

Anatomical and Palynological Investigations on *Rubia tinctorum* L. (Rubiaceae, Rubiaceae) from the Aegean Region of Turkey

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Abstract: The genus *Rubia* L. includes valuable species in terms of important agricultural, industrial, and pharmacological characteristics. Red dye obtained from the roots of *Rubia tinctorum* L., naturally found in the flora of Turkey and known as common madder, has been used as the source of a natural dye since ancient times. In this work, a preliminary report is given on anatomical and palynological traits of *R. tinctorum* distributed in the Aegean Region of Turkey examined by light and scanning electron microscopy. In the root anatomy, the cortex composed of multilayered parenchyma cells, the vascular tissue organized in collateral vascular bundles, 1(-2)-rowed ray cells, and the pith with a cavity at the center are observed. In the leaf anatomy, the bifacial and amphistomatic leaf, the dorsiventral mesophyll with one layer of columnar palisade parenchyma cells and a few layers of irregularly organized spongy parenchyma cells, and the midrib with a large collateral vascular bundle surrounded by parenchymatous bundle sheath cells are recognized. Pollen grains are shed as monads, small, mostly spheroidal in equatorial view, generally hexacolpate and have a microechinate-perforate exine ornamentation.

Keywords: *Rubia*, vegetative anatomy, pollen morphology, microscopy technique.

Türkiye'nin Ege Bölgesinden *Rubia tinctorum* L. (Rubiaceae, Rubiaceae) Üzerine Anatomik ve Palinolojik Araştırmalar

Öz: *Rubia* L. cinsi tarımsal, endüstriyel ve farmakolojik açıdan önemli türleri içermektedir. Türkiye florasında doğal olarak bulunan ve kızılboya olarak bilinen *Rubia tinctorum* L. eski tarihlerden beri köklerinden elde edilen kökboyası ile doğal boya kaynağı olarak kullanılmaktadır. Bu çalışmada, Türkiye'nin Ege Bölgesi'nde yayılış gösteren *R. tinctorum*'un ışık ve taramalı elektron mikroskobu ile incelenen anatomik ve palinolojik özellikleri üzerine bir ön değerlendirme raporu verilir. Kök anatomisinde, çok sıralı parankima hücrelerinden meydana gelen korteks, kollateral iletim demetlerinden oluşan iletim doku, 1(-2) sıralı ışın hücreleri ve merkezinde bir boşluk bulunan öz gözlemlenmektedir. Yaprak ayası anatomisi incelendiğinde, bifasiyal ve amfistomatik tipte yaprak, 1 sıralı silindirik şekilde palizat parankiması ile birkaç sıralı düzensiz dizilmiş sünger parankimasından oluşan dosiventral tipte mezofil ve parankimatik demet kını hücreleri tarafından çevrelenen büyük kollateral bir iletim demetine sahip orta damar bulunmaktadır. Polen tanecikleri tek tek dökülür, küçük boyutlu, ekvatorial görünümde sıklıkla sferoidal şekilli, genellikle heksakolpat ve mikroekinat-perforat ekzin süsüne sahiptir.

Anahtar kelimeler: *Rubia*, vejetatif anatomi, polen morfolojisi, mikroskopi tekniği.

1. Introduction

Rubiaceae (coffee family) is the fourth largest family of angiosperms with about 660 genera and 12000 species assigned into 42 tribes (Ehrendorfer, Barfuss, Manen, & Schneeweiss, 2018). *Rubia* L., the type genus for the Rubiaceae in the order Gentianales, possesses an Old World distribution (Yang, Sun, Ehrendorfer, & Nie, 2016). *Rubia* is the third largest genus of tribe Rubieae in the Rubiaceae with about 80 species around the world (Yang, Sun, Ehrendorfer, & Nie, 2016). Approximately half of these species are found in China, making it the most significant diversity center of this genus (Yang, Sun, Ehrendorfer, & Nie, 2016). The genus is distinguished from other members of this tribe by its 5-lobed corolla and fleshy fruits (Manen, Natali, & Ehrendorfer, 1994). *Rubia* species have been used for their red dyes known as alizarin in textiles, carpets, and other objects (e.g., *R. tinctorum* L., *R. cordifolia* L. roots). In addition to the commercial value, roots of *Rubia* species (e.g., *R. yunnanensis* Diels, *R. akane* Nakai) are medicinally used for the treatment of cancers, tuberculosis, rheumatism, hematemeses, metrorrhagia,

