



## Histochemistry of backcross 1 of oil palm seeds at different storage periods

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### ABSTRACT

Oil palm backcross 1 is the result of the crossing between *Elaeis oleifera* and *Elaeis guineensis*, followed by backcrossing to one of its parents. It has several advantages, including slow height growth and good oil quality, thereby having the potential to be developed. However, it also has a short seed shelf life, which might be inherited from *E. oleifera* that has relatively quick seed deterioration. This is problem to the breeding program, and there have not been many studies on the seed deterioration process. A histological examination can determine the composition of food reserves in seed endosperm tissue. Therefore, it is necessary to assess the histochemistry of seeds concerning the process of seed deterioration. Histochemical tests with Sudan III, Milon, and IKI reagents were used as histochemical tests of fat, protein and carbohydrate seed content with different seed storage periods, i.e. 4, 3, 2, and less than 1 year. The result showed that the fat content decreased during the storage periods. Based on carbohydrates and proteins staining, there were only very few substances, which were difficult to distinguish among the storage periods, so that this analysis could not be used as the determining indicator of seed deterioration. The fat content was a determining factor of seed deterioration and quality. The oil palm seed storage up to 3–4 years led to the reduction in the fat content in significant quantities compared to the fresh seeds stored less than 1 year as they still contained a lot of fat in the cell.

### INTRODUCTION

Oil palm seeds are classified as semi-recalcitrant because they show intermediate characteristics between orthodox and recalcitrant ones. Such seeds tolerate drying and long shelf life storage at low temperatures and can be desiccated to around 10–12% moisture content (Hong et al., 1996; Corley and Tinker, 2016; Norziha et al., 2017). Oil palm seeds have a relatively short shelf life at room temperature, but it is possible to find seeds that are still able to germinate

with a long shelf life up to 21 months at 22°C (Corley and Tinker, 2016). Storage of oil palm seeds for longer period is usually explored to conserve potential oil palm materials for future breeding programs. However, long time storage can decrease seed viability (Karneta et al., 2017), especially the seeds of *E. oleifera* that have a short shelf life, which is less than one year, and the seeds of *E. guineensis* Jacq. that have a quite long shelf life, which is around 24 months. As for backcross 1, there have not been many studies reported on the shelf life of the seeds.

Long time storage will affect the quality conditions of the seeds themselves. To maintain seeds ability to survive and germinate after long storage period, sufficient food reserve is required to supply energy for germination. Histological observation is one of the methods to determine the pattern of seed deterioration (Kok et al., 2015). The content of seed reserves can be observed by several methods, including seed biochemical tests and histological tests by making thin slices of endosperm and staining using special reagents. Test reagents to determine fat content can be done using Sudan III reagents, protein with Milon, whereas starch, sugar, and carbohydrates were tested using Schiff, PAS, and IKI reagents, respectively (Jensen, 1962).

The histology of the deterioration of oil palm seed content has not been much researched, despite it is important. Research on the histochemical observations of oil palm embryos is still limited to the histology of embryo formation from initiation to adult embryos (Kok et al., 2013; Kok et al., 2015). However, publications regarding the histochemistry of oil palm embryos from physiological maturity level, or as food reserves, to the deterioration or degradation process have not been published. Information regarding to the deterioration of the seeds, especially ones with exotic ancestral lineage from *E. oleifera* that generally has short seeds shelf-life, will be of value (Corley and Tinker, 2016; Setiawan, 2017). Indonesian Oil Palm Research Institute has developed backcross individuals from the crossing of interspecific hybrids (*E. guineensis* Jacq.  $\times$  *E. oleifera*) with the *E. guineensis* Jacq. parent. They are thought to have relatively short seeds shelf-life; the trait that has been shown by *E. oleifera* and its interspecific hybrids (*E. guineensis* Jacq.  $\times$  *E. oleifera*)

(Corley and Tinker, 2016; Montoya et al., 2013).

The aim of this study was to determine the histochemical description of the fat, protein, and carbohydrate content of oil palm endosperm of the seeds at different storage period and to determine the indicators of seed deterioration based on the histochemical description of the seed endosperm.

