MODIFIED RNA *in situ* HYBRIDISATION PROTOCOL FOR OIL PALM (*Elaeis guineensis* Jacq.) FRUIT AND INFLORESCENCE

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ABSTRACT

A modified RNA in situ *hybridisation method was tested on oil palm fruits and inflorescences. This method was efficient in preserving the morphology of tissues and preventing tissue damage due to dryness and shrinkage. The tissues were dehydrated with absolute 1-butanol followed by vacuuming with molten paraffin at 60°C. The* in situ *hybridisation analyses were carried out with sense and antisense probes of* ELF1-α *to examine and compare the condition of these tissues treated with the modified and the standard methods. The modified method showed that the permeability of tissues increased with absolute 1-butanol treatment and application of vacuum at 60°C facilitated penetration of the molten paraffin into the centre of the tissues. This resulted in well preserved tissues that remained intact during sectioning.*

Keywords: RNA localisation, oil rich tissue, 1-butanol, vacuuming.

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INTRODUCTION

The African oil palm (*Elaeis guineensis* Jacq.) is the highest oil-yielding crop grown on a commercial scale and is distributed throughout Malaysia and Indonesia (Jaligot *et al*., 2011; Tandon *et al*., 2001). These two countries alone account for almost 9 million hectares of oil palm plantation producing more than 80% of the world's palm oil (Kongsager and Reenberg, 2012). The commercial palms are known as *tenera*, a cross between large thick-shelled *dura* and small shell-less *pisifera*, and they have intermediate shell thickness. The oil palm fruits are drupes and form in a tight bunch. The pericarp of oil palm fruit comprises three layers, the exocarp (skin), mesocarp (outer pulp) and endocarp (a hard shell encloses the kernel) (Sambanthamurthi *et al*., 2000). The fibrous mesocarp is the source of palm oil and the kernel yields palm kernel oil (Tandon *et al*., 2001).

In situ hybridisation (ISH) is an invaluable tool in locating the expression of new genes or in detecting gene expression patterns. In the earlier experiments, radioactive nucleotides were used to synthesise probes due to their ability to detect very low levels of transcripts. Their major drawbacks are very long exposure times compared to non-radioactive labelled probe, hazardous and a relatively poor resolution, depending on the radioisotope used (Braissant and Wahli, 1998). As radioisotopes are hazardous, recently developed protocols have adopted the use of digoxigenin (DIG)-labelled RNA

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probes. These non-radioactively labelled probes have improved the ISH by shortening the time required for the experiment and provide images with a higher resolution compared to radioactive labelled probe (Engler *et al*., 1998; Braissant and Wahli, 1998). In higher plants, DIG system is widely used; however common problems such as high background noise and lack of consistency in signal specificity in different tissue types are common. Many factors such as probe quality, plant tissue fixation, pre-hybridisation, hybridisation and post-hybridisation tissue treatments affect the outcome of ISH (Shu *et al*., 1999; Wang *et al*., 2008). In order to maintain the tissue morphology and prevent the loss of mRNA from cells, tissue fixation is vital. However, in many ISH experiments, molten paraffin failed to penetrate plant tissues that are thick and contain water insoluble components such as waxy epidermis and oils. This will cause the tissue to become dry and brittle resulting in wax or paraffin failing to penetrate the tissue completely. To solve this problem, the tissue has to be softened, the oil and water components present in the tissue have to be removed without causing any damage to the tissue. Optimisation of the fixation step in ISH is crucial in maintaining the morphology of the tissue for the efficient detection of mRNA transcripts.

In this study, we have adapted methods from Coen *et al*. (1990) and Jackson (1992) with modification for oil palm inflorescences and fruits tissues. The oil palm fruit tissues contain a high level of oil components, fibrous mesocarp, waxy epidermis and hardy endocarp which prevent the infiltration of wax into the tissues. Our aim is to make the tissues more penetrable to paraffin and to prevent them from disintegrating, splitting, scraping or shredding during sectioning and ensure they remain intact after processing. This modified protocol enabled sectioning of paraffin embedded oil palm tissues up to 18 weeks after pollination (WAP) at 8 μm to 10 μm thickness.