epistaxis, contusion, and menoxenia in the Chinese folk medicine and healing wounds, inflammation, skin infections in the Indian traditional medicine (Meena, Pal, Panda, Sannd, & Rao, 2010; Zhao et al., 2011; Mouri, & Laursen, 2012).

In Turkey, five species of *Rubia* grow naturally: *R. davisiana* Ehrend. (endemic), *R. peregrina* L., *Rubia rotundifolia* Banks & Sol., *R. tenuifolia* d'Urv. and *R. tinctorum* L. The most significant and stable morphological feature for infrageneric classification of the genus are the leaf venation pattern. Sect. *Rubia* s.l. has single and pinnate veins whereas there are palmate major veins, branching from the leaf basis in sect. *Oligoneura* Pojark. Except for *R. davisiana* in sect. *Oligoneura*, the other Turkish *Rubia* species are classified in sect. *Rubia* s.l. There are some studies on the anatomy of some genera of Rubiaceae, as in *Alseis* (e.g., Campbell, Rabelo, & da Cunha, 2016), *Bathysa* (e.g., Nascimento, Gomes, & Vieira, 1996), *Psychotria* (e.g., Marques et al., 2015), *Rondeletia* (e.g., Kocsis, Darók, J., & Borhidi, 2004), *Rudgea* (e.g., Leo, Mantovani, & Vieira, 1997), *Rustia* (e.g., Vieira, Delprete, Leitão, & Leitão, 2001),

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Simira (e.g., Moraes, Barros, Silva Neto, Gomes, & Da Cunha, 2009), and *Virectaria* (e.g., Dessein, Jansen, Huysmans, Robbrecht, & Smets, 2001), as well as the pollen morphology of some species of Rubiaceae (Huysmans, Robbrecht, & Smets, E., 1994, 1998; Huysmans, Robbrecht, Delprete, & Smets, 1999; Piesschaert, Huysmans, Jaimés, Robbrecht, & Smets, E., 2000; Dessein, Scheltens, Huysmans, Robbrecht, & Smets, 2000; Dessein, Huysmans, Robbrecht, & Smets, 2002; Huysmans, Dessein, Smets, & Robbrecht, 2003). However, previously published accounts on the anatomy and palynology of *Rubia* are limited to general studies. Recent works on the anatomy and pollen morphology of almost all *Rubia* species are not available. Here, the study presents detailed anatomical characteristics of *R. tinctorum* for the first time. Some palynological properties of *R. tinctorum* from Belgium and *R. peregrina* from France are described (Huysmans, Dessein, Smets, & Robbrecht, 2003). However, in this work, new information about the pollen morphology of *R. tinctorum* growing in Turkey is provided. The main goal of the present study is to describe the stem and leaf anatomy and the palynology of *R. tinctorum*.

2. Material and Methods

Specimens of *R. tinctorum* were collected from its natural habitats during field studies in Usak and Kutahya provinces in Turkey. Sample details are provided in the following: Usak: Kasaboglu stream, June 2017, N. Ari 15, Usak: near Derbent village, July 2017, N. Ari 20, Kutahya: New Gediz road, roadsides, July 2017, N. Ari 23. The collected specimens were kept at the Laboratory of Plant Systematics and Phylogenetics at Usak University.

2.1. Light microscopy (LM) studies

A variety of methodologies exist for preparing plant organs for anatomical studies, many of which are sample dependent (e.g., Johansen, 1940; Keating, 2014). Here, for obtaining anatomical features of seven to ten stems and leaf specimens per population, material kept in 70 % ethyl alcohol was prepared and analyzed using traditional paraffin wax-embedding protocols with some modifications. They were embedded into paraffin, sliced into thin transverse sections with a thickness ranging from 8 to 15 μm , stained based on a double staining protocol using safranin and fast green, and then mounted in Entellan. Anatomical observations were performed on twenty-five slides.