## MATERIALS AND METHODS

The crossing was made to produce fruit bunches for the seed sources, which were carried out at the Jambi Bah Garden, PTPN IV, and the Indonesian Oil Palm Research Institute, Marihat North Sumatra. The research materials were oil palm seeds produced by the backcross 1 of *E. oleifera*  $\times$  *E. guineensis* Jacq. with various storage periods of four years, three years, two years, and less than a year. The scheme of the interspecific crossing and backcrossing of palm oil is presented in Figure 1. The seeds resulted from the crossing were processed according to the Indonesian Palm Oil Research Institute (IOPRI) seed processing standards, then the seeds were stored according to the treatments, which were 4, 3, 2, and less than a year using tightly closed clear plastic packaging. The packaging was then placed on the seed storage shelves at temperatures of 16-20°C and humidity of 79%. Before stored, the seeds were soaked in a 1% fungicide solution for 15 minutes. After that, the seeds were dried to an initial water content of about 13%, put in plastic bags, and placed on the seed storage shelves.

After storage, the histological observations were made on the seed samples by randomly selecting 5 seeds from each storage periods with 3 replications.

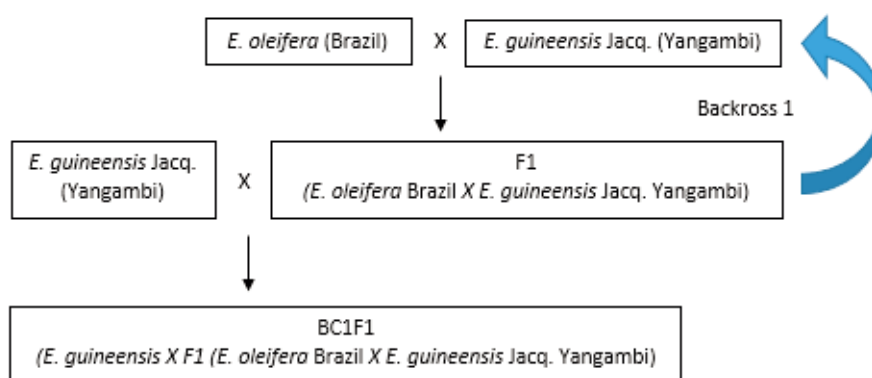


Figure 1. Scheme of interspecific crossing and backcrossing of oil palm

The seeds shells were then broken, and the kernels were taken for histochemical observations, including fats, proteins, and carbohydrates, using a procedure published by Jensen (1962). Histological observation of this method used the Sudan III reagents for fats, Milon reagents for protein, and IKI reagents for carbohydrates. Histological results were then observed using a light microscope with a magnification of 400 $\times$ .

The research was conducted in the Laboratory of Plant Development Structure, Faculty of Biology, Universitas Gadjah Mada. Histological observation for tissue used a preservative preparation from embryo tissue of backcross 1 of oil palm. The seed shell was broken, and then the embryo and its endosperm were carefully separated. Dry mounts of oil palm embryos were made through paraffin method. The first step was immersing the embryo using 70% alcohol. Fixation was done using FAA solution and kept for 24 hours. Washing and dehydration or tissues immersion in 70%, 80%, 90%, and 100% alcohol continuously every 30 minutes, was carried out gradually to avoid sudden changes in the cells. The next steps were dealcoholization, and paraffin block embedding with pure paraffin. The slices were then covered with Canadian Balsam and dried on a hot plate at 45°C (Jensen, 1962). The glass slides were labeled according to their treatment and observed under a light microscope.

