ANATOMICAL INVESTIGATIONS ON *OENOTHERA BIENNIS* L. USING OPTICAL MICROSCOPY AND SCANNING ELECTRON MICROSCOPY (SEM)

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Abstract. *Oenothera biennis* L. *(Evening Primrose or Evening Star) is a species of interest given by its medicinal and nutritive properties as well as for its economic value. It is also one of the earliest plant models in genetics and cytogenetics. Currently, O. biennis is cultivated mostly for its seeds, from which is extracted the Evening Primrose oil (EPO), rich in γ-linolenic acid. EPO is commercialized in diverse products, from nutritional supplements to cosmetics. The rest of the plant has medicinal value, being used to treat asthma, cough, intestinal pain, inflammation, and as an analgesic. The present study focuses on the morpho-anatomical characteristics of the main vegetative organs of O. biennis. For this study, the plant was collected from Macea village, Arad County, in the years 2015 and 2017. Root (lower part and upper part), stem (lower part and upper part), petiole and leaf samples were prepared according to current optical microscopy and scanning electron microscopy techniques, then observed and photographed with Novex-Holland optical microscope and Quanta 250 SEM microscope, respectively. The analyzed samples have shown the presence of a secondary structure at the level of central cylinder for both the root and the stem, a casparyan type endodermis, starchy liberian parenchyma, and numerous cells with calcium oxalate raphides. The pith had a thick, parenchymatic-cellulosic aspect, of meatic type. In the stem, the pith can present aeriferous cavities of irregular shapes.The root cortex presents what appear to be mucilage cavities. There has been observed a difference between the inferior part (towards the apex) and the superior part (towards the base) of the root and stem regarding the number of cells, their size, and their dispositions. Both the stem and the foliar limb present on their epidermis two types of trichomes: long and straight, short and curved. The foliar limb is amphistomatic and has additional secretory hairs. Our results contribute to a better understanding of this plant's structure and possible storage areas for active compounds of pharmacological interest.*

Keywords: *Oenothera biennis, morpho-anatomy, root, stem, leaf, petiole, SEM.*

INTRODUCTION

The *Oenothera biennis* L. species belongs to the *Onagraceae* family of the order *Myrtales* (SYSTMA *et al*, 2004). Its origins are in North-America, from where it spread in temperate and subtropical regions all around the world (WAGNER *et al*., 2007). The species grows on sandy soils, along the roads and railways, riverbanks, gravels, walls, ruderal places, and sometimes among crops (SĂVULESCU, 1957). The species has been extensively studied from a cytogenetic, genetic and taxonomic perspective, given some specific characteristics and high variability of the population that enables efficient exploration of phenomena usually difficult to detect (WAGNER *et al*., 2007).

There have been made cultivation and utility studies, especially in China, where it is mainly cultivated today (DENG *et al*., 2001), as well as investigations of the chemical components, mainly the lipids from seeds, and methods to improve the oil production (CALLAO *et al*., 2007; RATZ *et al.,* 2013, etc.). The variety of constituents described include steroids, terpenoids, fatty acids, flavonoids, tannins (SRIVASTAVA *et al*., 1998) oenothines (YOSHIDA *et al*., 1991), paraffin, resins, mucilage, invertines, pentosane, phytosterine (SĂVULESCU, 1957), xanthones and derivates (ATEEQUE *et al*., 2014).

Currently, *O. biennis* is cultivated for its seeds, from which it is extracted an oil, commonly known as EPO, rich in γ-linolenic acid, ω-6-polyunsaturated inessential fatty acid, ω-6-polysaturated essential fatty acid, linoleic acid, oleic acid, palmitic and stearic acid (National Toxicology Program, 2009). The seeds contain 10-17% oil, and the γ-linoleic acid can be up to 10% of the total fatty acid content. Unlike other fatty acid rich oils, this one is also resistant to oxidation (ZADERNOWSKI, 2002). The oil is mainly used in the pharmaceutic, nutraceutical, and cosmetic industry, and as a standard reference for ω-3 and ω-6 fatty acids analysis (SCHANTZ *et al*., 2013). Before the growing demand of EPO, the seeds were harvested from wild cultures and sold as ”wild sesame seeds” (DENG *et al*., 2001). The whole plant is edible and also makes good fodder for animals and birds (DENG *et al*., 2001).

