Preparation of leaf anatomy slide using modification protocols

A N Mardiyyaningsih* and E Daningsih

Pendidikan Biologi Study Programme, Universitas Tanjungpura, Jl. Prof. H. Hadari Nawawi, Pontianak, Indonesia

*Corresponding author e-mail: asria.nurdini.m@fkip.untan.ac.id

Abstract. Preparing leaf anatomy slide is a routine procedure in physiology and anatomy plant research. A standard microtechnique method frequently adopted for doing such procedure is Johanssen’s. However, for early user such as university students, executing this method may be challenging in term of using many chemicals in different stages which make it more pricey and need longer duration. This research attempts to analyse the possibility for using simpler protocol suggested by Gunarso to replace Johanssen method. It is conducted under a qualitative experiment design involving leaves from monocotyl and dicotyl classes as samples. Samples are analysed with safranin-fast green staining protocols. Result shows that there is no difference between Johanssen’s and Gunarso modified protocol (GMP) with respect to vivid observable of the cell membrane, the tissue structure, and the colour intensity. Hence, this new modification protocol is potential to replace the Johanssen methods in leaf anatomy slide

Keywords: Leaf anatomy slide, slide preparation protocol.

1. Introduction

Studying botany involves the needs for investigating the anatomy and physiology of plant [1]. The leaves, as one of the main organs of plants, are frequently become the object of investigation. The leaves serve three fundamental physiological process for plants: photosynthesis, respiration and transpiration [2]. The physiological process and anatomical structure of leaf are related, for that changes in the function frequently observed in the changes of its anatomical structure [3]. For example, the droughtness may lead to the change of the tissue thickness, i.e. mesophyll or palisade/spongy parenchyme [4] [5]. This anatomical change may be observed by preparing leaf anatomy slide through a microtechnique procedure is required to assist the production of slides to support the microscopy analysis of anatomical structure [6]. Thus, the basic skill for preparing anatomy slide is necessary for plant researcher in general, including the early researcher, such as the undergraduate students.

The sectioning or paraffin method is the routine procedure for producing plant anatomy slide. Compare to the freshly prepared slide, the permanent one is preferrable as it provides the chance to repeatedly observed the object over the time and differentiate the tissues effectively using combination of staining [7]. A standard microtechnique procedure frequently adopted for doing sectioning or paraffin protocol in plants is the Johansen method [6] [8]. Johansen used the combination of Tertiary butyl alcohol (TBA) alongside ethanol (EtOH) to reduce tissue hardening in the plant sample. Compared to the animal tissue preparation [9], the extra duration for sample soaking in the TBA-EtOH mixture at the dehydration step was conducted to ensure the maximum penetration of the chemical through the cell wall [10].

Although the Johansen’s is the routine protocol for producing leaf anatomy slide, for early user such as undergraduate students, executing this method may be challenging. It is not uncommon that preparing
the slides for their research become the first time students interact with the plant microtechnique. As it is, a simpler procedure with less material and shorter duration for working on the slides is preferable. The animal tissue preparation, which was suggested by Gunarso [9] could be the candidate for the easier and cheaper protocol. The modification of leaving the sample in the chemical was executed as a countermeasure for the drawback of cell wall presence in plant which will require extra time for going through.

This research is conducted to evaluate the possibility for undergraduate students for using simpler protocol with modification as suggested by Gunarso to replace the routine Johansen protocol.

2. Materials and Methods

This was a qualitative research design conducted in the Laboratory of Pendidikan Biologi Universitas Tanjungpura. The comparison of preparing samples between using Johansen method and the modification method of Gunarso was observed based on three parameters of good preparation protocol [9]: the clear distinction between cells, the tissue structure, and the colour intensity between different components of cell. The justification of general application the Gunarso modified-protocol (GMP) was conducted to the six different plants.

