Developmental and Cytochemical Features of Male Reproductive Organ in *Crataegus tanacetifolia* (Lam.) Pers.

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Abstract

In this study, the development of male reproductive organ was analysed in *Crataegus tanacetifolia* (Lam.) Pers., endemic to Turkey. Androecium is composed of 20 stamens which are attached at the base of the filaments. The anther wall formation follows the dicotyledonous type. The undifferentiated anther is ovoid-shaped, and the differentiation starts with the appearance of archesporial cells. Mature anthers are dorsifix and tetrasporangiate. The anther wall is composed of an epidermis, endothecium, two or three rows of middle layers and secretory tapetum. Endothecial cells show fibrous thickening. Tapetum is characterized by enlarged secretory types with binucleate cells, which presented an intense reaction with regard to proteins, insoluble polysaccharides and lipids. Features of chromatin condensation and nucleus disorders identified with the application of DAPI (4´,6-diaminido-2-phenylindole) point out programmed cell death. Epidermal and endothecial layers remain intact until anther dehiscence; however, middle layer and tapetum disappear during development. At the end of regular meiotic division, tetrahedral microspore tetrads are formed. Pollen grains are tricolporate, tectate and sphaeroidea. Exine is made up of lipoidal substances and proteins, but the intine includes insoluble polysaccharides. Further, cytoplasm of pollen grains are rich in proteins, lipids and insoluble polysaccharides.

Keywords: anther ontogeny, anther wall, microsporogenesis, Rosaceae

Introduction

*Crataegus tanacetifolia* (Lam.) Pers. belongs to genus *Crataegus* of the subfamily Maloideae of Rosaceae (Campbell *et al*., 2007). According to Davis and Browicz (1972), there are 17 species, one subspecies and a few varieties of *Crataegus*, which grow naturally in Turkey. *C. tanacetifolia* is an important endemic species to Turkey and widely spreads in Bolu, Karabük, Kastamonu and Ankara. *C. tanacetifolia* is economically valuable plant for having edible fruits.

Morphological characters of the male gametophyte and details of microsporogenesis are useful in taxonomic studies for defining the circumscription of taxa. There are some taxonomically important characters such as; variability in the number of anther layers, thickening in endothecium, number of rows in middle layer, arrangement of tetrads within the callosic wall, number of pollen pores or colpes and bi or tricellular pollen grains (Galati *et al*., 2006; Gotelli *et al*., 2006; Liu and Huang, 2003). Additionally, the number of tapetum and the type of tapetum have taxonomic value in the sense that all members of most angiosperm families have the same type (Davis, 1966; Johri *et al*., 1992).

Two main types of tapetum are recognized in plants; secretory and amoeboid type. Secretory type is considered to be the prevalent type in majority of plants. The tapetal cells remain accompanied with the anther wall until their degeneration and secrete substances to the anther locule. Four types of anther wall development were described by Davis (1966) based on the secondary parietal layers: basic type, dicotyledonous type, monocotyledonous type and reduced type. Many authors use this classification to explain anther wall ontogeny (Aybek *et al*., 2012; Bittencourt, 1996; Chehregani *et al*., 2008; Garcia, 2002; Hardy and Stevensen, 2000; Liu and Huang, 2003; Strittmatter *et al*., 2000).

The present research is the first study on the male reproductive organ of *C. tanacetifolia* (Lam.) Pers., endemic to Turkey. This paper provides knowledge on anther ontogeny, anther wall development, microspore development, pollen morphology and cytochemical features; by the application of light, fluorescence and scanning electron microscopy. Information on the development of male reproductive organ will help advance our understanding of reproductive behaviour and will thus contribute to attempts to solve taxonomic problems in *Crataegus*, a rather neglected genus in this respect.

Materials and Methods

**Material and morphological analysis**

Flower buds of *C. tanacetifolia* were collected from Bolu, Lake Abant Nature Park (Turkey), and flowers were morphologically analysed and picked under stereomicroscope (Olympus 970931).
using an automated sputter coater and then examined with a SEM (JEOL JMS-5910LV).

