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PASSIFLORA CAERULEA L. TREATED WITH *TRICHODERMA* PLANT BIOSTIMULANTS CONSORTIUM. MORPHO-ANATOMICAL CONSIDERATIONS

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Abstract: Plant biostimulants are an emerging category of inputs into technologies for plant cultivation, which activate plant metabolism and nutrient use efficiency. A microbial plant biostimulants consortium was applied on *Passiflora caerulea* L., a medicinal and nutraceutical plant grown in greenhouse conditions. The treatments were applied during *P. caerulea* vegetation, as a foliar treatment with a *Trichoderma* consortium suspension of 10^8 cfu/ml, equiv. to 10^{13} spores/ha. The treatment determined significant quantitative changes on morpho-anatomical features, on the leaf lamina (lamina with 10-20% thicker, palisadic cells with 10-20% longer, larger stomata and stomatal index increased by 15%), on the leaf petiole (the diameter ~30% larger, conducting bundles, 20% more developed, the adaxial conducting bundles, ~30% increase) and on the stem (the diameter with 15-20% larger, central cylinder with 15-20% bigger, xylem vessels of more than 50 µm diameter, with 20% more present). These morpho-anatomical features demonstrate the plant biostimulants effects of *Trichoderma* consortium. The results presented here sustain with morpho-anatomical data the accumulation the bioactive compounds, mainly polyphenols and flavonoids with an increased antioxidant activity, which we already reported. Larger stems and leaves of *P. caerulea*, allow accumulation at a higher level of bioactives compounds.

Key words: *Passiflora caerulea*, nutraceutical crop, plant biostimulants *Trichoderma* consortium, structural effects, stems and leaves.

Introduction

The present scientific work is referring to *Passiflora caerulea* L. plants, which have been treated during the vegetation period, with a suspension of *Trichoderma*-plant biostimulants consortium, consisting of two strains applied together. This multifunctional consortium was demonstrated to stimulate the early stage of plant development and to promote the synthesis and accumulation of biologically active compounds [RĂUȚ & al. 2014, 2015; ȘESAN & al. 2018].

The morpho-anatomical researches carried out on the *P. caerulea* plants, treated with *Trichoderma*, aim to identify the biostimulants effects of the treatment on the leaves and stems development, reported in our previous paper [ȘESAN & al. 2018, *in press*], emphasizing on the development of the conducting tissues and the leaf structures, involved in photosynthesis. To the best of our knowledge such approaches haven't been yet performed

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till now and represent the scientific novelty of this work, both at national and international level of plant morpho-anatomical domain.

In this respect, two sets of data were used in this work: (i) the data of the *Passiflora caerulea* untreated plants, published in 2016 in the scientific approach "*Passiflora* spp. – new nutraceutical crop in Romania" [ȘESAN & al. 2016; SAVIN & al. 2016] and considered the control plant and ii) the data of *P. caerulea* plants treated with *Trichoderma* consortium bioproduct. It has to be mentioned here that both experimental treatments, untreated plants (control) and plants treated with suspension of *Trichoderma* consortium, have been carried during the 2015 vegetation period and the anatomical investigations have been performed in the laboratory during 2015-2017.

Material and methods

Biological material consisted in leaves and stems of *Passiflora caerulea* plants, treated on the early vegetation period, with a foliar suspension of *Trichoderma* (10^8µfc/ml), in the watering standard of 200 l/ha [RĂUȚ & al. 2014, 2015; ȘESAN & al. 2018].

The *Trichoderma* consortium contained in two plant biostimulants strains of *Trichoderma*, both deposited on the National Collection of Agricultural and Industrial Microorganisms (NCAIM), Budapest, Hungary, *T. asperellum* T36b, NCAIM F 001434 and *T. harzianum*, Td50b, NCAIM F001412 [RĂUȚ & al. 2014, 2015]. Preparation of *Trichoderma* consortium suspensions for the foliar treatments: The two strains of *Trichoderma* have been cultivated in Petri plates on the solid PDA medium, incubated for 5 days at 28 °C, spores having been collected in sterile distillate water. For each *Trichoderma* isolates spores having been counted at the value 10^8 cfu. The mixing rate of two isolates for obtaining *Trichoderma* consortium was 1:1.