2.1. Materials

Sample of Hanjuang (Cordyline fructicosa) leaves were processed by both the Johansen and GMP. Leaves sample from other plants were used for confirming the possibility of applying the GMP. There were the leaves from dicots plants: Bougenville (Bougainvillea glabra Choisy), Pucuk Merah (Syzygium paniculatum Gaertn), and Puring (Codiaeum variegatum (L.) A. Juss), and the the leaves from monocot plants: Sri Rejeki (Aglonema crispum), Song India (Dracaena reflexa).

2.2. Methods

Each leaf samples were freshly cut from below the lowest branch. The leaves then cut into thin section of 2 x 2 cm. The preparation protocols of Johansen and GMP were conducted by the students under the supervision of the plant microtechnique instructor.

2.2.1. The Johansen method

This protocol is adopted from the routine procedure of plant sectioning methods as described in Prawasti et al.[8]

*Fixation and washing.* Samples were preserved using FAA (9 ml of ethanol 70%, 0.5 ml acetic acid, and 0.5 ml formaldehyde solution) for 24 hours. It was then washed using 70% ethanol solution for 60 minutes, which was repeated three times.

*Dehydration.* This was conducted through leaving the samples in a mixture of 1:1 TBA and parafin oil for 2 hours.

*Infiltration.* This step involved a mixture of chemical with a composition as follow:

- DJ 1: aq, etOH 95% and TBA = 5 : 4 : 1, samples were leaving for 2 hours
- DJ 2: aq, etOH 95% and TBA = 3 : 5 : 1, samples were leaving for overnight
- DJ 3: aq, etOH 95% and TBA = 15 : 50 : 35, samples were leaving for 2 hours
- DJ 4: etOH 95% and TBA = 45 : 55, samples were leaving for 2 hours
- DJ 5: etOH 100% (abs) and TBA = 25 : 75, samples were leaving for 2 hours
- DJ 6: pure TBA, samples were leaving for overnight
- DJ 7: pure TBA, repeated 3 times, each time samples were leaving for 2 hours

*Clearing.* This was conducted through leaving the samples in a mixture of 1:1 TBA and parafin oil for 2 hours.

*Infiltration.* This step involved a mixture of chemical with a composition as follow:

- IJ 1: TBA: paraffin oil: solid paraffin = 15 : 15 : 3, at room temperature, samples were leaving for 2 hours
- IJ 2: pure paraffin, samples were leaving for 1 hour. This step is repeated 3 times every 6 hours.
Embedding. Sample then embedded into a 2x2 cm block of paraffin which then allowed to set for 24 h at room temperature to solidify. Leaf positioned horizontally when put in the mould.

Sectioning & Affixing. The samples were sectioned at 10 μm thickness with a rotary microtome. The sectioned tape were straightened on the waterbath and placed on to the slides which were rubbed by albumin beforehand. The slides were dried out on the hotplate at 37°C for 24h.

Staining. Samples were stained with safranin-fast green. Both safranin and fast green solution were freshly made. Safranin solution were made by diluting 1 gr of safranin powder into 100 ml of aquadest, whilst separately fast green solution were prepared by diluting 0.5 gr of fast green powder into 100 ml of 96% ethanol [9]. Samples were deparaffinised with xylol and rehydrated in a descending series of ethanol (two minutes in each stage) and then stained with 1% safranin for 2 hours. The excessed safranin was washed by dipping the slides several times into aquades solution. The sectioned samples then dehydrated through a series of alcohol (two minutes in each stage) before dyeing with 0.5% fast green for 15 seconds. The excessed safranin was washed by dipping the slides several times into 96% ethanol. The samples then placed into absolute alcohol for 2 minutes, which repeated twice. Finally, the samples were dipped into xylol for 10 minutes, which repeated for 3 times.

