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ANTHER DEVELOPMENT AND MICROSPOROGENESIS IN DATE PALM (*PHOENIX DACTYLIFERA* L.)

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Abstract

Microsporogenesis and pollen morphology of *Phoenix dactylifera* L. was studied in this study. Anther, in different developmental stages, was removed, fixed in Formalin-glacial acetic acid-alcohol (FAA), stored in 70% ethanol, embedded in paraffin and then sliced at 8-10µm by rotary microtome. Staining was carried out with Hematoxylin-Eozin. Scanning electron microscope (SEM) was used to analyze the mature pollen grains. The pollen protein extracts of date palm were obtained from pollen by phosphate buffer saline (PBS). They were separated by 10% SDS-polyacrylamide gel electrophoresis. The anther wall is constituted of five cellular strata: epidermis, monostratified endothecium, middle layer formed by two cellular strata and the secretory tapetum. The microspore mother cells begin meiosis and form tetrads of tetragonal microspores. The mature anther wall consists of an epidermis and an endothecium. Mature pollen grains are two-celled and monosulcate, semitectate -reticulate. SDS- PAGE analysis of mature pollen grains showed protein bands of 10–110 kDa regions.

Key words: Anther development, Microsporogenesis, Pollen grain, *Phoenix dactylifera* L., SDS-PAGE, SEM.

Introduction

Being a member of *Arecaceae* or Palmae family, Coryphoideae subfamily, Phoeniceae tribe and phoenix genus, date palm (*Phoenix dactylifera* L.) is a tree species. It forms the Phoenix genus along with 13 other species (Clifford & Yeo 1985; Henderson *et al*., 2006; Gros-Balthazard *et al.*, 2013). In this genus, all species are dioecious, with male and female flowers on separate trees. DeMason *et al*. (1982) described the structure of the staminate and Pistillate flowers. Sweet-scented and normally having six stamens, the male flower is surrounded by waxy scale-like petals and sepals (3 each). Each stamen is composed of two little yellowish pollen sacs. With a diameter of about 3 to 4 mm, the female flower has rudimentary stamens and three carpels closely pressed together. In addition, the ovary is superior (hypogynous). Three sepals and three petals are united together so that only tips diverge. When flourishing, the female flowers show more yellow color, while the male ones show white color dust, produced on shaking. When the spathe bursts, the pollen sacs usually open within one or two hrs (De Mason *et al*., 1982; Zaid & De Wet, 1999). The anthers commonly open with the aid of mechanical tissue termed endothecium by creating tension in the maturing anthers and help in pollen dispersal (Esau, 1977; D'Arcy, 1996; Tebbitt & Maciver, 1999; Abid & Qaiser, 2004; Islam *et al.*, 2008). Pollen viability decreased gradually with time after release of anther. *In vitro* germination test of date palm pollen grains has showed that it can changed between 71.22% in cv. Khadrawy and 34.86% in cv. Hillawi after 12 month stored at -20°C (Maryam *et al*., 2015).

Previous literature on pollen morphology and anther wall development shows that more researches are required to be conducted yet. For anther development and pollen morphology, only few genera of *Arecaceae* have been described i.e., *Nypa, livistonia, hyphaene, chamaedora, cocos* (Radermacher, 1925; Mahabale & Chcnnaveeraiah, 1957; Kulkarni & Mahabale, 1974). Biradar (1968) studied anthers, ovules, female gametophyte and embryo of *Phoenix pusilla, P. acaulis*, and found 1-2 strata of middle layer. Mahabale & Biradar (1967) found successive type of division in microspores in *Phoenix sylvestris*. With Scanning Electron Microscopy (SEM), Soliman & Al-Obeed (2013) investigated the pollen morphology of eleven date palm cv of Succary, Menify, Sallag, Dikhiny, Nabout, Zamel, Serry, Khalas, Shagra, Safry, Maktumi and Kadary males (*Phoenix dactylifera* L.) and found morphological differences i.e. shape, size, pollen weight, germination percentage, length, width and the number of pores between males.

However, for date palm (*Phoenix dactylifera* L.), embryology and microsporogenesis (male meiosis), which lead to the formation of the microspores that further develop in to mature pollen grains, remains largely unexplored. The present study is the first research on anther wall development and microsporogenesis of date palm, which focuses on anther wall, microspore and male gametophyte development and pollen morphology in *Phoenix dactylifera*. This result could be used for evaluating the taxonomic relationships among *Phoenix* species.