**Results**

**Structure of the androecium**

Androecium of *C. tanacetifolia* consists of 20 stamens (Fig. 1a) which attached at the base of the filaments (Fig. 1c). Mature stamens are 5-8 mm in length and filaments are longer than the anthers (Fig. 2a). Mature anthers are yellow-brown, dorsifix and tetrasporangiate (Fig. 1b). Pollen grains are released by dehiscence of stomium (Fig. 2b).

<table>
<thead>
<tr>
<th>Anther length (mm)</th>
<th>Developmental stage</th>
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<tr>
<td>≤ 0.5 mm</td>
<td>Undifferentiated anther</td>
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<tr>
<td>0.5-0.75 mm</td>
<td>Sporogenous tissue</td>
</tr>
<tr>
<td>1 mm</td>
<td>Pollen mother cell</td>
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<tr>
<td>1.1-1.5 mm</td>
<td>Microspore tetrad</td>
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<tr>
<td>2-3 mm</td>
<td>Mature pollen stage</td>
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**Light and fluorescence microscope analysis**

Stamens were carefully excised and fixed in glacial acetic acid-alcohol and FAA (Formalin-glacial acetic acid-alcohol) solutions, and they were embedded in paraffin. Ultimately, they were sliced at 5-10 µm by Leica RM2235 rotation microtome. Sections were stained with Delafield’s hematoxilin.

For cytochemical analysis, sections were stained with periodic acid-Schiff (PAS) (Feder and O’Brien, 1968) for insoluble polysaccharides, Coomassie Brilliant Blue (Fisher et al., 1968) for proteins, Sudan Black B for lipids (Pearse, 1961) and Auramine O (Heslop-Harrison and Shivanna, 1977) for exine. For investigation of nucleus disorders, sections were stained with DAPI (4’,6-diamidino-2-phenylindole) (Schweizer, 1976).

For ultrastructural studies, stamens were fixed in Karnovsky fixative (5% glutaraldehyde and 4% paraformaldehyde) and embedded in epoxy resin. Next, ultrathin sections (~70 nm) were cut using a Leica Ultracut R and stained in 1% Toluidine blue (O’Brien et al., 1964).

The preparations were photographed with an Evolution LC color camera and attached to an Olympus BH-2 light microscope, and the images were analyzed though Image-Pro Express Version 6.0 scientific image processing and analysis software. Auramine O and DAPI preparations were investigated by Leica DM LB2 fluorescence microscope at respectively 440 nm and 365 nm wavelengths and photographed by Leica DFC 329 fluorescence camera.

**Scanning electron microscope (SEM) analysis**

For the study of anther and pollen morphology by SEM analysis, the material was fixed in 2.5% glutaraldehyde in 50 mM cacodylate buffer, pH 7.0 (Platt et al., 1983) and then dehydrated with an increasing ethanol gradient: from 70% up to 100%. Subsequently, the material for drying were kept in HMDS (hexamethyldisilazane) solution at room temperature (Topçuoğlu et al., 2009) and coated with 11 nm of gold by using an automated sputter coater and then examined with a SEM (JEOL JMS-5910LV).
the archesporial cells (Fig. 3b). They have prominent nuclei and dense cytoplasm which make them distinguishable from rest of the cells (Fig. 3c). The archesporial cells undergo periclinal divisions forming the primary parietal layer towards the epidermis and the sporogenous layer to the connective site (Fig. 3d). The cells of the parietal layer form concentric layers of wall endothecial, middle and tapetal layer (dicotyledonous type) by a series of periclinal and anticlinal divisions. At this time, the sporogenous cells enlarge and undergo mitotic divisions once generating pollen mother cells (PMCs). The anthers enlarge during the development, and they become tetrasporangiate (Fig. 3e). At the end of the development, mature pollen grains are released by dehiscence of stomium (Fig. 3f and Fig. 2b).

Structure and development of the anther wall

The anther wall of *C. tanacetifolia* consists of four layers from outer to inner: an epidermis, endothecium, two or three row of middle layers and a layer of tapetum (Fig. 4). Anther wall development is dicotyledonous. The middle layer and endothecium layer share the same origin. Initially, four wall layers have similar shapes and sizes.