The samples for anatomical analyses were collected on 23 of June 2015, from the Hofigal experimental field and preserved in 70% ethylic alcohol.

For histo-anatomical evaluation, the usual investigation and evaluation methods used in plant anatomy have been applied [ȘERBĂNESCU-JITARIU & al. 1983; SÂRBU & al. 2014; ȘESAN & al. 2016].

The *Passiflora* leaves and stems have been cross cut (anatomical knife), in the median zone of lamina segments, petiole and stems internodes from the median zone of the stems. Sections have been processed in accordance with the standard stages of the double staining technique [ȘERBĂNESCU-JITARIU & al. 1983]. Two differential and successive colorants have been used: Iodine green and Carmin Alum. To highlight the starch, IIK has been used. Paradermal sections were prepared, in order to observe the characteristics of the epidermis and the lacunose parenchyma cells, in apical view.

The microscopic analysis of the slides has been performed in normal and polarized lights (crystals study), with an optical microscope, DOCUVAL type. Microphotographs have been carried out, on the same microscope, using a Nikon D90 digital camera.

Results and discussions

Histo-anatomical evaluation

Leaves (PLATE I – II, Figures 1-9, Table 1)

Lamina. From the morphological point of view, the *Passiflora caerulea* lamina is simple, palmat-partite, with 5 unequally lobes (segments), ovat-lanceolate with acute apex, serrate border and penat nervation. The lamina segments are approximately 230-260 µm

width, hypostomatic and develop a dorsi-ventral structure. Tector or secretor trichoms were not observed on the level of the lamina epidermis (Figure 1, 2).

Median nervure of the leaf segment (500-550 µm width), has an adaxial part approximately flat and an abaxial one, with a proeminent semi-circular shape (Figure 1).

Epidermis presents proper epidermal cells, of a heterodiametric form (20-30 µm width x 60-80 µm length), with extern tangential wall thickened (~3-4 µm thickness) and covered with a cuticle of about 1-3 µm thickness (Figure 3, 5). At the level of the median nervure the epidermal cells are smaller and approximately isodiametric. Abaxial epidermis presents also stomata cells and stomatal annexes cells (Figure 4, 5).

Stomata (18 µm width x 24 µm length) are as anomocitic and anisocitic types and are present only at the level of abaxial epidermis (460- stomata / 1 mm^2).

Conducting tissues are organised in conducting bundles of collateral type: 1 – large bundle, disposed in the compact ground parenchyma of the median nervure of the leaf segment and 6-8 small bundles, located in the secondary nervures, at the mesophyll level (Figure 1). In all the conducting bundles, the xylem belt is adaxially oriented.

Mesophyll is differentiated in two zones: palisadic tissue, adaxially located and consisting in 1 – layer of vertically elongated assimilatory cells (90-120 µm length) and lacunose parenchyma, abaxially located and composed of spherical, slightly elongated cells and groups of more elongated cells (Figure 2, 4). Gaps of different sizes are present between the cells of the lacunose parenchyma.

Mechanical tissue consists in an angular collenchyma, observed / detected only in the median nervure of the leaf segment, where forms, 3-4 layers adaxially and 1-2 layers abaxially (Figure 1).

Calcium oxalate druses (15-25 µm diameter) are frequent in the structures of the lamina, where are arranged in rows, along the nervures (Figure 6).

Petiole (2.7 mm diameter) has a cylindrical form, is relatively circular in sectional view (Figure 7) and presents an adaxial shallow groove only in its lower zone. The petiole structure is of mono-symmetrical type, with separated conducting bundles.

Epidermis presents small cells, with an isodiametric shape in cross section, and extern tangential wall slightly thickened. Stomata are present, but tector or secretor trichoms missing (Figure 7, 8).

Mechanical tissue is represented by 3-4 subepidermal layers of angular collenchyma, with a discontinuous disposition (Figure 8).

Ground tissue consists of a lax parenchyma, composed of spherical and isodiametric cells, with thin walls (Figure 8).