Materials and Methods

Sampling: Materials employed in this study were male inflorescences (at different developmental stages) and mature pollen grains of *Phoenix dactylifera* L., which were collected during April from Bam, Iran (29° 6' 28" N/ 58° 21' 42" E).

Morphological studies: Male inflorescences were collected and were to release the flowers, their spathes were manually opened. Male flowers were fixed in FAA (Formalin–glacial acetic acid-alcohol). After the fixation process, samples were dehydrated during alcohol series, and after the process of paraffin saturation in toluene, they were embedded in paraffin. Sections of 8-10µm thickness were taken from the materials embedded in paraffin with rotary microtome. Hematoxylin-Eosin was employed for staining. They were mounted in Canada balsam, examined with a Zeiss Axiostar plus light microscope. Many samples were studied for each stage and photomicrographs.

Palynological studies: Purified pollen grains were mounted on 12.5mm diameter stubs. Then they were coated in a sputter coater with 25nm of gold palladium at an accelerating voltage of 15-25kV. SEM photographs were captured at 1000x, 4222x and 15000x magnifications from pollen grains, using a SEM (XL30, Philips, Holland). The terminology used here follows that of Punt *et al*. (2007) and Halbritter *et al*. (2007).

Protein studies: Extraction of pollen protein from date palm pollen grains was incubated overnight in Phosphate Buffer Saline (PBS, pH = 7.2) and centrifuged at 11000g for 45min at 4°C. Then they were dialyzed into PBS buffer overnight. Finally, according to the method of Laemmli (Laemmli, 1970), 10% SDS polyacrylamide gel electrophoresis was performed for the total soluble protein. By heating for 3-4min at 100°C before loading, soluble proteins were extracted in sample buffer (0.125M Tris–HCl (pH6.8); 4% SDS; 20% glycerol; 10% β-mercaptoethanol; 0.1% BPB). The amount of protein was 10µg per channel, and the total current applied was 14mA. The gel was run in Trisglycine buffer (pH8.3) with 0.1% SDS and calibrated with a marker protein obtained from Sigma. Using Bradford method (Bradford, 1976), the protein concentration of the pollen extract was determined.

Results

Anthers are tetrasporangiate and dehisce longitudinally. The anther of *Phoenix dactylifera* has a similar structure to that of angiosperms, according to semi-thin sections of floral buds (0.8mm long). Four pollen sacs connected by the connective at the center were observed in which a vascular bundle is surrounded by parenchyma tissue (Figs. 1 A and L).

In addition, our results indicated that the anther wall includes five layers from outer to inner: epidermis, endothecium, 2 or 3 rows of middle layer and a layer of tapetum (Figs. 1I and J). Middle layers are two or three rows on the outside of anther locule and 3 rows on the connective side anther. Tapetum is of secretory type, many of which had binucleate cells and degenerated in the mature anthers (Figs. 1F and H). Microspores and pollen grains are produced from pollen mother cells (PMCs), which are located in loculus anther (microsporangia or pollen sacs) of the flower. By dense cytoplasm and large nucleus, PMCs were recognizable (Fig. 1B). In early stages of meiosis, microsporocytes were separated with special wall, and following meiosis I, microspore dyads was resulted (Fig. 1C). Numerous tetrads of microspores appeared after meiosis II, tetrad shape was tetragonal and wall formation took place after each stage of meiosis (Fig. 1D). Microspores with formed walls were released following callose deposition. As a monad, the microspore divided, and then formed generative and vegetative cells (Fig. 1G). The tapetum cells were broken and their contents adhered to the pollen grain wall. It is noteworthy that middle layer in this species is degenerated in the late stage of microsporogenesis (Figs. 1J and K). Finally, epidermis and endothecium layers remained for anther wall (Fig. 1K). At this stage, anthers lost the septum and separated each sporangium from the theca and opened by longitudinal dehiscence, allowing the release of a large amount of morphologically well-formed pollen grains. The pollen grains were morphologically well formed and two-celled when shed.