The *Oenothera* species have been recommended in Argentina for crop rotations, because it’s a drought and pests resistant plant, with good yields even in arid or semi-arid areas (GAMBINO & VILELA, 2011). Compared to the wild species, cultivated *O. biennis* blooms later, accumulates more vegetative mass, and the senescence period is delayed (JARAMILLO & VILELA, 2015). *O. biennis* L. also has a long history of being cultivated as an ornamental plant in temperate regions (HALL *et al*., 1998). As a medicinal plant, it was used to treat asthma, cough, wounds, burns, and as an analgesic, to calm intestinal pain (Plants for a Future, 1996-2012), the palette being extended with time to various skin conditions, obesity, diabetes (DENG *et al*., 2001) and even psychological conditions such as dementia and schizophrenia (JOY *et al*., 2000; BARNES *et al*., 2002). Clinical studies show the potential of EPO in reducing the risk of diseases related to some (3-9) essential fatty acid deficiency, in atopic dermatitis, rheumatoid arthritis, high blood cholesterol, thrombosis, mastalgia, PMS (RATNAYAKE *et al*., 1989). A series of studies sustain the antioxidant and anti-inflammatory action of *O. biennis* extracts and oil (RATZ *et al*., 2015; GRANICA *et al*., 2013; SCHMIDT *et al*., 2003; SHAHIDI *et al*., 1997; BUDINCEVIC *et al*., 1995; MONSERRAT de la PAZ *et al*., 2014) as well as the antitumoral potential (PELLEGRINA *et al*., 2005; ARIMURA *et al*., 2003).

To our knowledge, no data are available regarding the histo-anatomy of the vegetative organs, except for some images of root collar in a stem anatomy atlas (SCHWEINGRUBER *et al*., 2011) and a small description of root in a plant archeology thesis (HATHER, 1988). Available optical microscopy, SEM, and TEM images and analysis concern only the reproductive parts of the plant, such as pollen, anthers, and ovary (NOHER de HALAC & HARTE, 1995; SODMERGEN *et al*., 1997; CHAPMAN & MULCAHY, 1997).

Considering the above-mentioned aspects, in the present study we were interested in investigating the internal structure of the main vegetative organs of *Oenothera biennis* L.

species, with regards to possible adaptative features and storage areas of bioactive compounds of economical and medicinal interest.

MATERIAL AND METHODS

For this study, samples of *Oenothera biennis* were harvested from Macea Village, Arad County, while in bloom, in July 2015 for the optical microscopy analysis and May 2017 for the SEM analysis. The voucher specimen was identified and preserved in ethylic alcohol 70% at the Institute of Life Sciences from „Vasile Goldiş" Western University of Arad.

Optical microscopy

Cross sections were performed through the main vegetative organs: root (lower part and upper part), stem (lower part and upper part), petiole and leaf, using a botanical razor, and a hand microtome. The samples were javelized with sodium hypochlorite for about 20-30 min., depending on the material, then washed with acetic water and distilled water. The sections were subjected to a double colouring process with iodine green (1 min.) followed by washing with 90° ethylic alcohol, and red carmine alaunate (20 min.) followed by washing with distilled water. The colored samples were fixed in glycerol-gelatine. The permanent samples obtained were photographed on a NOVEX Holland photonic microscope using an A95 Canon digital camera.

Scanning Electron Microscopy

Fresh samples of the main vegetative organs were prepared according to standard SEM techniques. The vegetal material was washed with distilled water to remove any dust particles. The excess water was removed, and small fragments were fixed on the carbon band. The fragments were then covered with a thin layer of gold particles using a sputter-coater. Samples were observed and photographed on a Quanta 250 Scanning Electron Microscope with its respective software, at the Electron Microscopy Laboratory of the Institute of Life Sciences from „Vasile Goldiş" Western University of Arad.

RESULTS AND DISCUSSIONS

Root

The root has a thick base and becomes extremely thin towards the apex. Some differences were observed in the internal structure close to both endings. In the primary structure were observed a casparian type endodermis and a tetrarch central cylinder. Secondary structures resulted from felogen and cambium activity were present.