2.2.2. The Gunarso modified-protocol (GMP)

This protocol is adopted from the routine procedure of animal sectioning methods as described in Gunarso [9] with a modification in the duration of dehydration, clearing and infiltration. Fixation and washing. Samples were preserved using FAA (9 ml of ethanol 70%, 0.5 ml acetic acid, and 0.5 ml formaldehyde solution) for 24 hours. It was then washed using 70% ethanol solution for 60 minutes, which was repeated three times.

Dehydration. The original protocol of Gunarso [9] advised to apply a series of 70% – 80% – 90% – absolute ethanol to completely dehydrate the samples, leaving the samples for 30 minutes in each solution. The modification were made to increase the duration into an hour for each dehydrated step. Clearing. The Gunarso modified-protocol was conducted by keeping the samples in a series of ethanol-xylol solution as follows:
- CG 1 : etOH 100% (absolute) and Xylol 3 : 1, samples were leaving for 1 hour
- CG 2 : etOH 100% (absolute) and Xylol 1 : 1, samples were leaving for 1 hour
- CG 3 : etOH 100% (absolute) and Xylol 1 : 3, samples were leaving for 1 hour
- CG 4 : pure xylol, samples were leaving for 1 hour, and change with xylol then kept for another 1 hour

Infiltration. In the Gunarso modified-protocol this step was conducted by keeping the samples in a series of ethanol-xylol solution in the oven at temperature of ±58-60°C as follows:
- IG 1: xylol and paraffin 3 : 1, samples were leaving for 1 hour
- IG 2: xylol and paraffin 1 : 1, samples were leaving for 1 hour
- IG 3: xylol and paraffin 1 : 3, samples were leaving for 1 hour
- IG 4: pure paraffin, samples were leaving for 1 hour, and change with paraffin then kept for another 1 hour

Embedding. Samples were embedded in paraffin with the procedure as descried in Johansen method. Sectioning & Affixing. The samples were sectioned at 10 μm thickness with a rotary microtome. The tape were were placed onto the slides with a help of albumin, in a procedure described as in the Johansen’s sectioning & affixing step.

Staining. Samples were stained with safranin-fast green with the same treatment with the staining process described in the Johansen’s staining procedure.

By comparing the two methods, it is clear that the differences of the two procedures were shown in the 3 stages: dehydration, clearing and infiltration. The TBA concentration in the Johansen dehydration stage was replaced by the gradual ascending of ethanol concentration in the GMP. Furthermore, the clearing and infiltration process in Johansen used the paraffin oil, which was replaced by xylol in the GMP. Table 1 shows the resume of the Gunarso modified-protocol and Johansen’s method.
Table 1. A comparison procedures between Johansens and Gunarso modified protocols for preparing leaf anatomy slides. The contrast were marked bold.

| Preparation step | Johanssen’s | Modified-Gunarso |
|------------------|-------------|------------------|
| Fixation         | 24 hours FAA | 24 hours FAA     |
| Washing          | 1 hour 70% ethanol, 3 changes | 1 hour 70% ethanol, 3 changes |
| Dehydration      | A series of ethanol concentration with a duration of 1 hour for each step | A series of a TBA-ethanol with a duration of 2 hours or overnight |
| Clearing         | A series of ethanol-xylol with a duration of 1 hour for each step | TBA and paraffin oil for 2 hours, 3 changes |
| Infiltration     | A series of xylol-paraffin with a duration of 1 hour for each step | A series of TBA-paraffin oil-paraffin, leaving for 2 hours |
|                  | Pure paraffin for 1h, 3 changes | Pure paraffin for 6 hours, 3 changes |
| Embedding        | Paraffin | Paraffin         |
| Sectioning       | Rotary microtome, 10 µm | Rotary microtome, 10 µm |
| Affixing         | Albumin | Albumin          |
| Staining         | Safranin-fast green | Safranin-fast green |

Each slides produced were numbered and given randomly to the two instructors. Instructors were evaluated the slides to assess the clarity of cell wall, tissue structure, and colour intensity.