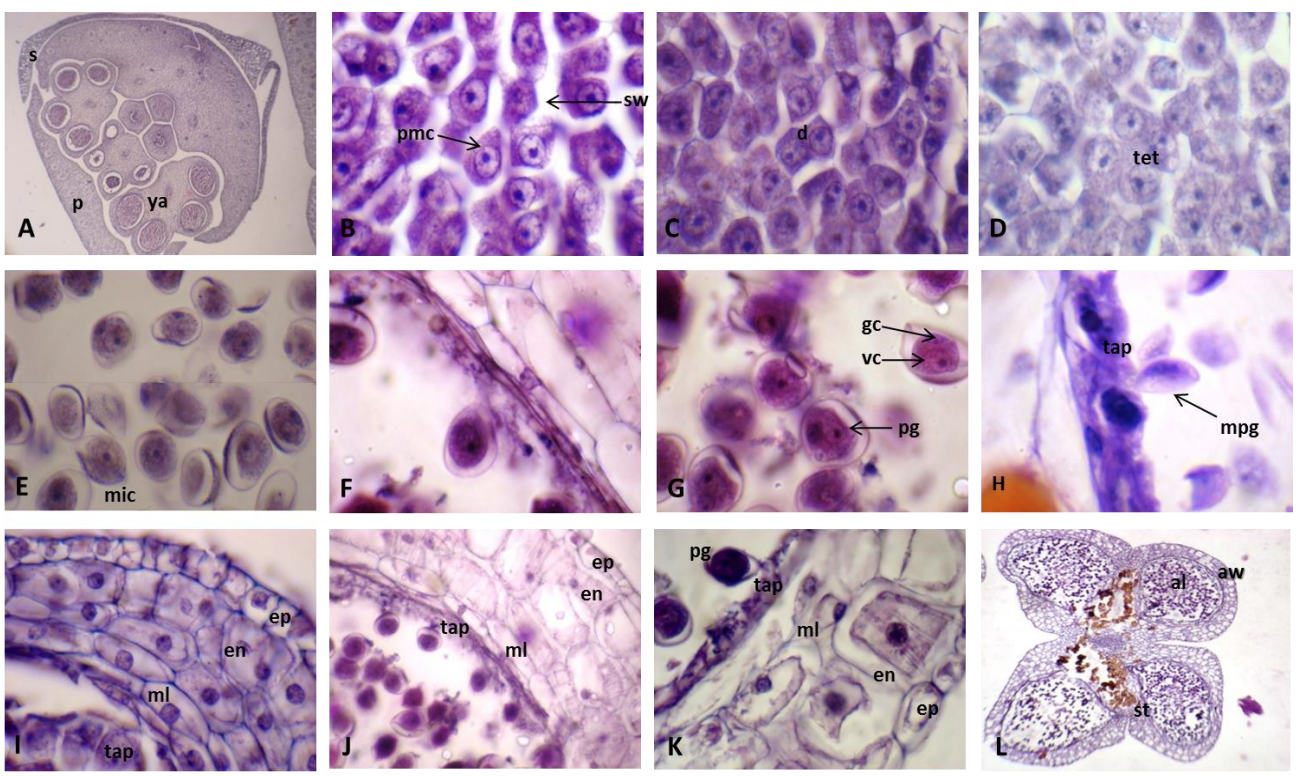

Fig. 1. Light micrographs of developing anther stained with Hematoxylin-Eosin. A: Transverse section of male yang flower; B-H: Microsporogenesis stages; I-K: Developmental stages of anther wall; L: Mature anther.
Abbreviations: al: anther locule; aw: anther wall; sw: special wall; ep: epiderm; en: endothecium; gc: generative cell; mic: microspore; ml: middle layer; mpg: mature pollen grain; p: petal; pmc: pollen mather cell; s: sepal; st: stomium; vc: vegetative cell; ya: young anther.