For palynological studies, the technique of Wodehouse (1935) was used so as to retain the natural form of pollen grains. After the grains were treated with 70% ethyl alcohol for the removal of oily substances, they were mounted in glycerin jelly stained with basic fuchsin. For species in monads, at least 30 pollen grains per sample were examined in equatorial and polar views. Polar axis (P), equatorial axis (E), colpus length (Clg), colpus width (Clw), exine thickness (Ex), intine thickness (In), and mesocolpium diameter (Me) were measured. Each value was expressed as mean \pm standard deviation.

For LM studies, anatomical and palynological material was examined and photographed by a Leica DM1000 light microscope (Leica Microsystems, Wetzlar,

Germany) at the Laboratory of Plant Systematics and Phylogenetics at Usak University.

2.2. Scanning electron microscopy (SEM) studies

For SEM investigations, the material was placed on aluminum stubs covered with carbon tape and sputter-coated with gold-palladium for five minutes. Then, the pollen grains were examined to determine the exine sculpturing at magnifications ranging from $\times 10000$ to $\times 20000$ with using a LEO-1430 VP SEM (Carl Zeiss SMT, Oberkochen, Germany) at an accelerating voltage of 20 kV at the Technology Application and Research Centre (TUAM), Afyon Kocatepe University, Afyonkarahisar, Turkey. The palynological terminology of Halbritter et al. (2019) was followed.

3. Results and Discussion

3.1. Stem anatomy (Fig. 1A-D).

The outermost layer of the root is covered by epidermal cells. Underneath the epidermis, the cortex comprises of multilayered parenchyma cells with thin walls. Vascular tissues are arranged in collateral vascular bundles. Vessel outline is angular or almost rounded. Rays are 1(-2)-seriate. In the central pith, the broken cells partially form a cavity. The peripheral limit of the pith cavity is lined with several layers of polygonal, thin-walled parenchyma cells with intercellular spaces. The stem anatomical properties of *R. tinctorum* are consistent with previous studies (e.g. Metcalfe, & Chalk, 1950).

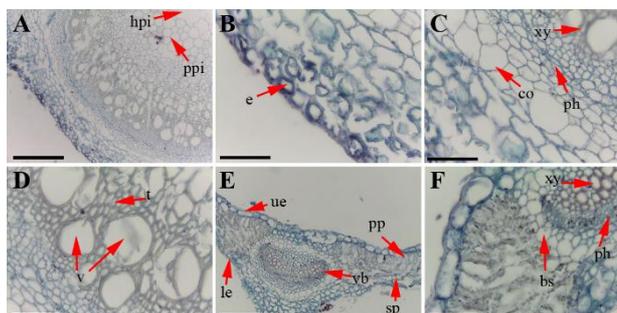


Figure 1. LM micrographs of cross-sections of *R. tinctorum* stems (A-D) and leaves (E-F). Abbreviations: bs: bundle sheath, co: cortex, e: epidermis, hpi: hollow pith cavity, le: lower epidermis, pp: palisade parenchyma, ph: phloem, ppi: parenchymatous pith cells, sp: spongy parenchyma, t: trachea, ue: upper epidermis, v: vessels, vb: vascular bundle, xy: xylem. Scale bars = (A, E) 200 μm , (B, C, D, F) 50 μm .

3.2. Leaf anatomy (Fig. 1E-F).

The leaf epidermis is one layered elongated to isodiametric cells covered with a fine cuticle. The upper epidermal cells are larger than the lower epidermal ones. The leaf is bifacial and amphistomatic. Stomata are placed slightly above the epidermal cells. The mesophyll is dorsiventral and comprises one layer of columnar palisade parenchyma cells and a few layers of irregularly organized spongy parenchyma cells. The presence of the dorsiventral mesophyll is congruent with the leaf properties for the family Rubiaceae (Metcalfe, & Chalk, 1950; Robbrecht, 1988). The midrib consists of a large collateral vascular bundle surrounded by parenchymatous bundle sheath cells. The vascular tissue in *R. tinctorum* shows a collateral arrangement described previously in other Rubiaceae species (Robbrecht, 1988). Bundle sheath extensions

surround the vascular tissue. The bundle sheath extending until the adaxial and abaxial epidermis is reported in some species of Rubiaceae (Dickison, 2000). These extensions can provide an additional mechanical support for the leaf (Moraes, Barros, Silva Neto, Gomes, & Da Cunha, 2009).

