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Another Wall and Pollen Development in *Averrhoa carambola* L.

Astija

Universitas Tadulako, Kemenristekdikti, Indonesia
Associate Professor, Research field: Plant Sciences

Abstract: ***This study reveals that anatomical development of anther wall and pollen in Averrhoa carambola L. flower. The developments, in different stages, were observed through a preparation by paraffin method, LR White resin method, and SEM. The preparation was observed using a light microscope. Results showed that anther derived from archesporial cells surrounded by epidermal cells. The archesporial cells divided into primary sporogenous cell and primary parietal cells. The former was then to form microspore mother cells that meiotically divided into dyad and tetrad, respectively. Pollen with one nucleus was released from the tetrad present in sporangia of the anther. The nucleus of the pollen was mitotically divided into pollen with two nuclei named vegetative and generative nuclei. The latter divided and differentiated into anther wall consisted of the epidermis, endothecium, two middle layers, and tapetum.***

Keywords: pollen, embryo sac, *Averrhoa carambola* L.

1. Introduction

Star fruit (*A. carambola* L.) is a plant included into Oxalidaceae and found in the tropical region such as Indonesia, Malaysia, and Filipina (Keller et al. 2012; Sánchez et al. 2010; Sawhney 2005). It is mostly like to grow in various soil types such as sandy soil, clay or rocky (Noor 1996)(Kakade et al. 2013). Star fruit (*A. carambola* L.) is widely used as fresh fruits or is processed in the form of drinks, sweets, jams and can also be used for drugs such as for treating fever, vomiting, diarrhea, attack bile, and sources of potassium and copper (Kakade et al. 2013; Li et al. 2016; Abeysekera et al. 2015; Otuki et al. 2011; Dasgupta et al. 2013; Deacute bora et al. 2013). Star fruit also contains vitamins A, B1, C and minerals in addition to carbohydrates and protein (Schreier 2009; Li et al. 2016). Star fruit has a distinctive flavor or aroma. The difference is because of the different contents of chemical compounds such as ascorbic acid, oxalate, benzoate (Schreier 2009).

Star fruit plants can be cultivated through vegetative ways such as graft and nursing, and generative way using seed (Das & Das 2013). The latter way was proceeded by pollination and fertilization (Sawhney 2005). The pollination occurs when pollen grains released from sporangium of the anther land on a stigma (Curie & Briat 2003; Arritt et al. 2002; Lord 2003; Attri et al. 2007). The pollen grain germinates and elongates within pistillous tissues to deliver the generative nuclei into an embryo sac of the ovule (Cheung 1996; Firmage & Dafni 2001; Hedhly et al. 2005; Smith et al. 2013; Cheung et al. 2010). Union of the generative nuclei with egg and polar nuclei produces embryo and endosperm, respectively (Cheung 1996; Firmage & Dafni 2001; Hedhly et al. 2005; Smith et al. 2013; Cheung et al. 2010; Chapman & Goring 2010; Cheung 2000; Chae & Lord 2011). As such, pollen grain plays an important role in the successful processes, in addition to pistillous tissues. However, only a few published account of star fruit pollen development are available. This lack of basic knowledge is more evident for the star fruit trees. Therefore, this study reveals an evidence regarding the anther wall and pollen development of the star fruit trees (Montoya et al. 2005).

2. Materials and Methods

Flowers of the star fruit (*Averrhoa carambola* L.), in different stages, were used in this study. To observe the development, flowers were made preparations preserved with paraffin method (Sass 1958). Various sizes of flowers from 0.2 mm to one day after anthesis were fixed in FAA (Formaldehyde, acetic acid, alcohol) with a ratio of 40% formalin: glacial acetic acid: alcohol 50% = 1: 1: 18 for 24 hours. The materials were aspirated to remove air from the tissues by using an aspirator. Furthermore, the materials were dehydrated in a series of Johansen Solution and were infiltrated with paraffin at 48 ^{o}C and planted into paraffin at 58 ^{o}C. The Materials slashed using a rotary microtome with a thickness of 5 micrometers. The incision was placed on a glass slide-etched Haupt's adhesive and formalin 4%. Glass object was then placed on the heater board at 42 ° C to expand paraffin containing the samples of the flower. The incision was colored with Mayer's Hemalum Dye. Finally, the object glass was etched with adhesive and covered with a glass lid. To observe the object, a light microscope was applied.