MATERIALS AND METHODS

Sample

Oil palm clonal fruits (Clone Pl64) ranging from a day before pollination (DBP) to 18 WAP, and oil palm immature and mature female inflorescences provided by the Malaysian Palm Oil Board (MPOB) were used in this study.

Riboprobe Synthesis

Riboprobes of Elongation Factor1-alpha (*ELF1-α*) were synthesised using the Ampliscribe™ T3 and T7 Flash™ Transcription Kit (Epicentre® Biotechnologies). The PCR product flanked by the T3 promoter sequence: 5′-AATTAACCCTCACTAAAGG-3′ and T7 promoter sequence: 5′-TAATACGACTCACTATAGG-3′ was labelled with 1X DIG RNA Labelling mix (Roche). Standard protocol of carbonate hydrolysis (Weigel and Glazebrook, 2002) was carried out and RNA was ethanol precipitated. The riboprobes were finally dissolved in 50% (v/v) formamide and stored at -20ºC. Dot-blot analysis was carried out according to the DIG Application Manual (Roche) to determine the concentration of riboprobe.

Fixation

Standard method. The fixation and embedding of tissues were carried out according to the standard protocol (Coen *et al*., 1990; Jackson, 1992). Tissues were fixed with EAF fixative mix [50% (v/v) of absolute ethanol, 5% (v/v) of acetic acid, 10% (v/v) of 37% formaldehyde and DEPC treated water], followed by vacuuming for 15 min and incubation of the sample in fixative solution for 1 hr at 4ºC. These steps were repeated three times prior to incubation of the samples in fixative solution for two days at 4ºC. Samples were then dehydrated in 70% (v/v) ethanol, 96% (v/v) ethanol and 100% (v/v) ethanol sequentially for 30 min each at room temperature (RT). Dehydrated samples were incubated in 75% (v/v) EtOH: 25% (v/v) xylene for 30 min followed by 50% (v/v) EtOH: 50% (v/v) xylene for 30 min, 25% (v/v) EtOH: 75% (v/v) xylene for 30 min and thrice in 100% (v/v) xylene for 15 min each. About 20 pieces of paraffin chips were added to 20 ml xylene and left overnight at 42ºC for penetration of paraffin into the plant material. The xylene with paraffin was replaced with molten paraffin (60ºC). The molten paraffin was refreshed twice daily for three days and followed by paraffin embedding. The paraffin blocks were stored at 4ºC until future use.

Modified method. The tissues were fixed in EAF fixative mix followed by vacuuming and dehydration with a series of ethanol as in the standard method. Then, the oil palm tissues were incubated in absolute 1-butanol (Merck) for a duration of two weeks or one month, depending on their tissue rigidity. The inflorescences and fruits from one DBP up to six WAP were incubated at RT in absolute 1-butanol for two weeks while the other fruit stages were incubated for one month in absolute 1-butanol. The solution was changed three times during this incubation period. After that, samples were incubated in 75% (v/v) EtOH: 25% (v/v) xylene for 30 min followed by 50% (v/v) EtOH: 50% (v/v) xylene for 30 min, 25% (v/v) EtOH: 75% (v/v) xylene for 30 min and thrice in 100% (v/v) xylene for 15 min each. About 20 pieces of paraffin chips were added to 20 ml xylene and left overnight at 42ºC for penetration

of paraffin into the plant material. The xylene with paraffin was replaced with molten paraffin and incubated at 60°C for three days. The molten paraffin was refreshed twice daily. The tubes were left open to evaporate traces of xylene. During this period, vacuum was applied 3 times at 60°C for 15 min each. The infiltrated tissues were paraffin embedded and stored at 4°C.