3.3. Pollen morphology (Fig. 2).

The pollen grains of *R. tinctorum* are shed as monads. Rubiaceae pollen grains are mostly dispersed as monads however permanent tetrads in some species (Dessein et al., 2005). Pollen size *R. tinctorum* is small as $P = 15.45-19.75$ (17.32 ± 1.03) μm and $E = 16.23-23.18$ (19.17 ± 1.42) μm . Pollen size of Rubiaceae varies from small to very large; however, many species have small to medium pollen grains ranging from 20 to 40 μm . (Dessein et al., 2005). In this study, the pollen shape of *R. tinctorum* is more or less circular in polar view while it is mainly spheroidal (very rarely suboblate) in equatorial view ($P/E = 0.85-0.95$). The pollen grains are isopolar, radially symmetrical, and mostly hexacolpate (occasionally pentacolpate, heptacolpate, or octacolpate). In a palynological study on Northwest European representatives of several genera of Rubiaceae (Huysmans, Dessein, Smets, & Robbrecht, 2003), findings concerning *R. tinctorum* pollen grains relatively confirm results of our study. However, our findings represent some differences in pollen size and the number of aperture. In this previous work, its pollen grains were found as $P = 19-22$ (20.5) and $E = 18-22$ (21) μm in size and spheroidal shape (P/E) and hexacolpate to octacolpate. The differences in size and shape can be assumed to be mainly due to variations in preparation. In the present study, pentacolpate pollen grains of *R. tinctorum* are first recorded. Colpi are distributed symmetrically. Colpus length is 9.63-14.98 (11.93 ± 1.60) μm and colpus width is 1.95-4.20 (2.99 ± 0.47) μm . Mesocolpium is 4.21-7.32 (5.67 ± 0.64) μm . Exine and intine layers are 0.45-0.91 (0.65 ± 0.12) and 0.23-0.48 (0.31 ± 0.06) μm in thickness, respectively. SEM investigations show microechinate-perforate surface ornamentation pattern (perforate with microspines).

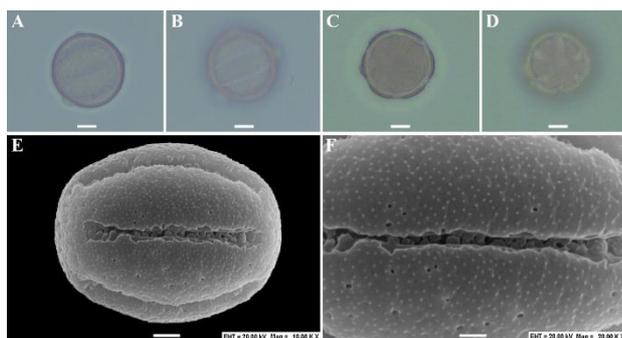


Figure 2. LM (A-D) and SEM (E-F) micrographs of *R. tinctorum* pollen grains. (A-B) Pollen grains in equatorial view, (C-D) Hexacolpate pollen grains in polar view, (E) Pollen grain in equatorial view, (F) Close-up showing microechinate-perforate surface sculpture and apertural region of pollen. Scale bars = (A-D) 5 μm , (E) 2 μm , (F) 1 μm .

The present paper is a preliminary study on anatomy and palynology of Turkish species of the genus *Rubia*. The results contribute to palynological knowledge of *Rubia* species distributed in Turkey. The combination of anatomical and palynological aspects may be valuable in the systematic of the genus *Rubia*. However, it is necessary to conduct further studies to obtain more comparative

information about anatomy and pollen morphology of other *Rubia* species.

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