In addition to paraffin method, the flowers were preserved with LR White resin method. The flowers were fixed for 2 hours in 2% paraformaldehyde, 1.5% glutaraldehyde, 2 mM $CaCl_2$ and 50 mM PIPES, pH 6.8 under vacuum (-50 kPa) to remove air from tissues of the flowers. The samples were then washed, 3 x 10 minutes, in 50 mM PIPES and stored at 4°C overnight. The samples then were washed 3 x 10 minutes in MQ H_2O. Tissue dehydration was performed through an ethanol series, 10% to 100%, in 10% increments for 45 minutes each at 4°C. The samples were stored overnight when they were in 70% and 100% ethanol and then washed twice in 100% ethanol. Infiltration was conducted using 20%, 40%, 60%, 80% and 100%LR White resin (ProSciTech) diluted with ethanol and changed every 2 hours. Embedding was conducted by placing each sample in a gelatine capsule (000size) filled with 200 µL LR White resin and polymerized at 60 ^{o}C for 3 days. Hardened capsules were trimmed with a hacksaw and a razor blade. The trimmed blocks were securely fastened into a Reichert

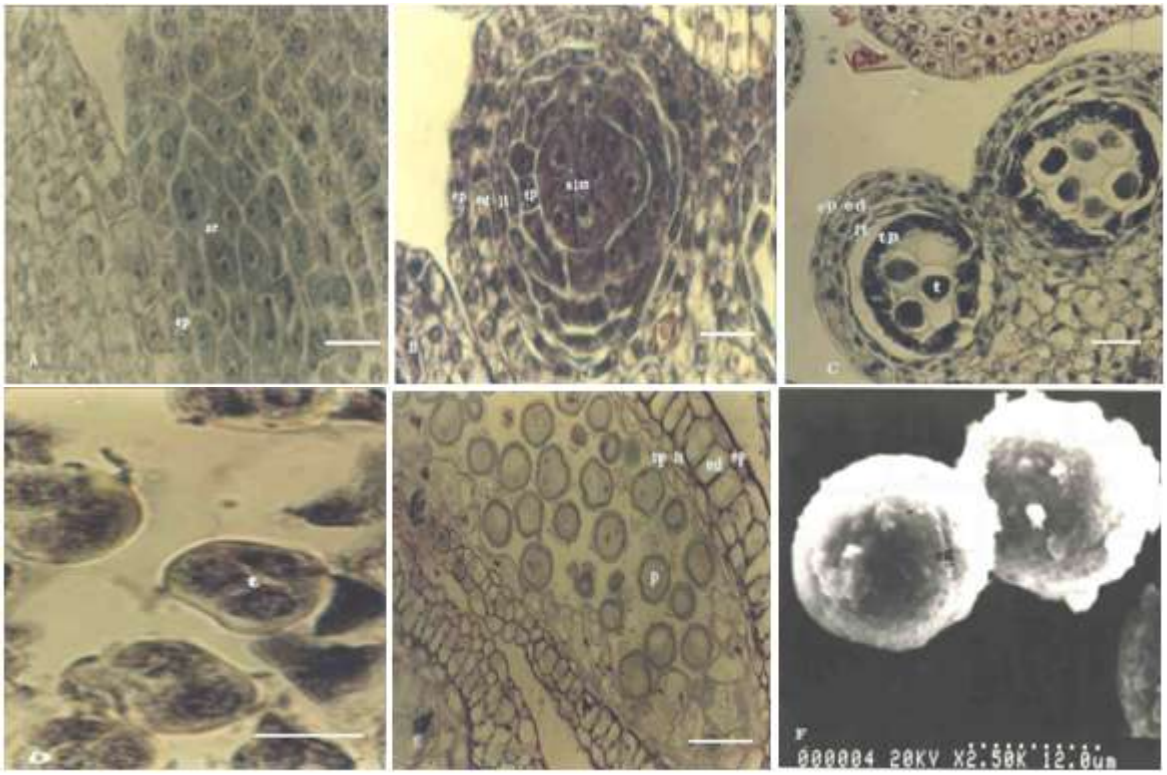
Ultracut E microtome. 1 µm thick sections were cut using 45° handmade glass blades (ProSciTech) from a Knife Maker Type 7801. Sections were floated onto water loaded into plastic knife boats (ProSciTech) fastened to the glass knife by dental wax. The sections were then transferred to a drop of MQ H_2O on a gelatine coated microscope slide. Two sections were placed on each slide and dried on a laboratory warming tray at 25°C under chloroform until sections were flattened.