Pre-hybridisation and Hybridisation

The tissues were sectioned at 8 μm to 10 μm by using a manual microtome (Leica RM3125, UK). The tissue sections were pre-treated based on standard protocols involving de-paraffinisation of section with xylene followed by dehydration in a series of ethanol solutions. The slides were incubated in 2X SSC for 15 min before immersing in Proteinase K solution [1 μg ml^{-1} proteinase K (Sigma) in a pre-warmed pronase buffer (50 mM Tris, pH 8.0, 5 mM EDTA, pH 7.5)] for 30 min at 37°C. The Proteinase K digestion was stopped by immersing the slides in freshly prepared 0.2 mg ml^{-1} glycine in 1X Phosphate buffered saline (PBS) for 2 min. The slides were washed in 1X PBS for 2 min twice before the sections were re-fixed in 4% (w/v) paraformaldehyde for 10 min. The slides were washed in 1X PBS for 5 min twice followed by acetylation of slides in 0.1 M triethanolamine (pH 8.0) and 0.5% (v/v) acetic anhydride for 5 min to reduce the background. The slides were rinsed twice in 1X PBS for 5 min each prior to dehydration in a series of ethanol solution.

The pre-treated slide was added with 100 μl of hybridisation solution [100 ng of denatured DIG-labelled *ELF1-α* probe in 50% formamide to a final volume of 20 μl and 80 μl of hybridisation buffer; 10 μl of 10X *in situ* salts (3 M NaCl, 100 mM Tris-HCl, pH 6.8, 100 mM NaH_2PO_4 buffer and 50 mM EDTA), 20 μl of 50% (w/v) dextran sulphate, 2 μl of 50X Denhardt's solution, 40 μl of deionised formamide, 1 μl of tRNA (100 mg ml^{-1}) and 7 μl of DEPC-treated water]. The slides were incubated overnight in a humidified container at 50°C.

Post-hybridisation and Detection

This step was carried out according to the optimised protocol of Ooi *et al*. (2012). The hybridised slides were washed in pre-warmed 5% (w/v) SDS in 40 mM sodium phosphate buffer followed by 1% (w/v) SDS in sodium phosphate buffer at 55°C for 15 min with slow shaking. Following that, the slides were rinsed with pre-warmed (37°C) 1X NTE buffer (0.5 M NaCl, 10 mM Tris-Cl, pH7.5, 1 mM EDTA) twice for 5 min and 1X TBS (0.1 M Tris-Cl, pH7.5, 0.15 M NaCl). The slides were cooled down at room temperature. Blocking solution [100 μl; 1% (w/v) Boehringer block A (Boehringer Mannheim, GmbH, Germany) in 1X TBS, diluted 1:2000 anti-DIG-AP, Fab fragments antibody (Roche Diagnostics GmbH, Germany)] was added onto each slide and incubated for 2 hr at room temperature. The slides were washed twice with 100 mM Tris-Cl, pH 9.5, 100 mM NaCl for 10 min each prior to staining. Staining overnight was performed in detection buffer containing 1:50 diluted nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP; Roche Diagnostics GmbH, Germany) at room temperature, in the dark.

Subsequently, the enzymatic reaction was stopped by rinsing the slides in distilled water followed by dehydration and rehydration of slides in a series of ethanol series. Finally, the slides were mounted with 50% (v/v) glycerol. All the slides were viewed and photographed with a camera attached to the LEICA DM6000 B light microscope (Leica, Germany) and processed using the Progress Research Pro Software (Leica, Germany).

RESULTS AND DISCUSSION

The oil palm fruits and inflorescences were subjected to both the standard *in situ* hybridisation (Jackson, 1992; Muller and Sheen, 2008) and modified methods. Both differentially fixed tissues were hybridised with DIG-labelled elongation factor 1-α (EL 687602) probe. The ELF1-α is a constitutively expressed gene and can be detected by most of the protocols (Gruber and Levine, 1997; Luo *et al*., 2013; Hart and Basu, 2009; Ooi *et al*., 2012).

