ORIGINAL ARTICLE

Anatomical observations on pistil morphogenesis and fertilization in salak (*Salacca wallichiana* C. Mart.)

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Received: December 26, 2020 Accepted: March 26, 2021

ABSTRACT Salak palms (*Salacca* spp.) are important fruit species in Southeast Asia, but the morphogenesis of their reproductive organs is unknown. To observe female gametogenesis, pistil morphogenesis of *S. wallichiana* and subsequent fertilization were observed. The ovary contained three ovule primordia three months before flowering (BF). The ovule contained one mono-nucleate megaspore at the center and three degenerated megaspores on the chalazal side at two months BF, and a mono-nucleate embryo sac (ES) at one month BF. The nucellus enlarged, and protruded beak-like from the integuments, forming a micropylar structure. The ovule was anatropous, unitegmic, and crassinucellate. Three weeks BF, the ES was at the 8-nucleate stage. Two weeks BF, an egg apparatus comprising one egg-cell nucleus and two synergid nuclei were developing on the micropylar side. The central cell contained one polar nucleus (PN). One week BF, the synergids and central cell became multi-nucleate and multiple-PN dispersed in the ES. Several days BF, one PN was again observed, and the remaining PN appeared to develop cell groups along the ES wall. Antipodals existed on the chalazal side. One day BF, ES contained two PN, and one day after pollination (DAP), the zygote and primary endosperm nucleus occurred on the micropylar side. One synergid remained functional and enlarged even after fertilization. Four DAP and thereafter, suspensor cells and an early embryo existed on the micropylar side, and the ES contained multiple free endosperm nuclei. In *S. wallichiana*, the ES likely indicates intermediate morphogenesis between *Polygonum* and *Oenothera* types, suggesting a unique development pattern among palms.

Key words: Calamoideae, Embryo sac, Megagametogenesis, Ovule, Rakam

INTRODUCTION

Salak palms (*Salacca* spp.), showing dioecy, belong to the subfamily Calamoideae of Palmae. Approximately 20 species are distributed in Southeast Asia (Furtado 1949; Dransfield et al. 2008). Among these, *S. zolacca* (Gaertn.) Voss and *S. wallichiana* C. Mart. are favored for fresh fruit consumption, and are cultivated widely in Indonesia and Thailand. Despite its economic importance, the morphogenesis of the reproductive organs of salak palms is still unknown.

Gametogenesis and subsequent embryogenesis have been observed anatomically in other important edible palms, such as coconut palm (*Cocos nucifera*) (Quisumbing and Juliano 1927; Reddy and Kulkarni 1989) and date palms (*Phoenix* spp.) (Mahabale and Biradar 1968; Biradar 1968; Biradar and Mahabale 1968), as well as ornamental palms, such as Indian doum palm (*Hyphaene indica*) (Mahabale and Chennaveeraiah 1958), and several species from the same genera including *Sabal*, *Licuala*, and *Livistona* (Rao 1958). These species, however, are limited

to only particular genera in Palmae, which include thousands of species classified into several subfamilies. In addition to salak palms, sago palms (*Metroxylon* spp.) also belong to the subfamily Calamoideae (Dransfield et al. 2008). In this subfamily, no previous studies on the anatomical observations of gametogenesis have been reported.

In the present study, to observe pistil morphogenesis of a salak (*S. wallichiana*), pistils were sampled from the spadices at various developmental stages from three months before flowering to the day of flowering. Ovule morphology was then observed anatomically. Subsequent morphological development at fertilization was also observed.

MATERIALS AND METHODS

To observe pistil morphogenesis, the following observations were conducted from May 2011 to May 2013 using the female cultivar 'Sumalee' salak, cultivated in a commercial orchard in eastern Thailand.