To observe the structure of pollen grains, a SEM (Scanning electron microscopy) was also used. In this method, the pollen grains were collected and fixated with glutaraldehyde 3% in buffer phosphate pH 6-7 for 24 hours. The pollen grains were washed with buffer phosphate three times, for five minutes each. After that, the pollen grains were fixated with 1% osmium tetroxide in buffer phosphate pH 6-7 for 1.5 hours and washed with cool water followed by serial alcohol 10% to 100% for 30 minutes each. The pollen grains were then put into serial mixtures of alcohol-amyl acetic with the proportion of 3:1, 2:2, 1:3 for 30 minutes each. The pollen grains were put into pure of amyl acetic solution three times for 15 minutes each. The following step was that the pollen grains were wrapped with clothes and then put into DCP-1 (Critical point dryer) equipment for drying the pollen. Later, the pollen grains were then attached to the stub for coating of gold, using GIKO IB 3 Sputter Coating for 20 minutes. Finally, those pollen grains were observed with SEM HITACHI 5.520 and photographed.

3. Results

Primordial anther was newly formed at flower sized 1 mm that consists of meristematic cells surrounded by a layer of protoderm. Furthermore, the hypodermic cells in that cells present at the layer of sub-epidermis differentiate to be an archesporial cell. The archespores divided periclinally and differentiated to be primary parietal and sporogenic primer cells (Figure A). The primary parietal cells divide periclinally and anticlinally to form anther wall. Flower buds sized 1.5 mm have formed the anther wall. The anther wall consists of the epidermis, endothecium, two middle cell layers and tapetum cell layer (Figure B). Meanwhile, sporogenous primary cells divide and form microspore mother cells. This stage was observed in flower bud sized 1.5 mm (Figure B). Microspore mother cells undergo meiosis to form dyads and tetrads. This stage was found in flower bud sized 2 mm (Figure C-D). The fourth microspores of tetrad then separate from one another to form pollen grains containing one nucleus (Figure E-I). This stage occurs at the flower bud sized 2.5 mm. At this stage, the pollen grains are still in the sporangia and had two nuclei including vegetative and generative nuclei (Figure F-G). The cell wall of pollen grain consisted of intine, an inner cell wall, and exine, an outer cell wall (Figure E). Exine consisted of a foot layer and endexine (Figure E, F). In contrast, Pollen grains were not found in staminodium due to unproduced microspore mother cells (data not showed).

At stadium of the flower sized 3.5 mm, a septum that separates two spaces or sporangium of each lobe of the anther ruptured resulting in the unification of the sporangium, and hence the pollen grains from the two spaces can be mixed in sporangia (Figure H). Once anthesis, the anther breakdown at 10.00 am. The rupture occurred in the longitudinal direction (introrse) and the pollen grains were released out of the anther through stomium (Figure I).