The standard method used tends to produce tissues that are brittle and dry which failed to keep the tissue structure intact. The cross sections of a fruit at 1 DBP (*Figure 1A I-II*) and immature female flower cross sections (*Figure 4B I-II*) that were treated with a standard method were broken at the central region and had many brown deposits. Some tissues in the central region were fragmented and detached too. In contrast, the morphology of tissues treated with the modified method (*Figure 1A III-IV* and *Figure 4B III-IV*) was intact and well preserved. Besides that, lesser brown deposits were found which cause less interference during observation of gene expression at targeted regions of the tissues. The longitudinal sections of fruit at 1 DBP (*Figure 1B I-II*) and immature female flower (*Figure 4A I-II*) showed that the stigma region of the tissues was totally damaged and disintegrated. While, the tissues treated with the modified method (*Figure 1B III-IV* and *Figure 4A III-IV*) showed an intact structure and the stigma canal region can be easily identified. The condition of the tissues in a more mature oil palm fruit showed the standard method is not suitable for preserving the tissue structure. The sections of fruit at one WAP (*Figure 2A I-II*) disintegrated and the brittleness of the tissue was quite obvious. The condition wors-

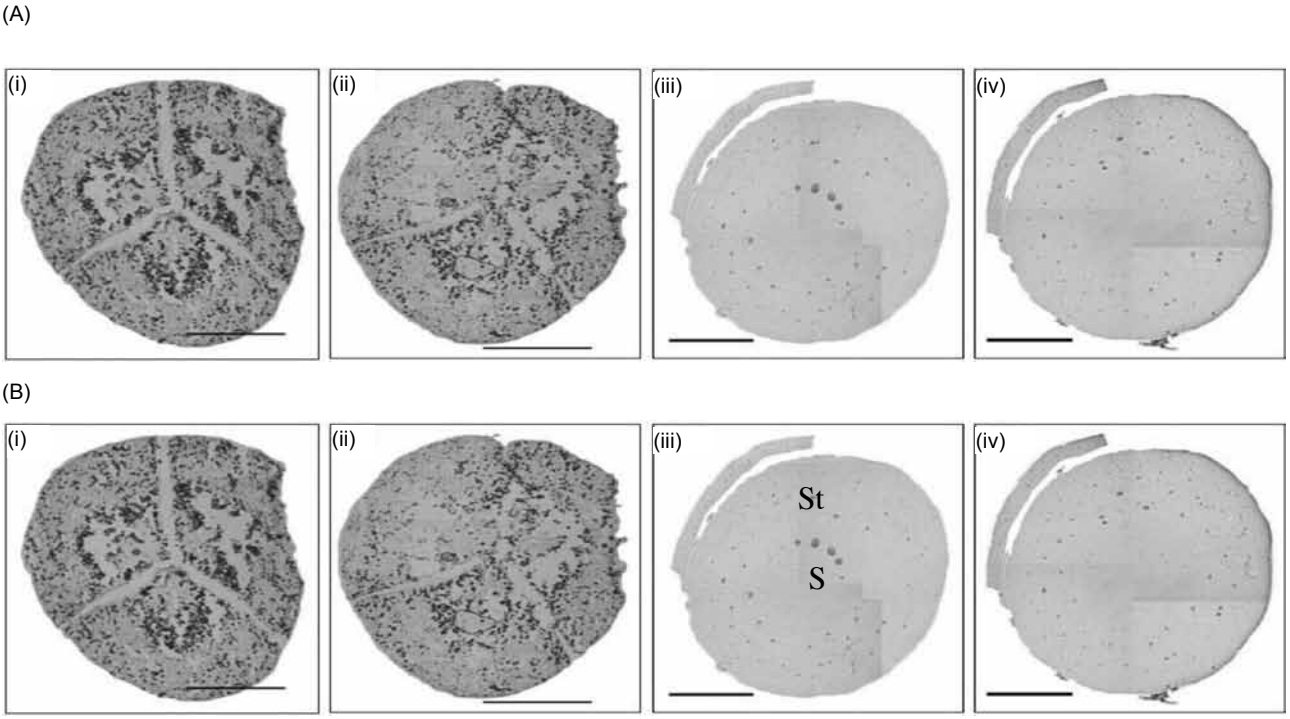

Figure 1. Comparison of in situ *hybridisation protocols on oil palm: (A) cross-section of fruit at a day before pollination and (B) longitudinal section of fruit at a day before pollination. The tissue section was hybridised with sense (I and III) and antisense (II and IV) of ELFα-1 riboprobes. Standard RNA* in situ *hybridisation method was applied for tissues I and II while tissues III and IV were carried out with a modified RNA* in situ *hybridisation method. Scale bar = 500 µm. SC, sytlar canal; St, stigma.*