Fig. 1. Floral morphogenesis of 'Sumalee' salak (*Salacca wallichiana*) Flower buds prior to flowering: a) 3 months before, spadix (a*) completely covered by bract; b) 2 months, spadix enlarging outside the bract; c) 1 month; d) 3 weeks; e) 2 weeks; f) 1 week; and g) a few days before flowering, individual buds were visually identified; h) at 1 day before flower opening; i) spadix at flowering; A) pairs of florets in each spadix were positioned in five spirals which were indicated by coiling color tapes; B) single pair of florets on the spadix. One flower had trifurcated stigma (α) and the other had no functional organ (β); C) individual pistillate flower; D) Ovary covered by trichomes. Scale bars = 1 cm (a–i, A, and B) and 1 mm (C and D).

Spadices at different developmental stages (Figs. 1.a–i) were randomly selected, and ten pistils at each stage were sampled using a scalpel. Sampled pistils were then immediately fixed in Copenhagen mixture (ethanol: glycerol: distilled water = 10: 1: 8, v/v/v; Bridson and Forman 1989). Other spadices at the same stage were tagged to estimate the day requirement for flowering. After rinsing the fixed pistils with distilled water overnight, they were dehydrated in an alcohol series and embedded in paraffin wax in the same manner as described by Matsuda et al. (2011).

Embedded pistils were sectioned transversely or longitudinally at 8–10 μ m thickness. The sections were arranged on microscope glass slides pre-coated with Haupt's adhesive (gelatin: distilled water: phenol: glycerol = 1: 100: 2: 15, w/v/w/v; Jensen 1962). The sections on the slides were dewaxed in lemosol and then stained with 0.05% w/v toluidine blue, in a solution of 0.1 mol/L citric acid and 0.2 mol/L disodium hydrogen phosphate mixture (12:8, v/v) (pH 4.1). The stained sections were rinsed again with distilled water overnight, dehydrated in ethanol and lemosol, and mounted to create permanent preparations

using polystyrene resin (NEW M-X, Matsunami Glass Ind., Ltd., Osaka, Japan).

The morphology of the stained pistils was observed under an optical microscope to examine the process of pistil morphogenesis. To determine the position of degenerating cells during meiosis of the megaspore mother cells, five other pistils were randomly sampled from the above-described spadices regarded as two months before flowering (Fig. 1.b). The sampled pistils were sectioned in the same manner as described above. The sections were stained with a 0.1% aniline blue solution containing 0.1 M K_2PO_4 , and callose deposition indicating cell degeneration was observed under a fluorescence microscope (CyScope, Partec GmbH, Saarbrücken, Germany).

To observe pistil morphogenesis from pollination to fertilization, ten spadices just after flower opening were randomly selected. These spadices were then pollinated at 1000–1100 h by dusting with fresh pollen mixed with talcum powder (1:10, v/v) using a paintbrush as described by Matsuda et al. (2020). Twenty pistillate florets per spadix were sampled and fixed in a Copenhagen mixture at one, two, four, and seven days after pollination. Another two

spadices just after flower opening were randomly selected and bagged not to be pollinated, and these florets were sampled and fixed at one and two days after flower opening. After removing the sepal, petal, and trichome (Figs. 1.C and D) from these sampled florets and rinsing the pistils with distilled water overnight, the pistils were sectioned as described above. The sections were stained with toluidine blue solution and mounted to create permanent preparations for microscopic observations.

RESULTS

Morphogenesis until flowering

The spadix was enwrapped with bracts three months before flowering (Fig. 1.a). Half of the spadix protruded from the bracts two months before flowering (Fig. 1.b), and the entire spadix was visible one month before flowering (Fig. 1.c). Between three weeks and one week before flowering, each floret was enlarged and the petal tips became clearly visible (Figs. 1.d–f). A few days before flowering, the spadix thickened and the floret petals were

more visible (Fig. 1.g). Apical half of each floret emerged one day before flower opening (Fig. 1.h–i). The spadix included 40–180 pairs of a pistillate floret and a floret containing degenerated reproductive organs (Fig. 1.B); the floret pairs were positioned in five spirals on the spadix surface (Fig. 1.A).