The suber towards the tip is composed of 5-6 overlayed, tangentially elongated cells (Figure 1). As we get closer to the root base, the suber becomes thinner because the external layers were exfoliated. The phelloderm is 7-8 cell layers thick, with round cells and meatuses, some cells are particularly larger and contain calcium oxalate raphides. The abundance of raphide crystals in vegetative organs is a well known feature of the *Onagraceae* (RAVEN, 1979; CARLQUIST, 1975). In the cortex we have observed some cavities that appear to be mucilage cavities, but further investigations are needed to confirm (Figure 1).

The central cylinder has, towards the superior part, a thin ring of secondary phloem with starchy parenchymatous cells. Towards the inferior part, the secondary phloem rings become thicker and the parenchymatous cells form radially rows (Figure 2). Large, tangentially elongated cells with calcium oxalate raphides become more numerous close to the root base (Figure 3). Towards the apex, the secondary xylem has many vessels of various diameters randomly spread, few woody fibers with relatively thick and lignified walls, especially between the vessels near the cambium and cellulosic parenchymatous cells, aspects which coincide with the description made by Hather (1988), except for the high concentrations of tyloses in xylem which are absent in our sample. The difference in the wood vessel diameter is thought to be related to the seasonal moisture availability (CARLQUIST, 1975) and the presence of tyloses are considered a plant response to stress (MICCO *et al*., 2016). Towards the root base, the secondary xylem is very thick, with few vessels in the central area and more cellulosic parenchymatous cells, and 2 concentric rings with many vessels separated by woody fibers and few cellulosic parenchymatous cells. Between the 2 xylem rings stands a thick band of cellulosic parenchyma with cells forming radially rows.

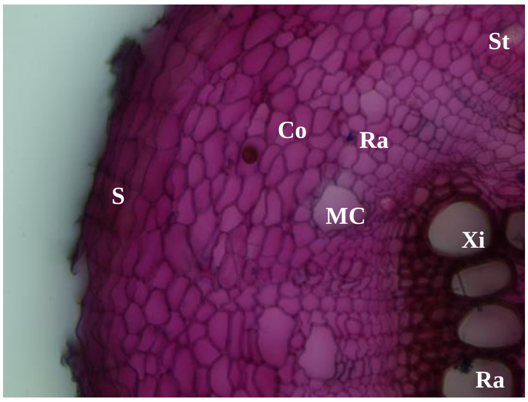

Figure 1. Inferior part of the root (apex). Cross-section. Aspect of suber, parenchyma cells and wood vessel with raphides. S- suber, Co- cortex, MC- mucilage cavities, Xi- xylem, Ra- raphide, St- starch (x 100).

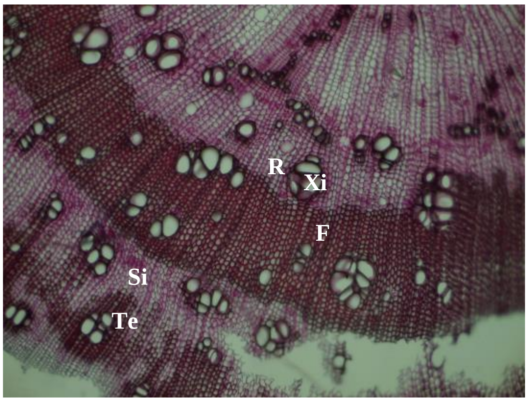

Figure 2. Superior part of the root (base). Cross-section. Multiseriate rays of parenchyma cells.Wood vessels forming small groups. Xi- xylem, R- ray, F- fiber, Te – tension wood Si- sieve tubes (x 50).

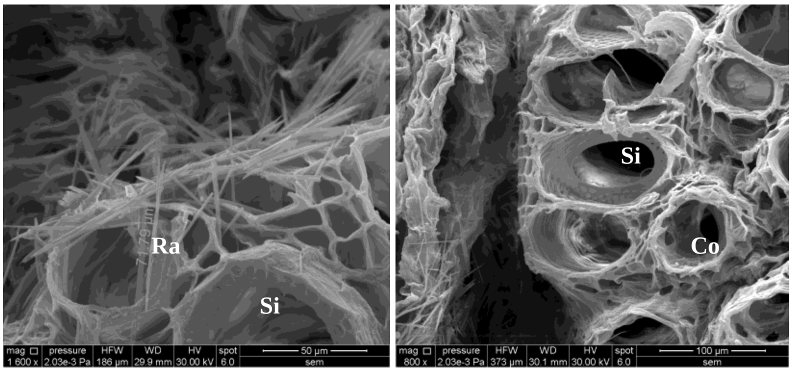

Figure 3. Root vascular bundle with calcium oxalate raphides. Ra- raphide, Si- sieve tube, Co- companion cell.