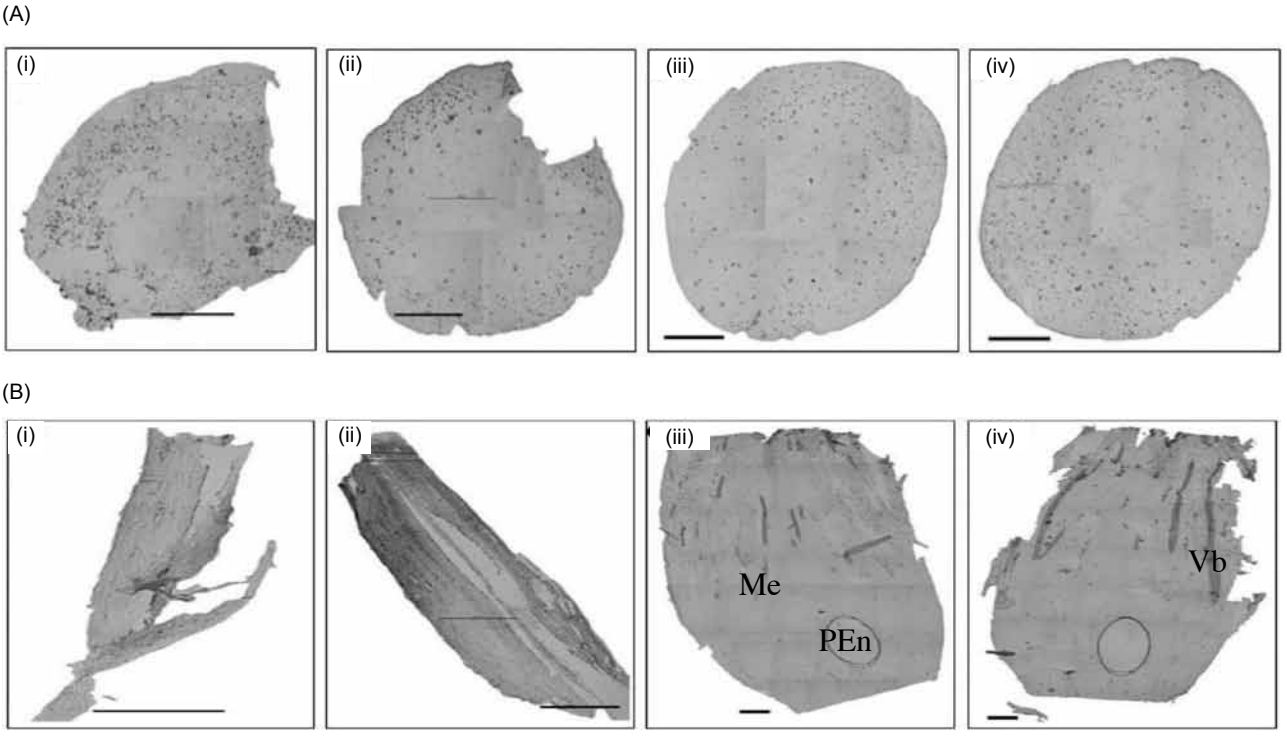

Figure 2. Comparison of in situ *hybridisation protocols on oil palm: (A) cross-section of fruit at one week after pollination and (B) longitudinal sections of fruit at two weeks after pollination. The tissue section was hybridised with sense (I and III) and antisense (II and IV) of ELFα-1 riboprobes. Standard RNA* in situ *hybridisation method was applied for tissues I and II while tissues III and IV were carried out with a modified RNA* in situ *hybridisation method. Scale bar = 500 µm. Me, mesocarp; PEn, pre-endocarp; Vb, vascular bundle.*