Three months before flowering, the formation of three ovule primordia was observed in one of a pair of florets, and these were developing in a circle at the transverse section (Figs. 2.Aa and Ab). Internal organs from the remaining floret had been degenerated, and gametogenesis was not noted. Two months before flowering, a functional megaspore and its nucleus were observed at the center of each ovule in one of a pair of florets (Fig. 2.Ba). Three aborted megaspores showing callose deposition were observed on the chalazal side (Fig. 2.Bb), and stigma and style formation commenced (Fig. 2.Bc). In the other one of the pair of florets, the aborted ovary contained rudiments of the aborted ovule primordia (Fig. 2.Bd). One month before flowering, the trichome began forming on the ovary surface in a functional floret (Fig. 2.Ca). The ovule was anatropous, unitegmic, and crassinucellate, and contained a mononucleate embryo sac (Fig. 2.Cb). The nucellus

Fig. 2. Microscopic observations of pistil morphogenesis of 'Sumalee' salak (*Salacca wallichiana*) fluorescence (Bb) and optical (the remaining) microscopy: Aa) the transverse section of single pair of flower buds at 3 months before flowering. Three ovule primordia (arrow heads) were observed in one bud, and the other bud had no ovary; Ab) The longitudinal section of ovule primordium (op); Ba) female gamete, composed of one-nucleate embryo sac at 2 months before flowering; Bb) female gamete (fg), and aborted megaspore triad in which cell walls accumulated callose (arrow heads); Bc) stigma (stg) and style (stl) formation on the ovary (ovr); Bd) ovarian rudiment of one of the pair of flower buds; Ca) trichome forming on the ovarian (ovr) surface, and stigma (stg) forming sulci at 1 month before flowering; Cb) micropyle (mp) formation, and uninuclear embryo sac (es) enlarging inside nucellus (nu) covered by integument (int); (continues) Scale bars = 100 μ m (Aa) and 50 μ m (the remaining).

Fig. 2. (continued) Da) eight-nucleate at 3 weeks before flowering. Arrow heads indicate nuclei. Antipodal cell walls forming; Db) ovule with aborted embryo sac; Dc) trichome formation on the ovary; Dd) sulci inside stigmatic wall deepening; Ea) egg apparatus forming in the micropylar side of embryo sac (es) at 2 weeks before flowering. Egg nucleus (en) connected to synergids (sy); Eb) polar nuclei (pn) integrating; Fa) egg nucleus (en) with synergid in which nuclei multiplying at 1 week before flowering; Fb and c) polar nuclei (pn) multiplying into free nuclei (arrow heads); Fd) secretory papillae forming on the stigmatic surface. (continues) Scale bars = 50 μ m

enlarged, protruded beak-like from the integuments, and formed a micropylar structure.

Three weeks before flowering, the embryo sac contained eight nuclei (Fig. 2.Da), and 33 % of the observed 21 ovules contained aborted embryo sacs (Fig. 2.Db). The trichome on the ovary surface was elongated (Fig. 2.Dc), compared with those at one month before flowering (Fig. 2.Ca). Sulci began to develop on the stigma surface (Fig. 2.Dd). Two weeks before flowering, the egg apparatus formed in the embryo sac; egg nuclei and two adjacent synergid nuclei were observed at the micropylar side (Fig. 2.Ea). One large polar nucleus existed at the center of the embryo sac (Fig. 2.Eb). Similar to three weeks before flowering, 27 % of the observed 30 ovules contained aborted embryo sacs at two weeks before flowering. One week before flowering, synergids adjacent to the egg nucleus contained multiple nuclei (Fig. 2.Fa). Similarly, the central cell contained multiple polar nuclei (Fig. 2.Fb), and some of these were free and dispersing toward the cell wall (Fig. 2.Fc). The formation of papillae was observed on the stigma surface (Fig. 2.Fd).