Stem

The stem presents a secondary structure as well. In cross - section, the upper part of the stem has a roughly circular shape, with irregular ribs. The smallest ribs bear trichomes. In the lower part of the stem, the ribs are few and slightly attenuated (Figure 4).

The epidermis has tangentially elongated cells, with thickened internal and external walls in the upper part and less thickened in the lower part. With unicellular hairs, some long and perpendicular to the stem surface, some short and claviform, with the tip bent parallel to the stem surface. The long trichomes are papillate and thin-walled, while the small hairs have a smooth surface. Some trichomes can appear striate-ridged, feature signaled in some *Oenothera* species (KEATING, 1982). Rare stomata have been observed as lightly prominent on the surface (Figure 6).

The cortex has a unistratified hypoderma, tangentially elongated. Between some ribs were observed 2-3 layers of collenchyma. Cortical parenchyma is of meatic type, with cells tangentially elongated in the lower part and containing fewer raphides than the upper part.

The central cylinder presents pluristratified, discontinuous pericycle with polygonal, thin-walled cells. External phloem, with sieve tubes has starchy parenchyma cells and companion cells. As well as a discontinuous ring of internal phloem islands formed only of sieve tubes and companion cells, called interxylary phloem by Carlquist (1975). The presence of interxilary phloem, a characteristic of *Myrtales* family, but not of all *Onagraceae*, has been observed in other *Oenothera* species along with the phloemic parenchyma. The formation of interxylary phloem may be dependent on rates of photosynthate conduction, space availability, and flower formation, as massive flowering draws rapidly on stored sugars (CARLQUIST, 1975).

The secondary wood has vessels often forming rays, with thin walls and woody fibers with walls that started to thicken and lignify. The primary wood forms a thin, discontinuous ring, with vessels displayed in rays, thick and lignified walls, separated by cellulosic wood parenchyma. In the lower part, the meatic pith has large cells in the axial area, with some cells that got disorganized and formed irregular shaped aeriferous cavities.

In *Oenothera* spp. the presence of interxylary phloem and secondary xylem is put on spatial considerations, the possibility of rapid translocation of starch from storage fibers, and a derivation from perennial ancestors with large stems (CARLQUIST, 1975).

Where phloem bands parenchyma are abundant, starch tends to accumulate there, and where parenchyma in interxylary phloem bands is minimal, the starch tends to accumulate in libriform fibers and ray cells (CARLQUIST, 1975). Many *Onagraceae* species, especially perennials and those that grow in dry areas, form characteristic deposits that appear dark and maybe granular in microscopic samples. These are thought to be tannins. And some areas that appear very dark, solid and gummy, such as droplets or some accumulations that block light. In other *Oenothera* species, this was reported in ray cells (CARLQUIST, 1975) (Figure 5).

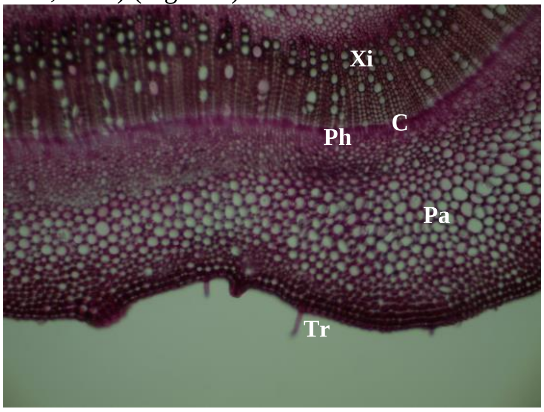

Figure 4. Upper part of the stem. Aspects of ribs with trichomes. Tr- trichome, Pa- parenchyma, Ph- phloem, C- cambium, Xi- xylem (x 50).