ened for the two WAP (*Figure 2B I-II*) whereby most part of the tissue was ripped off and shrivelled. While the modified method, kept the tissue architecture well preserved in both (*Figure 2A III-IV* and *Figure 2B III-IV*). The formation of pre-endocarp and the presence of vascular bundle in the mesocarp region were clearly observed in *Figure 2B III-IV*. For the standard method, the cross-section of a fruit at two WAP (*Figure 3A I-II*) and the longitudinal section of a fruit at four WAA (*Figure 3B I-II*) were totally damaged and the cells had collapsed. The tissues did not adhere well to the slides due to extreme dryness of the tissues. However, the modified method was able to preserve the tissue well and the anatomy was visible (*Figure 3A III-IV* and *Figure 3B III-IV*). Intact and well preserved epidermis and mesocarp were detected in these tissues. As in longitudinal tissue of a fruit at four WAP, the ring of endocarp was clearly observed. The data for the remaining tissues up to 18 WAP is not shown as the tissues prepared using the standard method were in extremely bad condition and failed at sectioning. The tissues were hard and excessive dryness caused difficulties in sectioning. With the modified method, the fruit tissues up to 18 WAP were successfully sectioned and the tissues were still intact even after hybridisations were carried out. As the oil palm fruit ages, the mesocarp becomes more fibrous and the endosperm increases in size. After 10 WAP, the endosperm hardens and oil deposition starts and is completed by 16 WAP. The endocarp hardens further throughout the development phase and by 16 WAP the endocarp is a hard shell enclosing the kernel (Kok *et al.*, 2013). The hard nature of the endocarp is attributed to the presence of sclereids, with thick and highly lignified cell walls. The sclerenchymal fibre bundles and tracheal elements of the mesocarp start lignifying and oil starts to accumulate in the parenchymatic cells as the fruit develops (Reis *et al.*, 2012). Thus, for the older oil palm fruits, the infiltration process was unsuccessful using the standard method. In order for the infiltration process to work efficiently, the oil content needs to be removed from the fruits and replaced by a matrix to avoid tissue shrinkage.

The common procedure in dehydrating, clearing and embedding tissue is to replace the water present in the specimen with alcohol. The alcohol is then replaced by a volatile fluid such as xylene. However, there is certain limitation to the standard technique which makes it unsuitable for plant materials containing lignified elements. The specimen tends to harden and shrink even in complete dehydration if left in high concentration of alcohol too long (Wilson, 1930). All the water must be removed from the tissue

