LC for seeds at 16°C than RT suggests that more maintenance on the membrane re-structural processes to cope with germination readiness. Moreover, the slight increasing pattern of MC for seeds at 16°C, 40% RH (6 DAS) was found coincidently at the point before the increase of their respiration rate (8-12 DAS). This further evidencing that the rise of MC was unlikely due to water generates from the intense respiration, but rather due to the enhancement of water driven through cells as their structural morphology changed. The rise of respiration rate reflected that there might be more compensation processes to accommodate the disrupted metabolisms. However, those disturbances may not lead to worse visible injuries or changes in growth and development rates. The physiological processes are reversible to stability[24] and further revealed through the higher GRI in seeds at 16°C, 40% RH than the control at the end of storage.

As evidenced by SEM images, the consequences of retaining aqueous phase within seed cells along 12 DAS at 16°C, 80% RH manifested by the characteristic of wrinkled, less thick cell walls structure with more lesions detected on plastid membranes (at the meristematic axes) [Fig. 3c(i-iii)]. Even though the seeds at 16°C, 80% RH had a lower respiration rate than the control, it had a strong leakage that might lead to DNA synthesis attenuation[25]. As there was less deposition of insoluble reserve materials in cells, the vacuolated cells might change (reduce) their structural volume due to the osmotic solute gradients. This supported by the lesser damages at the cotyledon cells, but cells plasmolysis occurred towards 12 DAS [Fig. 3c(v-vi)]. The increasing trend of LC coupled with high seed MC at 16°C, 80% RH suggested the slower repair mechanisms that become more inoperative along with the increasing "unregulated" metabolisms[26], which justifies their decreasing pattern of germination characteristics and seedling performances towards the end of storage.

Classified as the chilling temperature, seeds at 14°C, 40 and 80% RH revealed similar poor seed germination and seedling performances. The chemical (e.g. oxidative stress) rather than physical (i.e., structural) damage is inferred from the observation that membrane abnormalities were most frequent in the slowly dried axes[27]. As confirmed by SEM observations, the hypertonic symptoms presented at the cotyledon cells (4-12 DAS) [Fig. 3d(v-vi)] of seeds at 14°C, 40% RH, indicating that there might be the osmotic solutes gradients transition. The process caused the cells to shrunken and gradually induced more water uptake. Comparatively, seeds at 14°C, 80% RH displayed more turgid cotyledon cells [Fig. 3e(iv-v)]. However, the high MC at early storage strengthens the capillary water forces, further explains the ruptured cells covered over major surface areas of cotyledon cells, including cells within the tissues [Fig. 3e(iv-vi)]. More water retention detected at the meristematic axes as characterized by lesion signs on plastid membranes [Fig. 3e(ii-iii)]. Those lesions that appeared as early as at 4 DAS indicate the increased leakage and followed by a sharp declining pattern of FGP.

According to Wen[7], *Livistonachinensis* embryos that underwent the physiological effects of seed production with acquisition and loss of cryo-tolerance demonstrated the re-differentiation after de-differentiation in the subcellular organization, which caused the increase followed by a decrease in total soluble sugar content. Subcellular re-organization has also occurred in this study. The seeds underwent the physiological changes of developing dehydration sensitivity and decreasing pattern of total soluble sugar along 12 DAS. Since recalcitrant embryos are in the germination mode of growth, the possible primary source of soluble sugar synthesis is starch, a common food reserve in seeds to maintain energy metabolism during hydrated storage[11]. SEM micrographs also indicated that cells (at the meristematic axes) did not seem to have been abundantly packed with starch grains, which is a typical feature of actively metabolizing recalcitrant seeds. Plastids-containing starch that was a characteristic of the seed cells of all storage treatments were no longer evident, mostly at 4 DAS (seeds at 14°C) and 8 DAS (seeds at 16°C) onwards. A marked decrease in starch deposits may well have provided the sugars required for the growth processes following germination[28].

Seed with a low level of metabolic activity during post-ripening may retain their viability[8] and a similar pattern was expressed for seeds at RT in this study. In addition to serving as a respiration substrate, sugars such as sucrose and glucose are also carbon sources to produce metabolites such as amino acids, lipids, proteins and complex carbohydrates[29]. In this study, seeds at RT exhibited the lowest respiration rate and consistently accumulated the lowest soluble sugars. In parallel with the highest FGP and increasing GRI of seeds at RT, there might be the rapid inter conversions of sucrose to simple sugars (hexoses), while those hexoses lead to sucrose synthase to maintain their cell homeostasis[30]. The negative correlations between glucose, sucrose and RFOs with GRI in this study proved that seed viability preservation occurred with the efficient utilization of soluble sugars.