Epidermis consists of single row of rectangle, flattened and ordered cells (Fig. 5a). It does not include trichomes and stomas. At the beginning of the development, epidermal cells remain small (Fig. 5a). Concurrent with the progression of meiosis in PMCs, the epidermal cells grow and tangentially elongate (Fig. 5c). As from tetrad stage, epidermal cells lose their rectangular appearance, and they start to look flattened (Fig. 5e). At mature tetrad stage, epidermis takes the largest and more flat form, preserving their vitality until the period of anther dehiscence (Fig. 5h, i).

The single layer of endothecium consists of flattened cells at sporogenous tissue stage (Fig. 5a). The cells radially elongate (Fig. 5c) and develop until the period of anther dehiscence. However, as from tetrad stage, cells show wall fibrous thickening which increases in the course of cell aging (Fig. 5e). At mature pollen stage, endothecial layer and thickening of the wall attain the maximum development (Fig. 5h), and mature pollen grains discharge by dehiscence of stomium, which is the thinnest region of anthers (Fig. 3f and Fig. 2b). Endothelial layer preserves its vitality until the period of anther dehiscence like epidermis layer (Fig. 5i).

The middle layer is two-three rows and ephemeral. At sporogenous tissue stage, it consists of flattened cells (Fig. 5a). After the first division of the pollen mother cells, middle layer enters a slow atrophy process (Fig. 5c). As from tetrad stage, middle layer cells lose volume (Fig. 5e), and the layer becomes compressed, crushed and completely invisible at mature pollen stage (Fig. 5h, i).

*C. tanacetifolia* has secretory tapetum, which consists of single layer of cells (Fig. 5a). They have larger volume, denser cytoplasm and larger spherical nuclei than the rest of cells of the anther wall (Fig. 5b).

Tapetal cells extend radially into the anther locus, from sporogenous tissue stage (Fig. 5d) to end of the tetrad stage (Fig. 5e). Until the end of the tetrad stage, width, length and area in the
pushed towards the inner tangential wall of tapetum by a large vacuole, which is formed in this stage.

Tapetum nucleus is stained more brightly and intensely than nuclei of the other anther wall layers with DAPI. Until the beginning of the tetrad stage, nucleus is ordered, spherical, with blue fluorescence, and nucleolus is distinctive (Fig. 9a, c, d, e, f). From the tetrad stage, disorder in tapetal nucleus starts to be visible (Fig. 9b). Initially, nucleus loses its proper sphere shape, invagination increases and chromatin condenses (Fig. 9g, h, i). At the end of the tetrad stage, with the decrease of the cell volume, the nucleus shrinks. But nucleolus is still evident. In addition, disorders and nuclear fragmentations of nucleus are more apparent. At the end of this stage (end of tetrad stage – start of young pollen stage), nucleolus disappears, fluorescence of nucleus decreases, and nucleus disintegrates (Fig. 9j, k). Determination of chromatin condensation and nucleus disorders are characteristics of programmed cell death.

Various cytochemical methods were applied to anthers at different developmental stages. During development, epidermis is rich in insoluble polysaccharides (Fig. 10a, h), but poor in protein and lipid content (Fig. 10a-d and Fig. 10i-l). Endothecium and middle layer are poor in insoluble polysaccharides, proteins and lipids; however, endothecial wall thickenings are rich in these compounds. From tetrad stage, tapetal cells accumulate a large amount of insoluble polysaccharides, proteins and lipids (Fig. 10c, g, k), as well as cells disintegrate at the end of the tetrad stage (Fig. 10d, h, l).
Fig. 10. Cytochemistry of anther wall layers at different developmental stages. a. Sporogenous tissue stage, b. PMCs stage, c. Tetrad stage, d. Mature pollen stage stained with Coomassie Brilliant Blue. e. Sporogenous tissue stage, f. PMCs stage, g. Tetrad stage, h. Mature pollen stage stained with PAS. i. Sporogenous tissue stage, j. PMCs stage, k. Tetrad stage, l. Mature pollen stage stained with Sudan Black B. Bar: 10 µm (a,j,v,d,e), 20 µm (f,g), 50 µm (h,k,l)

Morphology and cytochemistry of pollen grains

In order to determine the pollen morphology, pollen grains were examined by light and scanning electron microscope and the measurements were presented at Table 2 and Table 3.