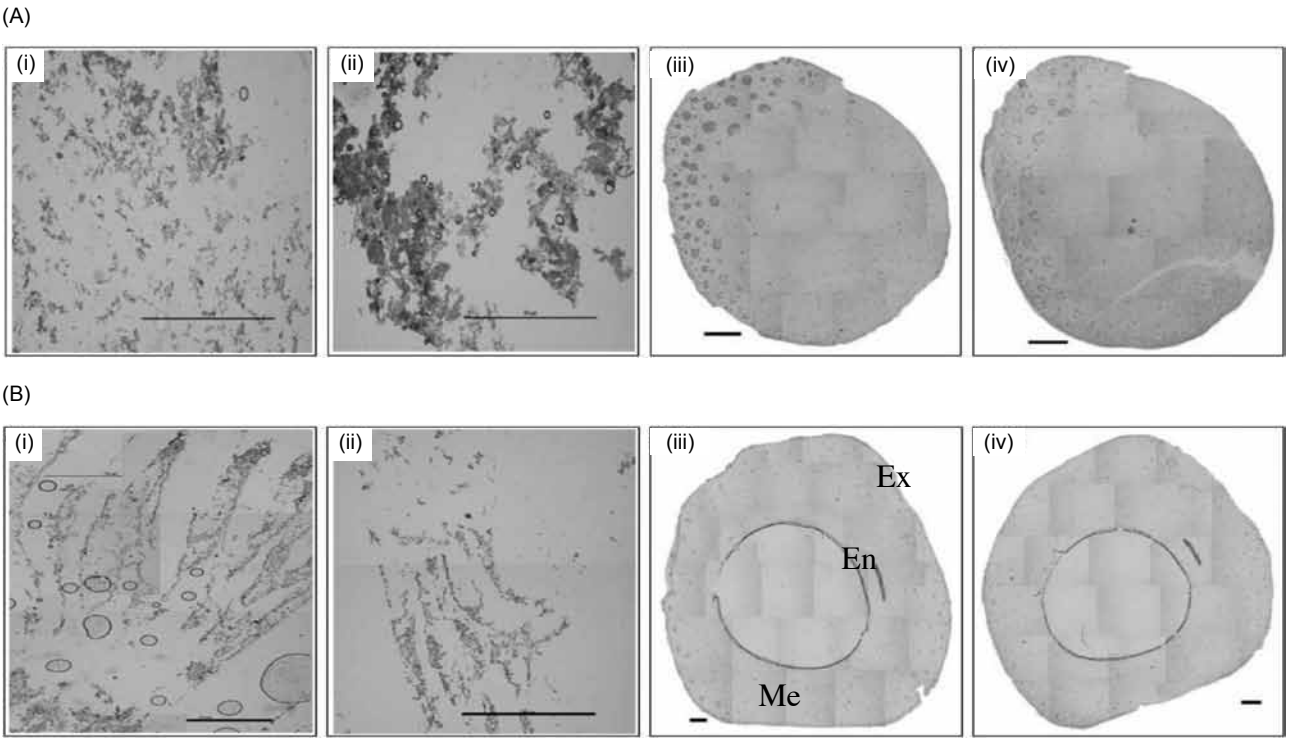

Figure 3. Comparison of in situ *hybridisation protocols in oil palm: (A) cross-section of fruit at two week after pollination and (B) longitudinal section of fruit at four weeks after pollination. The tissue section was hybridised with sense (I and III) and antisense (II and IV) of ELFα-1 riboprobes. Standard RNA* in situ *hybridisation method was applied for tissues I and II while tissues III and IV were carried out with a modified RNA* in situ *hybridisation method. Scale bar = 500 µm. ME, mesocarp; En, endocarp; Ex, exocarp.*

