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 dorsivenral 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.

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, & Schneweiss, 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, hematemesis, 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. davisania Ehrend. (endemic), R. peregrina L., Rubia rotundifolia Banks & Sol., R. tenutifolia 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. Oligonema Pojark. Except for R. davisania in sect. Oligonema, 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), Bathysya (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 Vireuctaria (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, Jaimes, 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 Kütahya provinces in Turkey. Sample details are provided in the following: Usak: Kasaboglu stream, June 2017, N. Arı 15, Usak: near Derbent village, July 2017, N. Arı 20, Kütahya: New Gediz road, roadsides, July 2017, N. Arı 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 from 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 (Ch), exine thickness (Ex), intine thickness (In), and mesocolpium diameter (Me) were measured. Each value was expressed as mean ± 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 ×1000 to ×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, uc: upper epidermis, v: vessels, vb: vascular bundle, xy: xylem. Scale bars = (A, E) 200 μm, (B, C, D, F) 50 μm.](image-url)

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. Celpus 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).
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