3. Results and Discussion

Figure 1 shows cross sections of hanjuan leaf (*Cordyline fruticosa*) which were prepared by Johanssen’s and Gunarso modified protocol (GMP). Both slides shows the fairly clear tissue structure of monocot leaf. There are no clear distinction of the colour of the cells although both slides are stained by safranin-fast green.

![Figure 1](image)

Both Johansen and GMP preparation procedures could preserve the tissue structure well. This evidence in the clarity of the cell wall which allow the tissue topography observation. Since other steps
in the preparation were the same, the possible differences would come from the different treatment of three steps: dehydration, clearing and infiltration. Apart from fixative, these three steps were important as they ensure the non-polar environment for samples could be embedded in the paraffin and then being sectioned\cite{6, 9, 10}. The different cells were clearly identified. Some of them were recognised for their position (the upper and lower epidermis), the bigger cell size (the bundle sheath), and the fill-in cells (mesophyll)\cite{11}. However, xylem and phloem were not easily notified since the fast green did not give the counterstain to safranin. With the safranin-fast green, xylem and phloem should be stained differently as they composed of different substrat. The lignified xylem should appear brilliant red with Safranin, whilst the cellulosic cell wall of phloem appears brilliant green\cite{6}. Thus, the two procedures were lacking on supporting the Johansen Safranin-Fast Green staining procedure. The less colour intensity was thought due to the ineffective application of Safranin-Fast green procedure. It is suggested to look over the steps in this procedure as well as to try out another easy to apply safranin-haematoxylin staining protocol as described by Abogadallah et al\cite{12} or by replacing the safranin-fast green protocol with the more cheaper natural dye staining procedure\cite{13}.

Although the GMP did not give far better result compare to Johansen method, this new protocol has a good chance to replace the routine protocol. A three repeated examination of the slides quality produced by the two methods (Table 2) shows no difference in the marking. However, the Johansen method needs to prepare more chemicals (TBA and paraffin oil) compared to GMP (xylol). Furthermore, for students researcher who need to apply sectioning method of plant microtechnique, the GMP is more easy follow and need less time to complete. Subsequently, this advantage overweigh the aplication of Johansen method in this research.

**Table 2.** A qualitative examination of cross section of tissues of Hanjuang leaf slides by two protocols: Johansens’ and GMP. The examination was running for three separated slides prepared by the two protocols.

| Component         | Johansens | GMP            |
|-------------------|-----------|----------------|
|                   | R1        | R2  | R3  | R1  | R2  | R3  |
| Cell wall         | I         | I    | I    | I    | I    | I    |
| Tissue structure: |           |      |      |      |      |      |
| Upper epidermis   | I         | I    | I    | I    | I    | I    |
| Mesophyll         | I         | I    | I    | I    | I    | I    |
| Bundle sheath     | I         | I    | I    | I    | I    | I    |
| Xylem             | NI        | NI   | NI   | NI   | NI   | NI   |
| Phloem            | NI        | NI   | NI   | NI   | NI   | NI   |
| Lower epidermis   | I         | I    | I    | I    | I    | I    |
| Colour intensity  | F         | F    | F    | F    | F    | F    |

**Notes:**
R1 = 1\textsuperscript{st} slide  
R2 = 2\textsuperscript{nd} slide  
R3 = 3\textsuperscript{rd} slide  
I = identified  
NI = not identified  
S = Strong  
F = Fair  
L = Low

The possibility to generally apply the GMP protocol for producing leaf anatomy slides for different species were observed through trailling out the procedure over the six plant species as described in the methods. Figure 2 shows the cross section of slides for each representatives of dicots and monocots leaf.
Figure 2. Cross sections of monocots and dicots leaves prepared by GMP at 400x. The structure of tissue shows the U upper epidermis, M mesophyll, VS vascular bundle, SP spongy parenchyme, PP palisade parenchyme, L lower epidermis. The dicots samples of leaves were *Bougainvillea glabra* Choisy (A), *Syzygium paniculatum* Gaertn (B), and *Codiaeum variegatum* (L.) A. Juss (C). the monocots samples of leaves were *Aglonema crispum* (D), *Dracaena reflexa* (E), and *Cordyline fruticosa* (F).