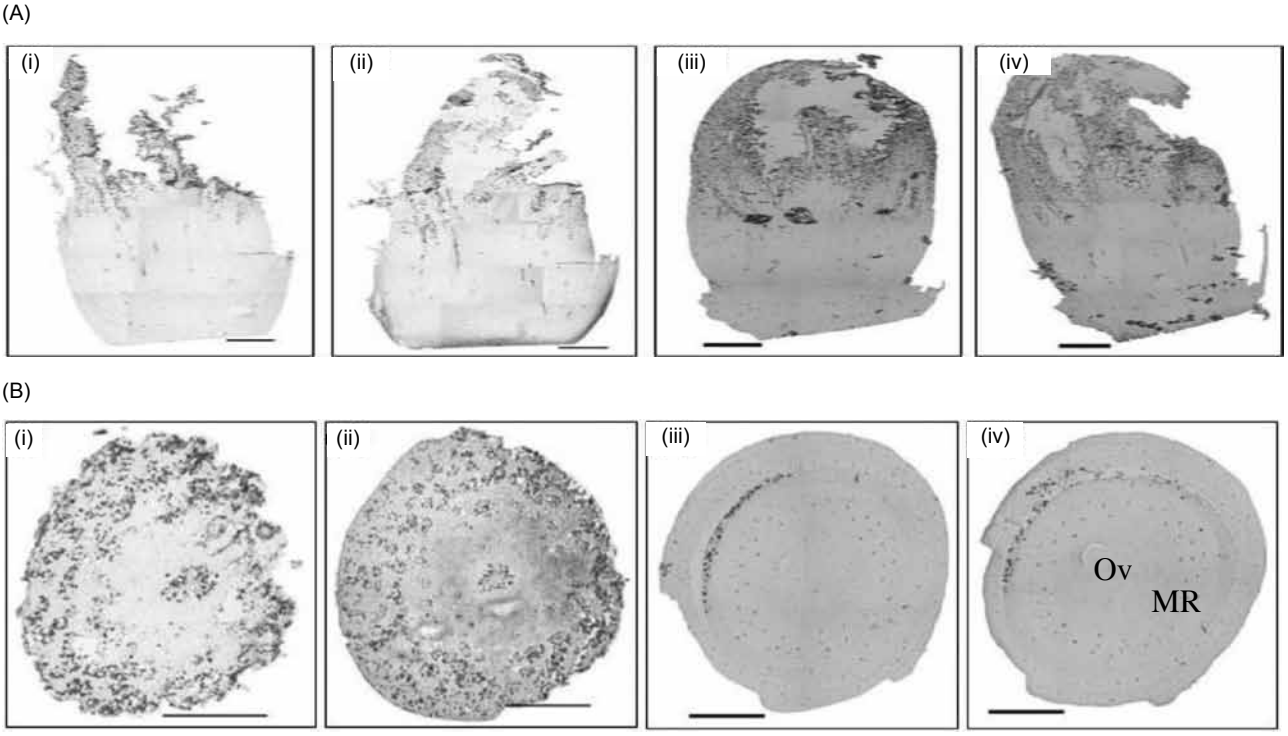

Figure 4. Comparison of in situ *hybridisation protocols on oil palm: (A) longitudinal section of fruit at immature female flower and (B) cross-section of fruit at immature female flower. The tissue section was hybridised with sense (I and III) and antisense (II and IV) of ELF riboprobes. Standard RNA* in situ *hybridisation method was applied for tissues I and II while tissues III and IV were carried out with a modified RNA* in situ *hybridisation method. Scale bar = 500 µm. Ov, ovule; MR, median region of the mesophyll.*

to ease the penetration of xylene and paraffin. Thus, butyl alcohol can be used for dehydration since it is a slow dehydrant causing less shrinkage and hardening of the tissue (Spencer and Bancroft, 2013).

The butanol treatment after the fixation and ethanol dehydration steps obviously improved the tissue rigidity. In animal studies, butanol has been used to reduce the hardening and shrinkage of lightly chitinised arthropods (Carson and Hladik, 2009). Besides that, the canine endocardium blocks were dehydrated with t-butanol to reduce shrinkage compared to the usage of high concentration of ethanol for scanning electron microscopy (Wheeler *et al*., 1975). In plant, butanol has been used as dehydrant solvent in many histological studies (Lu and Vasil, 1985; McCully, 1967; Chengalrayan *et al*., 2001) and *in situ* hybridisation analyses (Aharon *et al*., 2003; Jabnoune *et al*., 2009; Alvarez *et al*., 2006).

Apart from that, the tissue must be infiltrated with paraffin before embedding. The paraffin is not miscible with water and alcohol. Thus, the tissue is gradually dehydrated with alcohol as discussed above. Then, the clearing agent (xylene) which is miscible with alcohol and paraffin is infiltrated through the tissue. The size, penetrability and components of the tissue dictate how quickly this can occur. A vacuum system can be applied to improve the efficiency of wax infiltration by speeding up the removal of the clearing agent (Nowacek, 2010). The tissue texture becomes dry and hard when the paraffin failed to fill in the whole tissue particularly in the centre region of the tissue section. By applying vacuum to the treated tissue, the fluid paraffin can be easily penetrated to the hard tissues and this step has been proven by Wang *et al.* (2008) in their studies.

CONCLUSION

We conclude that the modified RNA *in situ* hybridisation method reported in this study is effective in preserving the structure and anatomy of oil palm fruits and inflorescences. This method might be applicable for other higher plant species with tissues rich in oil content, fibre and/or with waxy cuticles.

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