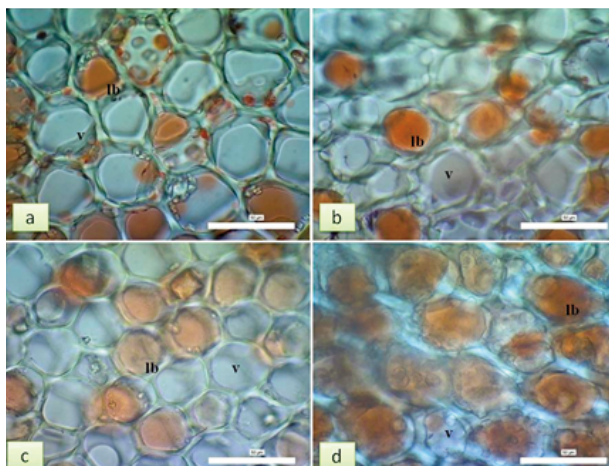
Endosperm tissue mounting for fresh histochemical analysis was prepared by breaking the seeds coat with hammer and separating the kernel from the shell. The kernel in fresh condition was cut (5  $\mu$ m thick) using sliding microtome. The slices were collected and immersed in petridish containing 50% ethyl alcohol for three minutes. The slices were placed on a glass object and dropped with IKI staining reagents for starch or carbohydrate observation, Milon reagent for protein observation, and Sudan III reagent for fat observation. IKI reagent was prepared by dissolving 2 g KI in 100 ml of water, which was then dissolved with 0.2 g of iodine in KI solution. After dropping the reagent, it was covered with slide cover and heated on a hot plate at the temperature of 45°C (Jensen, 1962). Afterward, glass objects were labeled according to their treatments and observed under light microscope.

## RESULTS AND DISCUSSION

Fat is stored within cells as fat drops (oil storage bodies or spherosomes) with a diameter of 0.2-0.6 microns. Sudan III would show red to orange color in cell tissue with fat content (Jensen, 1962). Observation showed that seeds stored for 4 years had very small colored cell parts, indicating that there was little amount of fat remain in endosperm. In the seeds stored for 3 years, the size of the colored cells tended to be larger but in small number. In the seeds stored for 2 years, the cells were not fully colored, but it is clearly seen that the orange colored parts were clearer than in the seeds stored for 3 and 4 years. Fat filled whole cell tissues in fresh seeds stored less than a year (Figure 2).

Based on the results of histochemical observations, it was found that the longer the seed stored, the less the fat content in endosperm cell tissue. Kok et al. (2013) stated that the fat content in oil palm seeds reached 55% of the total dry weight of the seeds, followed by carbohydrate content of 18% and protein content of 17%. Bewley et al. (2013) stated the composition of fat, carbohydrate, and protein content in oil palm reached 49%, 28%, and 9%, respectively. Seeds with a high fat content have a limited shelf-life because seeds with high fat composition are very susceptible to oxidation (Morello et al., 2004; Shaban, 2013).

Seed carbohydrate in the form of polysaccharide or starch, which was observed using the IKI test, will produce a red to purple color (Jensen, 1962). Carbohydrates occur as large molecules detected with histochemistry on the outside surfaces of all types of cell, as stored or secreted substances within some cells, and in the extracellular matrix (Kiernan, 2017). Histochemical test on oil palm endosperm showed a yellowish orange to brownish color (Figure 3). This shows that oil palm endosperm contained only small amount of carbohydrates. Based on the results of histochemical observations, all cells were orange to brown in all storage periods from 4 years to less than 1 year (Figure 3). Based on carbohydrate content at different storage periods, it was very difficult to distinguish carbohydrate content in the form of starch and starch grains. These results are



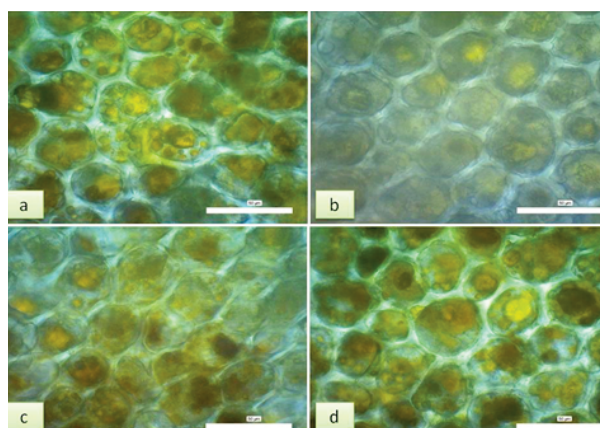
**Figure 2.** Histology of seed endosperm fat content using Sudan III reagents at different storage periods: a) 4 years; b) 3 years; c) 2 years; d) less than 1 year. Observation used a microscope with a magnification of 400×. lb= lipid bodies; v= vacuola.