As shown in Figure 2 and 3, the GMP protocol could well preserved the leaf tissues, either in dicots or monocots, thus showing the different tissues component distinctively. For dicot plants, the palisade parenchyme could be sharply differentiated from the spongy parenchyme due to the different shape.
Meanwhile the same supporting feature of GMP protocol was not really clear. From the Figure, we can also infer that the GMP shows clearer tissue structure for some species, i.e. Bougenville (A), Puring (C) and Sri Rejeki (D). Since the microtechnique procedure usually specific for a part of plants \cite{14} as well as the species \cite{6}, it is still need a further research of how the GMP will allow this. However, so far the GMP could be generally applied to the both dicots and monocots leaves, and well enough supporting research which aims to measure the tissue thickness of a leaf related to some specific circumstances, such as conducted by Surya et al \cite{15}

4. Conclusion
The modification of Gunarso’s animal tissue preparation technique (GMP) has the potency to replace the routine Johansen’s plant tissue procedure procedure as it shows no distinction to observable cell walls border, the tissue structure and the colour intensity of the Safranin-fast green staining. Due to the limitation of the counterstain bond to the slides, so far, the GMP allows the measurement of the thickness of the leaves or thickness of specific tissues, the palisade and spongy parenchyme.

Acknowledgement
The authors wish to thank Faculty of Keguruan dan Ilmu Pendidikan (FKIP) Universitas Tanjungpura to partially fund the research with the DIPA scheme. We would also like to thanks students of Pendidikan Biologi who carried out the protocols as the early researchers: Pangesti Ayu Wandari, Minati, Galih, Inka Febriyanti, Intan, and Nadia Fransiska.

References
\[1\] Salisbury F B, and Ross C W 1995 Fisiologi Tumbuhan Vol. I (Bandung: ITB Press)
\[2\] Culter D F, Botha T and Stevenson D W 2007 Plant Anatomy an Applied Approach (Victoria: Blackwell Publishing)
\[3\] Lewis, M. C. 1972 60(237) 25-51
\[4\] Suharti, Mukarlina and Gusmalawati D 2017 Jurnal Probiont 6(2) 38-44
\[5\] Boughalleb F, Abdellaoui R, Ben-Brahim N, and Neffati M 2014 Central European Journal of Biology 9(12) 1215-1225
\[6\] Ruzin S E 1999 Plant microtechnique and microscopy (New York: Oxford University Press, Inc)
\[7\] Anggraeni R, and Christijanti W 2015 Unnes.J.Biol.Educ 4(3) 256-261
\[8\] Prawasti T, Sulistyaningsih Y, Dorly, Juliandi B , and Juliarni 2014 Penuntun Praktikum Mikroteknik Jurusan Biologi FMIPA (Bogor: IPB)
\[9\] Gunarso W 1989 Bahan Ajar Mikroteknik (Bogor: Depdikbud, Dirjen Dikti, PAU Ilmu Hayat, IPB)
\[10\] Johansen D 1940 A Plant Microtechnique (London: McGraw-Hill Book Company, Inc.)
\[11\] Esau K 1977 Anatomy of Seed Plants, (New York: J. Wiley)
\[12\] Abogadallah G. M, and Nada R 2019 An improved protocol for single-step differential staining of plant tissues with safranin-haematoxylin, prepared print ResearchGate 330994736
\[13\] Noor R 2020 Jurnal Lentera Pendidikan 5(2)
\[14\] Barbosa A. C., Pace M. R., Witovisk L., and Angyalossy V 2010 IAWA Journal 31(4) 373-383
\[15\] Surya S and Hari N 2017 International Journal of Pharmaceutical Science and Research 2(3) 11-14