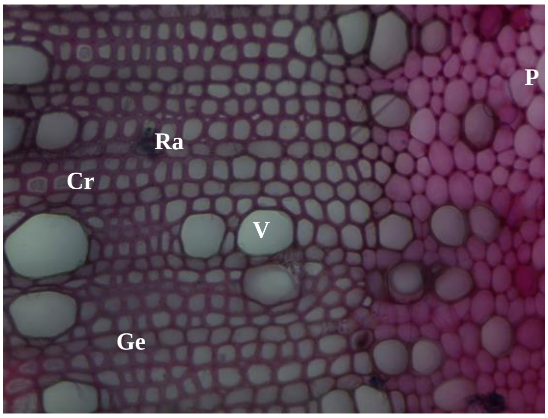

Figure 5. Lower part of the stem. View of vessels, xylem, some gelatinous fibers and cells containing raphides. P- pith, V- vessel, Ra- raphide, Ge- gelatinous fiber, Cr- crystal surrounded by mucilage (x 200).

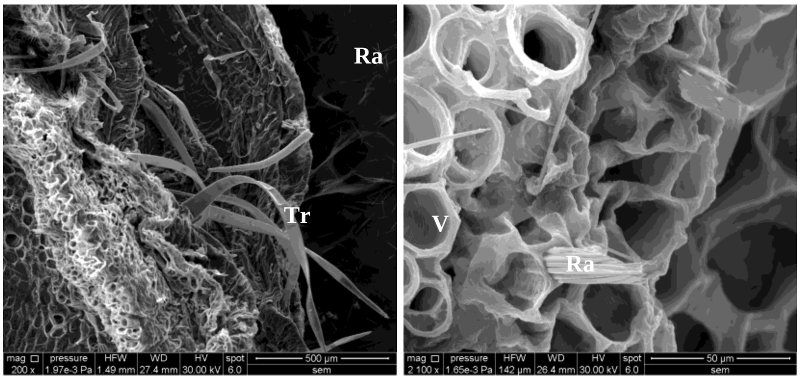

Figure 6. Aspects of stem trichomes (left) and vessels with needle-like raphide bundles (right). Tr- trichome, Ra- raphides, V- vessel (sieve tubes with raphides).

Lamina and petiole

The median vein is particularly prominent on the abaxial side and slightly curved on the adaxial side (Figure 7). The epidermis has isodiametric cells with thick internal and external walls and covered by a thin cuticle. The types of trichomes that were previously discussed are present on this level as well. The trichomes observed at this level are thin-walled and bear small irregular protuberances on their surface, what Keating (1982) mentioned as papillate surface, and nodose trichome with knobby surface by Voigt (2007) (Figure 9). A feature mentioned possible in some *Oenothera*, as trichomes are not present in a third of the species (KEATING, 1982). The leaf has additional secretory hairs, that are rare, very short, and with large, bulging glands. The presence of unicellular, clavate, inclined, and bent, cone shaped, nodose trichomes on the adaxial side, and curved, thread-shaped trichomes on abaxial side was mentioned before by Voigt (2007). The parenchyma is colourless, with large meatuses between cells. Angular collenchyma offers support under both epidermises. The vascular bundles are of open, bicollateral type. It was observed the formation of a periphloemic sclerenchyma arc, with polygonal, cellulosic cells. The external phloem forms small islands separated by parenchyma cells. The wood has vessels displayed as radial rows separated by cellulosic, woody parenchyma (Figure 7).

The leaf is amphistomatic, a normal characteristic of the genus (KEATING, 1982), with stomata prominent on the surface (Figure 9). The mesophyll is differentiated in a bilayered palisade tissue and approximately 5 layered spongy tissue with cells containing calcium oxalate raphides (Figure 8). The dorsiventral structure, rounded margins, differentiated mesophyll and the presence of adaxial phloem are considered features of a primitive state, the broad arc of midvein a transitional feature, while the presence of stomata on the adaxial part also, the unicellular trichomes, and the double layered palisade are features of a more advanced state of evolution (KEATING, 1982).