consistent with observations made previously on oil palm *E. guineensis* Jacq. and several other palms, including *Phoenix dactylifera* (Aslam et al., 2011), macaw palm (*Acrocomia aculeata* (Jacq.) Lodd. ex Mart) (Moura, 2010), and *Euerpe edulis*, which have a protein and lipid shelf tissue in the form of protein bodies and lipid bodies, but not in starch.

Milon reagents is one of the oldest tests for protein. This test is based on the amino acid content of tyrosine and tryptophan (Jensen, 1962). Seed storage proteins are accumulated in the storage protein vacuoles (Zafra et al., 2018). Large to small globular proteins are found in the fat layer that surrounds the protein (Bewley and Black, 1985). Proteins are stored in protein body units of 1–20 microns in diameter attached to the lipoprotein membrane.

Based on the observations, it can be seen that the protein bodies in endosperm are mostly globoid crystals and only a few are crystalloids. The globoid crystals and crystalloids form of protein is the same as the proteins that make up the endosperm on *Phoenix dactylifera* (Aslam et al., 2011). Crystalloid proteins originate from external endosperm cells and form food reserves in endosperm (Panza et al., 2004).

The protein in oil palm endosperm is relatively low. This can be seen from the small amount of color absorbed in the process of histochemical staining. Cells that contain lots of protein will generally absorb the dye and turn red. Based on the results of this research, it can be seen that the absorption of color was very slight in all periods so it became very difficult



**Figure 3.** Histology of carbohydrate at the endosperm of oil palm using 5% IKI at different storage periods: a) 4 years; b) 3 years; c) 2 years; d) less than 1 year. Observation used a microscope with a magnification of 400×.

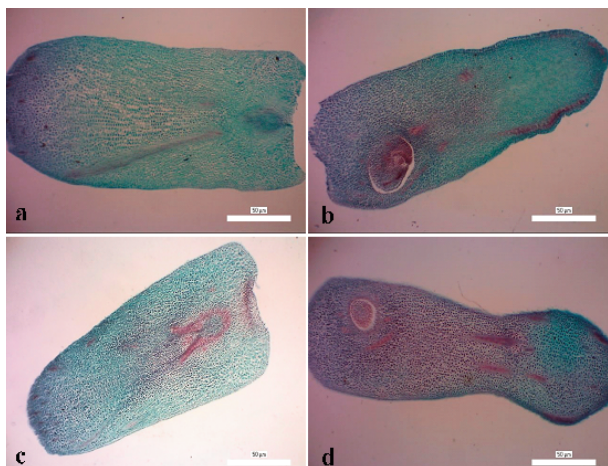
to be used to distinguish different storage periods.

Histological results of embryonic tissue showed green and red staining. The cells that absorbed strong red dye were cells with solid cytoplasm and actively metabolizing cells, while green cells were dead cells. From these observations, it is clear that seeds stored for 4 years did not produce color (the cells already died). Meanwhile, the seeds stored for 3 and 2 years still have colored red cells. The condition of seeds stored less than a year were relatively fresh so that the absorption of red color was much clearer, meaning that cells were still life (Figure 4).

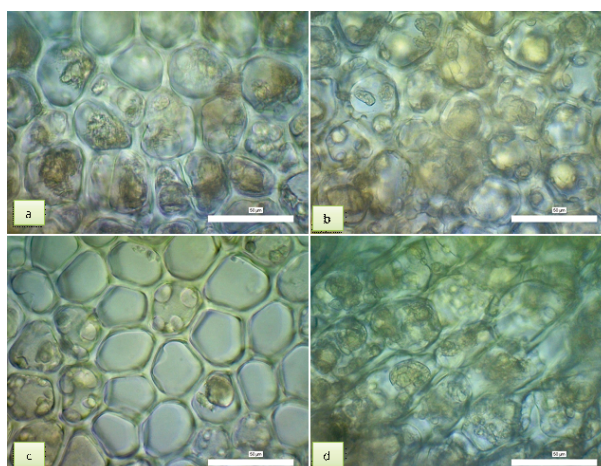
Seed food reserves were formed optimally when the seeds reached their physiological maturity and the chemical composition of seeds was greatly influenced by genetic factors, such as differences in species and varieties. However, after seeds were harvested and stored, it was found that the longer the shelf life, the more decreasing the quality of food reserves in the seeds (Bewley et al., 2013).