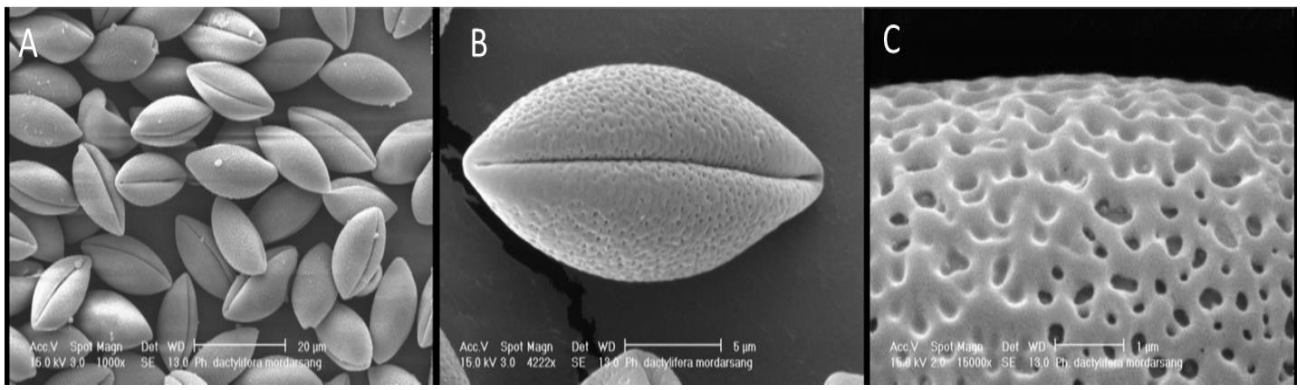

Fig. 2. SEM micrographs of pollen grain of *Phoenix dactylifera* L.

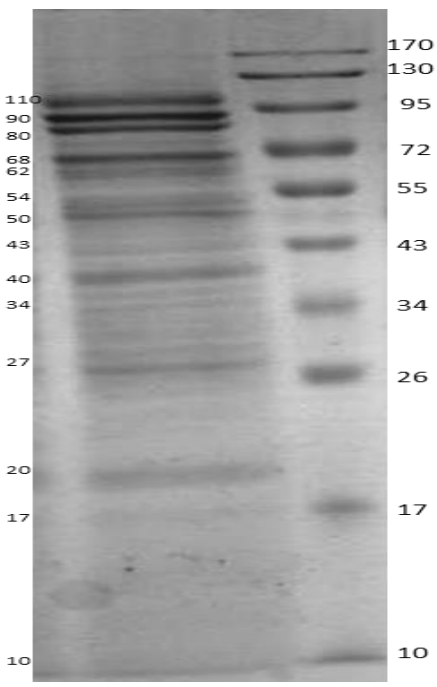

Fig. 3. Electrophoresis profile of the total protein extracted from *P. dactylifera* L. pollen grains (*left*) and the molecular marker (*right*) (sizes are in kD).

Pollen morphology: SEM observations showed that pollen grains of date palm are elliptical-oblate and monosulcate and exine is reticulate (Figs. 2a-c). The longest and the shortest axes were 21µm and 9µm, respectively.

Bradford assay conducted on total protein content, extracted from *P. dactylifera* pollen grains, showed protein concentration of 19mg/g. Electrophoresis profile of pollen grains proteins revealed many bands in the range of 10–110kDa. The protein bands were separated with a little distance, continuously. These included 3 major bands of 110, 90 and 80kDa, and 11–13 other bands, which are shown in Fig. 3.

Discussion

In Arecaceae family, anther wall ontogeny has been studied in only a few genera (Chan & Lim, 2011). This is the first report on anther development in *Phoenix dactylifera*.

In this study, we described detailed development of anther wall and microsporogenesis of pollen grains in date palm. The anthers were tetrasporangiate. The wall of the young anther consists of an epidermis, endothecium, middle layer, and the tapetum. However, at maturity, it comprises only epidermis and endothecium. Anther wall formation is a basic type, although for monocotyledon, in general, the formation of the walls almost always proceeds according to the monocotyledonous type (Davis, 1966; Dahlgren & Clifford, 1982). Our results are consistent as the results of Davis (1966), who established these characters for this family. Dahlgren & Clifford (1982) reported the presence of a unique cellular stratum forming the middle layer in monocotyledons. However, *P. dactylifera* usually has two middle layer strata, which is in agreement with the results of Mahabale & Biradar (1967) on *Phoenix sylvestris*; Biradar (1968) on *Phoenix pusilla, P. acaulis*; Gonzalez-Cervants *et al*. (1997) on *Chamaedorea elegans* (*Arecaceae*) and Robertson (1976) on *Jubaeopsis caffra* that had found two middle layers in anther wall (Robertson, 1976).