Pollen grain of *C. tanacetifolia* is tricolporatae, tectatae and sphaeroidea (Fig. 12f and Fig. 13a). The pollen wall is composed of intine, innermost layer and exine, outermost layer (Fig. 12a). Exine of mature pollen grain is thicker than intin and exine pattern is rugulate type (Fig. 13b). Cytoplasm of a mature pollen grain is filled with insoluble polysaccharides, proteins, lipids (Fig. 12b, c, d). Exine is made up of lipoidal substances and proteins (Fig. 12c, d, e, g), but intine is composed of insoluble polysaccharides (Fig. 12b).

Discussion

In *Rosaceae* family, anther wall ontogeny and microsporogenesis have been studied in only a few genera (Hamideh et al., 2012; Sumner and Remphrey, 2005). This is the first report on anther wall ontogeny and microsporogenesis in *Crataegus tanacetifolia* (Lam.) Pers., endemic to Turkey.

In *Crataegus* species, flowers have usually 10 (*C. transmississippiensis*) or 20 stamens (*C. submolis* and *C. canadensis*) (Phipps, 2012). *C. tanacetifolia* belongs to the type with 20 stamens.

Generally, one specific type of the anther wall development is found in each family. The anther wall formation in *Rosaceae* was reported as dicotyledonous type (Davis, 1966). In the study of *C. babakhanloui* (Hamideh et al., 2012), anther wall development was reported as dicotyledonous type, and this report is correlated to our study.

Watson and Dallwitz (1992) reported that the members of *Rosaceae* family have monosporangiate (e.g. *Alchemilla*), bisporangiate and tetrasporangiate anthers. The anthers of *C. tanacetifolia* are tetrasporangiate.

In most angiosperms, the anther wall consists of four layers from outer to inner: an epidermis, an endothecium, one or two rows of middle layer and tapetum. The outermost cell layer of the anther is epidermis, which in most plants, is slightly modified during development (Rezanejad, 2008) as in *C. tanacetifolia*. As mentioned by Watson and Dallwitz (1992) in *Rosaceae*, anther epidermis of *C. tanacetifolia* is persistent and it preserves its vitality until the period of anther splitting as in *C. babakhanloui* (Hamideh et al., 2012).
Anther epidermis comprises simple and small cells in *C. tanacetifolia*.

As mentioned by Watson and Dallwitz (1992) in *Rosaceae*, endothelial cells show wall thickening in *C. tanacetifolia*. It also preserves its vitality until the period of anther dehiscence, as in *C. babakhanloui* (Hamidieh et al., 2012).

The row number of middle layer is a taxonomically important character. In *Rosaceae*, anther wall has more than one middle layer (Watson and Dallwitz, 1992). Although, the middle layer of *C. babakhanloui* consists of two rows (Hamidieh et al., 2012), and the number of middle layer in *Prunus armenica* (Rosaceae) (Julian et al., 2011) is two to four, middle layer in *C. tanacetifolia* is composed of two or three rows.

The type of tapetum may also have taxonomic value in the sense that all members of most angiosperm families have the same type (Davis, 1966; Johri et al., 1992). Tapetum is identified as secretory type in *Rosaceae* by Watson and Dallwitz (1992), and it is parallel to this report in *C. tanacetifolia*. However, *C. babakhanloui* differs from other species of *Rosaceae*; by having two types of tapetum, a periplasmoidal type and a secretory type (Bhojvani and Soh, 2010; Hamidieh et al., 2012; Lernsten, 2004).