The seed period is one of the factors causing the seed deterioration. Oil palm seeds with different shelf lives have different histological characteristics of fat, carbohydrate, and protein content. The histological characteristics can be used as a parameter of seed quality because by knowing the composition of fat, carbohydrates, and protein, the seed deterioration of seeds can be detected.

The fat content, which is the largest food reserve in oil palm seeds, was a determining factor in the level of seed deterioration and quality. The shelf life



**Figure 4.** Histology of seed embryo tissue with 40x magnification at different periods of seeds: a) 4 years; b) 3 years; c) 2 years; d) less than 1 year



**Figure 5.** Histology of protein in seed endosperm at different storage periods: a) 4 years; b) 3 years; c) 2 years; d) less than 1 year. Observation used a microscope with a magnification of 400x

of oil palm seeds for up to 3-4 years led to a very large reduction in fat content as seen from cell tissue histology with very few cells colored red to orange (Figure 2). The results of observing fat content at different shelf lives of seeds produced very clear staining differences so that the results of histological observations can be used as the indicator of seed deterioration.

Carbohydrates and proteins that are part of seed food reserves in oil palm endosperm were in relatively very small amount. It was seen from the histology of cell tissue that absorbed very little dye (Figures 3 and 5). This result is similar to the histology of zygotic embryo from another seeds like macaw palm (Moura et al., 2010) and cacao (Dangou et al., 2002). Moreover, it is similar to the queen palm seeds (*Syagrus romanzoffiana* (Cham.)) that has very low protein content (Iossi et al., 2016).

Histological observations of carbohydrate content represented by observations of starch (Figure 3) and of protein observations (Figure 5) show the same staining, making it very difficult to distinguish different shelf life treatments for seeds. This is because food reserves in the form of polysaccharides are very rarely found except on the cell wall. Similar results were also reported by Moura et al. (2010) on the polysaccharide content observation in embryos and endosperm using PAS test on macaw palm plants. Therefore, histochemical results from carbohydrate and protein observations are not representative if used as a determinant of backcross 1 of oil palm oil seeds at different shelf lives.

The more mature the cell tissue, the more obvious its effect on seed deterioration, seen from histological observation. In the histology of embryos stored for a very long time, which was up to 4 years, all the embryonic cells deceased. It can be seen from the entirely green coloring pattern. On the contrary, embryos stored for less than a year, which tended to be fresh, produced a slightly green color pattern with the majority of red, indicating the cell is still active (Figure 4).

Based on the results of this study, the seeds of backcross 1 were easily deteriorated with a relatively short shelf life. It can be seen from the content of food reserves, including fat, protein, and carbohydrates, which were rapidly degraded, especially in the seeds that were stored for more than a year. Therefore, the results of this study can be used as a basis for determining the seed deterioration of backcross 1 to be used as a consideration in the seed storage management both for seed production and germplasm requirements. In general, the seeds of *E. guineensis* Jacq. can be stored up to more than 48 months, thus the storage for backcross 1 seeds must be more considered due to its relatively short shelf life, which is less than a year.

**CONCLUSIONS**

The histochemical content of fat is shown in orange, which fills the cell portion at the shelf life of less than 1 year, and the fewer seeds cells are colored at longer shelf life. Carbohydrate and protein histo-

chemistry on endosperm of the backcross 1 is very small so that the staining is very vague, and there is no difference between seed storage duration. Histochemical description of fat content of seed endosperm can be used as the indicator of deteriorating seed.

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