Tapetal cells were binucleated. Mahabale & Channaveeraiah (1957) had observed similar binuclent tapetal cells in *Hyphaene indica*, Rao (1959b) observed them in members of *Alecineae*, *Cocoineae and Sabalae* and also Kulkarni & Mahabale (1974) in *Livistona chinensis*. The amoeboid or plasmodial type of tapetum is described as the most common type for monocotyledons (Dahlgren & Clifford 1982). In *Arecaceae* family, the secretory type is more frequent as in *P. dactylifera* and the tapetum cells degenerated completely at the late microspore stage. These results agree with the results of Mahabale & Biradar (1967) on *Phoenix sylvestris*, Kulkarni and Mahabale (1974) on *Livistona chinensis* and Gonzalez-Cervants *et al.* (1997) on *Chamaedorea elegans*, who found that the tapetum is of secretory type. Furnes & Rudall (1998, 2001) revealed that the tapetum in *Arecaceae* is only of secretory type and degenerates at the late stage of microsporogenesis.

The arrangement of microspore tetrads is of tetragonal type, that were consistent with Mahabale & Biradar (1967) on *Phoenix sylvestris* and Mahabale & Channaveeraiah (1957) on *Hyphaene indica*, but this arrangement in palms is very variable. The tetrahederal, T-shape and liner forms of tetrad in palms has been

described previously, for example, Rao (1959a), Kulkarni & Mahabale (1974), Dahlgren & Clifford (1982), Harley (1999), Harley & Baker (2001).

The middle layers in this species are degenareted in the late stage of microsporogenesis, which is not common in palms.

In palms, simultaneous cytokinesis or simultaneous and successive cytokinesis is very common in microspores (Sannier *et al*., 2006), but in date palm microspores meiosis only begun with successive cytokinesis. In addition, similar successive type of division was seen in *Phoenix sylvestris* by Mahabale & Biradar (1967), *Hyphaene indica* by Mahabale & Channaveeraiah (1957) five species of *calamus* by Kumar & Ramaswamy (2003). Furthermore, this finding was inconsistent with the results of Sannier *et al*. (2006), Furness & Rudall (2001) reported successive cytokinesis in palms. Pollen grains are two-celled when shed. It has been reported for *Phoenix sylvestris* and *Caryota urens* as well (Johri *et al*., 1992). Kumar & Ramaswamy (2003) identified that pollen grains of *Calamus stoloniferus, C. nagbettai* and *C. travancoricus* are 2-celled when shed. However, in *C. rotang* and *C. gamblei*, the pollen grains are 3-celled upon shedding.

In date palm, pollen grains were monosulcate and the exine was reticulate. In *Phoenix sylvestris* investigated by Mahabale & Biradar (1967), and *Hyphaene indica* by Mahabale & Channaveeraiah (1957), exine was smooth and monosulcate. Rao (1959a) observed reticulate thickening on pollen grains in *Areca* and *Caryota* species. In addition, monosulcate pollens were seen in *Livistona*, *Sabal*, *Licuala* and *Johannestijsmannia lanceolata* (Mahabale, 1967; Chan & Lim, 2011). Furthermore, Soliman & Al-Obeed (2013) described a monosulcate pollen type and reticulated exine in eleven varieties of date palm. The size measured in this study, i.e., 21µm × 9µm, is close to size reported by Almehdi (24µm × 11µm), Kwaasi (22µm × 12µm) and Soliman and Al-Obeed in different cultivars as well 17.20-21.40µm × 6.9-10.20µm (Kwaasi, 2003; Almehdi *et al*., 2005; Soliman & Al-Obeed, 2013). Here it seems that this difference of pollen grains' size could be related to genetic variability on different cultivars, and it could be used in biosystematics researches. The mature pollen grains are shed at the two-celled stage as *Phoenix sylvestris*, *P. pusilla*, *P. acaulis*, *P. reclinata* (Mahabale & Biradar, 1967; Biradar 1968).

According to the results of the Bradford (1976) method, proteins of pollen grains were high (19µg/g) and this could be seen in the electrophoretic profile. A result like this was reported by Chakraborty *et al*. (2004) on *Phoenix sylvestris*. They have shown that *P. sylvestris* pollen extracts have 14-17 proteins in SDS-PAGE (Chakraborty *et al*., 2004).

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