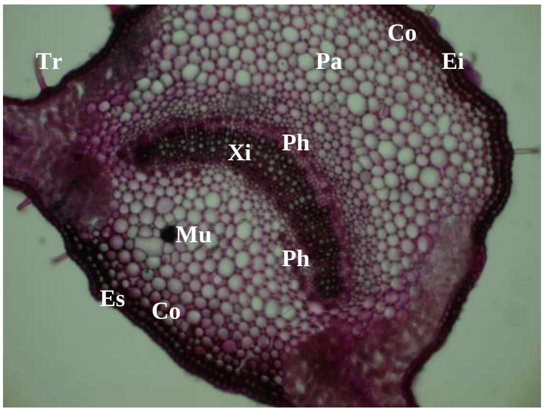

Figure 7. Median vein of the lamina. Es- upper epidermis, Ei- lower epidermis, Co- collenchyma, Pa- parenchyma, Tr- trichome, Xi- xylem, Ph- phloem, Mu- mucilage (x 50).

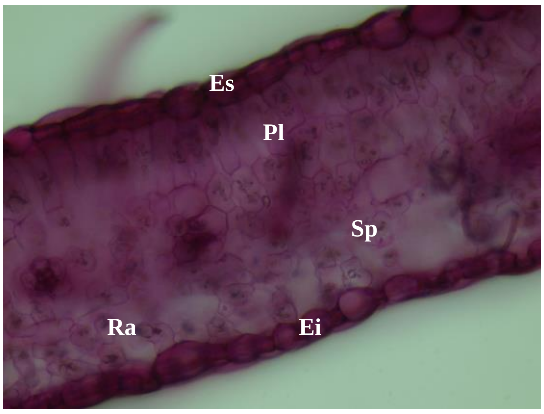

Figure 8. The mesophyll and epiderma. Numerous cells containing calcium oxalate raphides. Es- upper epidermis, Ei- lower epidermis, Pl- palisade, Sp- spongy tissue, Ra- raphides (x100).

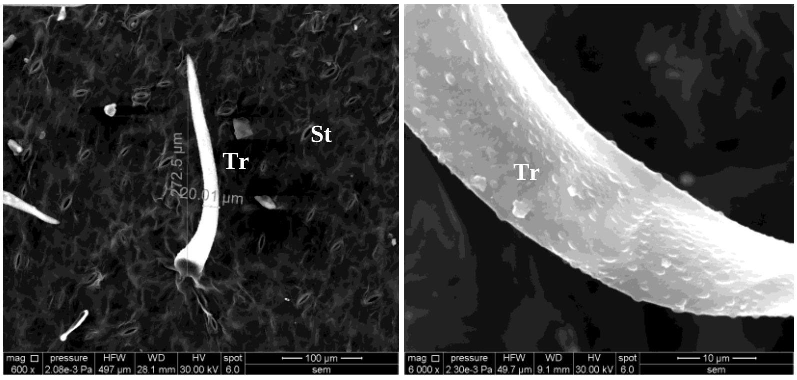

Figure 9. Foliar limb trichome and stomata. Lower epidermis aspect (left). Detail of adaxial trichome surface (right). Tr- trichome, St- stomata, Gh- glandular hair.

CONCLUSIONS

There have been observed aspects characteristic to the *Onagraceae* such as the abundance of calcium oxalate raphides in various types of cells in all vegetative organs, starch deposits, and multiseriate rays. We made observations of internal phloem, or interxylary phloem in this species, a feature that has evolved in some species of *Onagraceae* and has implications in its relation to other Myrthalean families and the photosynthate translocation within the wood. As well as some features that have been previously reported for other species of the genus, such as the presence of thin-walled trichomes with irregular protuberances on their surface, on stem and leaf.

The root and stem present secondary growth and some differences between the lower and upper parts of the organs regarding the number of cells, their size, and dispositions. The stem and leaf have unicellular trichomes, some long and perpendicular to the surface, and some short and bent parallel to the surface. Both the stem and leaf have prominent stomata. Cavities, which appear to be mucilage cavities are present in the root cortex, but additional investigations are necessary. The leaf has additionally rare, short, secretory hairs. Characteristic deposits that appear dark in microscopic samples have been observed in ray cells and vascular bundles and may represent storage locations of secondary metabolites.

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