Several days before flowering, the egg-cell wall

formed around the egg nucleus (Fig. 2.Ga), one large polar nucleus formed in the central cell, and multiple nuclei were observed on the embryo-sac wall and formed new cells (Figs. 2.Gb and Gc). These nuclei on the embryo-sac wall appeared to derive from the free polar nuclei dispersed in the embryo sac at one week before flowering (Fig. 2.Fc). Multiplied antipodal cells were observed on the chalazal side (Fig. 2.Gd), and the stigma surface was covered with secretions containing cytoplasm (Fig. 2.Ge).

One day before flowering, a filiform apparatus was visible surrounding the multiplied cells of synergids (Fig. 2.Ha). Structures similar to the filiform apparatus were also observed adjacent to the antipodal cells on the chalazal side (Fig. 2.Hb). Two polar nuclei were observed in the central cell (Fig. 2.Hc). Among the 30 observed ovules, 57 % contained a normal egg apparatus and polar nuclei, 13 % ovules were missing the egg apparatus, and the other 30 % contained aborted embryo sacs. Secretions extended to the bottom part of the locule one day before flowering (Fig. 2.Hd), compared with those on several days before flowering, which were limited to the stigma surface (Fig. 2.Ge).

Fig. 2. (continued) Ga) egg-cell wall (ecw) forming at a few days before flowering; Gb) a part of polar nucleoplasm (pn) reintegrating, and the other free nuclei forming cells on the embryo-sac wall (arrow heads); Gc) polar nucleus (pn) formation. Cell layers derived from polar nuclei was formed on the embryo-sac wall; Gd) antipodal cells multiplying; Ge) stigmatic surface covered with secretion (sec); Ha) filiform apparatus forming in the multi-nucleate synergids (sy) at 1 day before flowering; Hb) antipodals forming filiform apparatus (fa) like synergids; Hc) polar nuclei (pn) dividing; Hd) secretory papillae on the bottom of locule; (continues) Scale bars = 50 μ m

On the day of flowering, one large polar nucleus was observed adjacent to the egg apparatus (Fig. 2.Ia). Several cell groups developed on the embryo-sac wall, and concaves existed between these cell groups (Fig. 2.Ib). These concaves connected with the cytoplasm distribution inside the embryo sac. Among the 24 observed ovules, 58 % contained normal egg apparatus and polar nuclei. One day before flowering, the egg apparatus was absent in 17 % ovules, and the other 25 % ovules contained degenerated embryo sacs on the day of flowering.

The embryo-sac morphogenesis process was described from two months before flowering to the day of flowering (Fig. 3).

Morphogenesis after pollination

One day after pollination, pollen grains had germinated on the stigma (Fig. 2.Ja), and the pollen tubes elongated into the secretion near the locule wall (Fig. 2.Jb). The pollen tube then extended into the micropyle and reached one of a pair of synergids (Figs. 2.Jc and Jd). The

embryo sac contained a large mononuclear zygote at the micropylar side (Fig. 2.Je), and one larger primary endosperm nucleus containing two nucleoli was observed adjacent to the zygote. One day after pollination, the synergids enlarged and the inner nuclei further multiplied (Fig. 2.Jf), compared with those on one day before flowering (Fig. 2.Ha). Two days after pollination, a multinuclear early embryo was observed adjacent to one cell group, which appeared to be haustorial organs connecting the early embryo toward the embryo-sac wall at the micropylar side (Fig. 2.Ka). One of the pairs of synergids degenerated (Fig. 2.Kb). The membrane and nucleoli of the primary endosperm nuclei became unclear (Fig. 2.Kc), and the nucleoplasm seemed to decompose and disperse toward the cell groups on the embryo-sac wall (Fig. 2.Kd). Four days after pollination, free nuclei containing three nucleoli formed on the embryo-sac wall (Fig. 2.La), and similar free nuclei were located apart from the wall in the embryo sac (Fig. 2.Lb). Four days after pollination, suspended cells adjacent to the multiplied early embryo cells were observed on the micropylar side (Fig. 2.M).