Tapetal classification based on nuclear number (Cooper, 1933) has merit in taxonomy. In *Rosaceae*, the tapetum is either constantly uninucleate or else binucleate to multinucleate (Buss, 1971). In *C. tanacetifolia*, at sporogenous tissue stage, a tapetal cell has usually single nucleus with a single nucleolus. While meiosis continues at pollen mother cells, mitotic division occurs at tapetal cells, as mentioned by Julian et al. (2011) in *Prunus armenica* (Rosaceae). Tapetal cells become 2-4 nucleated at tetrad stage in *C. tanacetifolia*.

The cells of secretory tapetum degenerate towards the end of pollen development (Pacini et al., 1985), and tapetal cells undergo programmed cell death (Papini et al., 1999; Vardar and Unal, 2011; Wu and Cheung, 2000). The time of the degeneration varies greatly among species. Although tapetal cells have completely degenerated at young pollen stage in *C. babakhanloui* (Hamidieh et al., 2012), degeneration progresses rapidly and ends at the end of the tetrad stage in *C. tanacetifolia*. In *Lathyrus undulatus*, tapetal cells undergo degeneration at vacuolated pollen stage and completely disappear at bicellular pollen stage (Vardar and Unal, 2011). Programmed cell death takes place in the variety cells of reproductive organs including megaspores, synergids, antipodals, nucellus, suspensor and tapetum. Papini et al. (2014) and Vardar and Unal (2011) demonstrated programmed cell death in the tapetal cells using DAPI, TUNEL and ultrastructural techniques. Vacuolisation, cell shrinkage, chromatin condensation and nuclear degeneration are some of the characteristics of programmed cell death.

The tapetum has been considered as the nutritive tissue for the developing pollen (Pacini, 1994). According to our results, the tapetal cells of *C. tanacetifolia* accumulate proteins, carbohydrates and lipidoid substances during development; exclusively at tetrad stage. Cytological results of *C. tanacetifolia* anthers showed that the cells of epidermis, endothecium and middle layer give weak reaction to proteins, polysaccharides and lipids. Endothecial thickenings in *C. tanacetifolia* give intense reaction for proteins, polysaccharides and lipids at mature pollen stage.

In *Rosaceae*, microsporogenesis is simultaneous (Watson and Dallwitz, 1992) as in *C. tanacetifolia*. As a result of meiotic division, tetrads are formed in *C. tanacetifolia*, as in *C. babakhanloui* (Hamidieh et al., 2012). In *Rosaceae*, pollen grains are shed at 2-celled stage in 14 genera (Watson and Dallwitz, 1992) likewise in *C. tanacetifolia*.

The general outline of the pollen in the genus *Crataegus* is usually transversely elliptic in equatorial view and trilobed to subcircular in polar view, as in most other *Rosaceae* (Byatt, 1976; Eide, 1981; Moore et al., 1991; Reitsma, 1966). In general, there is no significant variation in size between the taxa and within the taxa of Turkish *Crataegus* (Dönmez, 2008). In *C. tanacetifolia*, pollen grains are not very big in size. According to Dönmez (2008), the most frequent aperture type is tricolporate in *Crataegus* taxa, and *C. tanacetifolia* is correlative to this report.

Several researchers reported that polysaccharides, proteins and lipids exist in pollen cytoplasm (Bedinger, 1992; Hess, 1993; Li et al., 1995). In *C. tanacetifolia*, cytoplasm of pollen grains are rich in polysaccharides, proteins and lipids. Exine is made up of lipoidal substances and proteins, but intine is composed of insoluble polysaccharides.

**Conclusion**

In *Crataegus tanacetifolia*, androecium is composed of 20 stamens. Mature anthers are tetrasporangiate and the anther wall formation follows the dicotyledonous type. The anther wall is composed of an epidermis, endothecium, two or three rows of middle layers and secretory tapetum. Epidermal and endothelial layers remain intact until anther dehiscence; however, middle layer and tapetum disappear during development. Tapetum is characterized by enlarged secretory types and chromatin condensation and nuclear disorders are identified in tapetal cells. By regular meiotic division, tetrads are formed. Pollen grains are tricolporate, tectate and sphaeroidea.

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**References**