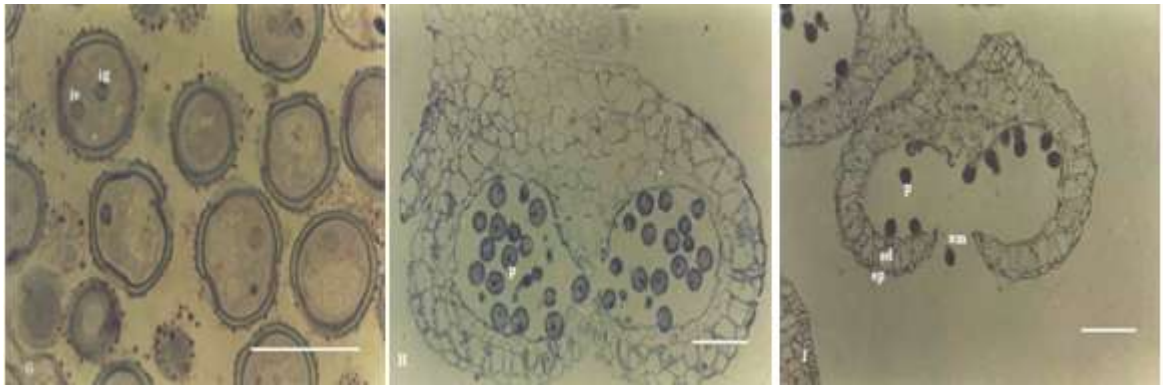


Figure: Anther wall and pollen development in *Averrhoa carambola* L. A, cross-section of anther showing initial anther consisting of epidermis (ep) and archesporial cells (ar). B, cross-section of anther showing a formation of anther wall including epidermis (ep), endothecium (ed), middle layers (lt) and tapetum (tp), and mother microspore cell (sim). C, cross-section of anther showing an anther wall and tetrad stage (t). D, an high magnification of tetrad stage was taken from figure C. E, cross section of the anther showing pollen grains in a sporangium using LR White resin with 1 µm thick sections. F, an ultrastructure of pollen grain using SEM (Scanning electron microscopy). G, detail of pollen grains, using LR White resin with 1 µm thick sections, showing pollen grain contained vegetative and generative nuclei. H, cross-section of anther showing pollen grains present at sporangia of the anther in which septum was rupturing. I, cross section of anther showing pollen grains were introrsely released out from the sporangium of the anther through stomium.

4. Discussion

The anther of the star fruit (*A.carambola* L.) consists of the epidermal layer, endothelial layer, two middle layer and tapetal layer (Figure B). The model anther was similarly found in *Feijoa sellowiana*, Myrtaceae ((Zou et al. 2016). The middle layer would be pressed causing the layer missed out when the anther is mature (Figure H and I). Tapetum cells appear irregular and whorls in the innermost layer of the anther walls bordering sporangia. Tapetum type was secretory that was indicated by the remained tapetum present at its original position (Figure E). The secretory tapetum type had been reported in several species such as *Brachypodium distachyon* (Sharma et al. 2015), *Lycopersicum esculentum* (Polowick & Sawhney 1993), *Primula obconica* (Stevens & Murray 1982), *Cichorium intybus* (Chehregani, Abdolkarim; Mohsenzadeh, Fariba; Granad 2011). During development of the microspore, the tapetum secreted nutrients from those cell surfaces to space or sporangia containing pollen grains used for microspore growth. Lead to nutrient secretion, the tapetum might be a change of their size to be smaller (Figure B and E).

Microspore mother cells divide meitotically to be dyads and tetrad. Type of the formed tetrad is tetrahedral with simultaneous cytokinesis (Figure C and D). This type is generally found in several species such as *withania somnifera* (Ghimire & Heo 2012), *Feijoa sellowiana* (Zou et al. 2016), *Larix leptolepis*, *Lycopersicon esculentum* (Brukhin et al. 2003). Pollen grains released from the tetrad had a single nucleus. Further, the nucleus of the pollen divided mitotically to form vegetative and generative nuclei (Figure G). The type is uncommonly observed in some species. However, it is similar reported in *Lycopersicon esculentum* (Brukhin et al. 2003) and *Feijoa sellowiana* (Sharma et al. 2015). Pollen grains have cell wall consisting of intine and exine layers and have three germination pore (Figure G). The exine layer is tectate-columellate, comprising a foot layer and endexine (Figure F and G). The pollen grains were released introrsely from sporangium of the anther through stomium when the anther was fully mature (Figure I).

5. Conclusion

The anther wall of star fruit (*A. carambola* L.) had a wall consisting of a layer of epidermal cells, the endothecium cell layer, two middle layers and tapetum cell layer. The anther contains pollen grains with two generative and vegetative nuclei. The pollen grains are developed from microspore mother cells. The developments of microspore mother cells are preceded by the meitotic division to be dyad and tetrad. The pollen grains containing the two nuclei were introrsely released from the anther through a pore (stomium).

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