Fig. 2. (continued) Ia) polar nucleus (pn) approaching the egg cell at the day of flowering. Arrow head indicates egg nucleus; Ib) mature embryo sac had four concave portions (arrow heads) on the wall. These concaves connected with the cytoplasm distribution inside embryo sac; Ja) pollen germination on the stigma observed at 1 day after pollination; Jb) pollen tube (pt) elongation through the locule toward the micropyle (mp); Jc and d) pollen-tube (pt) penetration into micropyle (mp) and synergid (sy); Je) zygote (zy) and primary endosperm nuclei (pen); Jf) functioning synergid (fs) enlarging and another synergid degenerating (sd); Ka) haustoria (ha) and early embryo (eem) with several nuclei at 2 day after pollination; Kb) functioning multi-nucleate synergid (fs) and degenerated synergid (ds); (continues) Scale bars = 50 μ m

In florets without pollination, stigma secretion disappeared two days after flower opening (Fig. 2.Na). The cytoplasm in the cells of the embryo-sac wall and egg apparatus were densely stained and the individual cells became difficult to identify, indicating degeneration (Fig. 2.Nb). No nuclei or organs were observed in the embryo sac, which was filled with equable cytoplasm.

DISCUSSION

Morphogenesis until flowering

The present study is the first report of anatomical observations of gametogenesis in useful palm species belonging to the subfamily Calamoideae. In many species of Palmae, for example *Cocos nucifera* (Quisumbing and Juliano 1927; Reddy and Kulkarni 1989), *Phoenix* spp. (Mahabale and Biradar 1968; Biradar 1968; Biradar and Mahabale 1968), and several species of *Sabal* and *Livistona* (Rao 1958), embryo sacs were reported to develop

according to the *Polygonum* type (Maheshwari 1950). In the present study, embryo sacs of *S. wallichiana* developed according to a similar type to *Polygonum*; the megaspore mother cell divided into four megaspores, and only one of those continued to develop into an embryo sac containing an egg apparatus (two synergids and one egg cell), polar nuclei, and antipodals (Figs. 2.B–I, and 3.g). However, the megaspore at the micropylar side and not the chalazal side developed into the embryo sac (Figs. 2.Ba, 2.Bb, and 3.a). In *Oenothera*-type morphogenesis (Maheshwari 1950), after the megaspore mother cells divided into four, one megaspore at the micropylar side developed, and the embryo sac contained an egg apparatus (two synergids and one egg cell) and polar nuclei, similar to the present observations in *S. wallichiana*, although without antipodals. Therefore, embryo sacs of *S. wallichiana* were considered to develop according to an intermediate morphogenesis process between *Polygonum* and *Oenothera* types.

Synergids enlarged and became multinuclear during embryo-sac morphogenesis (Figs. 2.Fa, 2.Ha, and 3.e–h), similar to *Ammania baccifera*, Lythraceae (Joshi and

Fig. 2. (continued) Kc and d) multi-nucleate primary endosperm decomposing into nucleoplasm which dispersing toward the wall; La and b) tri-nucleoli free nuclei (fn) formation near the embryo-sac wall surface at 4 days after pollination; M) developing early embryo (eem) cells with suspensor cells (spc) derived from synergids following 4 days after pollination; Na) stigmatic secretion disappeared at 2 days after flower opening; Nb) ovule with degenerating embryo sac. Scale bars = 50 μ m

Venkateswarlu 1935). Among other plants, no previous observations of such synergid development have been reported. Furthermore, in *S. wallichiana*, nuclei that were likely to be derived from polar nuclei formed cell groups on the embryo-sac wall (Figs. 2.Fb, 2.Fc, 2.Gb, 2.Ib, 3.e, and 3.f). No similar observations were reported in the other species with monosporic embryo sacs, including *Polygonum* and *Oenothera* types. In species with tetrasporic embryo sacs, including the *Penaea* and *Plumbago* types, in which megaspore mother cells do not divide before embryo-sac morphogenesis, the formation of cells derived from megaspore nuclei was observed on the embryo-sac wall (Maheshwari 1950). However, in *Asplundia polymera*, Cyclanthaceae, several parts of the nucellus cells were reported to form bulges on the embryo-sac wall (Johri et al. 1992b). Accordingly, the cell groups on the embryo-sac wall of *S. wallichiana* in the present study were possibly derived from not only polar nuclei but also nucellus cells along the embryo-sac wall.