The membrane alterations for seeds at 16°C attributed more leaked substrates or enzymes for further metabolic activity. However, those seeds seemed to benefit from the adverse conditions. In the slowly desiccated developing seeds of *Machilusthunbergii*, an increase in sucrose concentration may indicate their protective role[31]. While DNA repair enzymes are unstable during storage and lose their function, resulting in gradually delayed repairing mechanism and germination, these enzymes could still regenerate during the dehydration-rehydration cycles[32]. More rapid dehydration-rehydration processes of the seed cells contributed to the higher viability and vigour for seeds at 16°C, 40% RH than seeds at the higher RH (80%)[9,13]. The extension of hydrated storage duration caused vigour performance lags behind viability due to more impaired biochemical changes that occurred and this pattern depicted by seeds at 16°C, 80% RH. The discrepancy between seeds quality seems to be universal not only due to dehydration tolerance but also due to the ready access of respiratory substrates for increasing the metabolism associated with radicle emergence[33]. Thus, starch hydrolysis also functioned to produce sucrose to reduce dehydration sensitivity in the metabolically active embryo during hydrated storage[11], including the terms to which starvation of sugars could be avoided.

In general, low temperature increases soluble sugar levels[30]. The cytoplasm viscosity declines at a slight chilling due to increased colloid dispersion (due to increased LC) and structural formations decay[24], which explains the higher glucose, sucrose and RFOs until 6 DAS for seeds at 14°C. The higher MC for seeds at 14°C, 80% RH suggested for the faster deterioration to have occurred. The osmotic treatment of seeds stored at the chilling temperature was first manifested 'imbibitional chilling' events indicated by the higher membrane disruptions (highest LC) until 12 DAS. The secondary disorders followed as membrane disruptions allow more enzymes to be contacted by their substrates, leading to biochemical changes in seeds[34]. Subsequently, the final sharp increase of MC showed for seeds at 14°C, 80% RH might be due to more damaged cells and high cell volume changed which caused the higher water uptake in seeds. The long-term and robust chilling lead to viscosity status to be increased due to the coagulation of structural proteins[24], by the decreasing pattern of the glucose, sucrose and RFOs (8-12 DAS).

Furthermore, low temperatures slowed down certain enzymes' activity that dissociation of multimeric enzymes, protein-lipid and hydrophobic interaction disorders, reversible changes in the kinetic properties of enzymes and allosteric control[11]. Thus, the higher soluble sugar accumulations exhibited for seeds at the chilling temperature (14°C) indicate the metabolism's slow-down. According to Connor and Sowa[31], recalcitrant *Quercus alba* acorns (embryonic axis) showed high sucrose accumulation coupled with declining viability. They suggested that sucrose was no longer used for growth and development or that the enzyme activity in deteriorating seeds or both of the starch were broken down. The continued sucrose accumulation in the drying embryos and cotyledons acted secondarily as a glyco-protectant, preventing both desiccation damage of cell membranes and cell collapse. Corroboration to this study, seeds at 14°C, 40% RH have similar symptom as LC maintained among the lower pattern with increased storage durations, indicating functioning repairing mechanisms probably involved.

However, the mechanism combination of high sucrose concentrations and high MC did not work to maintain viability that quickly fell after few days for *Q. alba* acorns[31]. Similarly, the increasing trend of MC, together with the higher sucrose until 6 DAS for seeds at 14°C, also showed the reducing trend of FGP and lowered GRI than the other treatments. Since the recalcitrant seeds have already lost viability at higher MC, the contribution of soluble sugars to strengthen the intracellular structure or against the lateral contact between membranes can be less critical than the sucrose hydrolysis which provides a readily available respiratory substrate. The process might be necessary to sustain a continuous development imperceptibly for germination, followed by establishing seedlings under favourable natural conditions.

CONCLUSION

Conditions of RT and 16°C, 40% RH appeared to be convenient along 12 days of cacao seeds storage. Their mild dehydration patterns contributed to the least seed microstructural changes and thus, lessened cell damages. Though with much lower impaired respiration, the high LC of seeds at 16°C, 80% RH increased their dehydration sensitivity at 8 DAS. The intense respiration and low metabolic events of seeds at 14°C accelerated the loss of vigour and their eventual decline in viability during the extended storage (>6 DAS).

SIGNIFICANCE STATEMENT

This study discovered the alternatives of the easy-handled short-term storage of the locally produced recalcitrant cacao seeds that can be beneficial for improving the seed producer's awareness and understanding of maintaining cacao seed quality using the specific storage method and duration. This study will help the researchers uncover the critical areas of emphasizing the windows of the short-gap of storage in producing and maintaining the high quality of cacao seeds that many researchers were not able to explore. Thus, a new theory on the effects of the specific storage conditions towards locally produced cacao seeds may have arrived in the future. The continuous evaluations on the other seed biochemical changes, including factors that contribute to oxidative stress, may help further to elucidate the deterioration development of cacao seeds during storage.

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