Ovules of *S. wallichiana* were anatropous (Fig. 2.Cb), similarly to many other species of Palmae, for example, *C.*

nucifera (Perera et al. 2010), *Sabal* spp. (Rao 1958), betel palm (*Areca catechu*), and fishtail palm (*Caryota urens*) (Rao 1959). In contrast, ovules of some species of Palmae, for example, footstool palm (*Livistona rotundifolia*) (Rao 1958), *Bactris major* (Rao 1959), and African oil palm (*Elaeis guineensis*) (Adam et al. 2005), were reported to be orthotropous. Although *S. wallichiana* displayed unitegmatic ovules (Figs. 2.Cb and 3.a), most species of Palmae, including the above-cited species, had bitegmatic ovules (Johri et al. 1992a). Among plant species, micropyle is generally constructed by integuments (Bhojwani et al. 2015); however, in *S. wallichiana*, nucellus cells protruded from the integuments to construct the micropylar structure (Figs. 2.Cb, 2.Jd, 3.a, and 3.b). Such a structure is called a beak-like nucellus (Bhojwani et al. 2015), and has been reported to be observed only in a few species of several families, such as Euphorbiaceae (Weniger 1917; Singh 1969), and Cucurbitaceae (Mann and Robinson 1950).

Fig. 3. Embryo-sac morphogenesis in *Salacca wallichiana*: a) two months before flowering; b) one month before flowering; c) three weeks before flowering; d) two weeks before flowering; e) one week before flowering; f) several days before flowering; g) one day before flowering; h) the day of flowering.

Morphogenesis after pollination

In *S. wallichiana*, one of the synergids enlarged with cell division after pollination, and continued to remain functional even after fertilization (Figs. 2.Jf, Kb, and M). In most plant species, synergids exhibit a short life after pollination and degenerate before fertilization; in contrast, synergids of *Allium angulosum* and *A. pulchellum* were reported to continue to function by multiplying the nuclei (Bhojwani et al. 2015), similar to the present observations in *S. wallichiana*. Syamasundar and Panchaksharappa (1975) observed that the synergids of *Allium cepa* also enlarged and the thick, large nuclei were functional after fertilization. They also noted that the synergids functioned by supplying nutrients to the egg cell, zygote, and early embryo. Our present observations indicated that the synergids of *S. wallichiana* functioned as well during and after fertilization, although haustorial organs that seemed to supply nutrients were observed in the early embryo (Fig. 2.Ka). Suspensor cells that were observed four days after pollination (Fig. 2.M) resembled the previously observed synergids (Figs. 2.Jf and Kb) in terms of position and

morphology. This indicated that the suspensor cells of *S. wallichiana* were possibly derived from synergids.

In many species of Palmae, for example, *C. nucifera* (e.g., Abraham and Mathew 1963), and *Phoenix* spp. (e.g., Biradar 1968), the endosperm commenced to develop in the form of multiplying free nuclei, and the cell walls of endosperm then formed along the surface of the embryo-sac wall. Similar to *S. wallichiana*, the endosperm after fertilization was composed of free nuclei (Fig. 2.L). Mature seeds of *S. wallichiana* contain a hard endosperm, and the free nuclei are considered to form cell walls and advance cell division, for hardening of the endosperm one week after pollination.

ACKNOWLEDGEMENTS The authors highly appreciate the Sukjit Orchard and Chanthaburi Horticultural Research Center for their kind support and advice on the field experiment. This study was partly supported by a Grants-in-Aid for Scientific Research (KAKENHI No. 24658028 and 15K07290) from the Japan Society for the Promotion of Science.

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