Conducting tissues are organised in 10-12 conducting bundles of opened collateral type (vascular cambium is present) of different sizes: an adaxial large bundle (550 µm width x 700 µm length) and 10-12 smaller bundles (Figure 7, 9). Each of the conducting bundles has amilifera sheath (Figure 9).

Calcium oxalate druses are frequent in the petiole structure, being located both in the phloem parenchyma and ground parenchyma.

Stem (PLATE III, Figures 10-11, Table 2) has a cylindrical shape, irregular-ribbed, of about 2.8-3.3 mm diameter and developed a secondary structure (Figure 10).

Epidermis consists of approximately isodiametric cells in cross section, with slightly thickness walls, covered with a cuticle of 4-5 µm thickness.

Cortex is differentiated in an extern subepidermal zone, consisting in an angular collenchyma and an intern zone, represented by a lax parenchyma. The angular collenchyma it is more developed (3-4 layers) at the level of ribs. The last layer of the cortex is represented by the amilifera sheath (Figure 11).

Central cylinder is voluminous (2.5-3.0 mm diameter) and has in the center a large pity cavity (1.0-1.2 mm diameter), formed by the disorganization of the central zone of the ground parenchyma. From the activity of the vascular cambium are formed the secondary conducting tissues. These are organized in two rings: an outer ring of secondary phloem and an inner ring of secondary xylem (xylem vessels up to 150 µm diameter). The primary xylem is located in the inner part of the secondary xylem ring, near the pith cavity and the primary phloem at the periphery of the secondary phloem ring. Packs of sclerenchymatic periphloemic fibres of different sizes, accompany the phloem (Figure 11).

Calcium oxalate druses are abundant in the structure of the stem and are located in different types of cells as epidermal, cortical, phloemic parenchyma cells.

Storage cells (PLATE IV – Figures 12-14).

Possible storage cells for phytochemical products were identified in the lamina of *Passiflora caerulea*. These are some of the cells of the lacunose parenchyma and the adjacent cells of the stoma (Figure 12, 13, 14).

Comparative aspects

The comparative analyses of the morpho-anatomical data obtained from the two types of *Passiflora caerulea* plants, untreated plant (control plants – ŞESAN & al. 2016) and treated plants with *Trichoderma*, have allowed the demonstration of the biostimulatory effects of the treatment on the development of the leafs and the stems.

Some quantitative changes were observed in treated plants as compared to untreated plants (Tables 1-3).

Table 1. Analyzed *Passiflora caerulea* leaf parameters (control and treated with *Trichoderma* consortium bioproduct)

Leaf parameters	Measurements / size	
	Control (ŞESAN et al. 2016)	Treated with *Trichoderma* consortium bioproduct
Median nervure of leaf lobe	300-350 µm thickness	500-550 µm thickness
Leaf lamina	180-220 µm thickness	230-260 µm thickness
Cells in adaxial (superior) epidermis	20-30 µm width x 60-70 µm length	20-30 µm width x 60-80 µm length
Cells in abaxial (inferior) epidermis	20-40 µm width x 40-80 µm length	20-30 µm width x 40-80 µm length
Epidermis – extern wall	3.5 µm thickness	3.4 µm thickness
Cuticle	1.2 µm	1.3 µm
Stomata dimensions	10 µm width x 22 µm length	18 µm width x 24 µm length
Stomatal index	390 stomata/mm^2	460 stomata/mm^2
Palisadic cells dimensions	9-10 µm width 60-80 µm length	15-16 µm width 90-120 µm length
Median nervure bundle dimensions	150-160 µm width 170-180 µm length	200-230 µm width 220-240 µm length
Petiole diameter	1.5 mm (1.4-1.7 mm)	2.7 mm (2.5-3.0 mm)
Petiole bundle	8-9	10-12

Petiole adaxial bundle	250 µm width x 300 µm length	550 µm width x 700 µm length
Druses size	15-20 µm diameter	15-25 µm diameter

Table 2. Analyzed *Passiflora caerulea* stem parameters (control and treated with *Trichoderma* consortium bioproduct)

Stem parameters	Measurements / size	
	Control (ŞESAN et al. 2016)	Treated with *Trichoderma* consortium bioproduct
Stem diameter	2.4-3.0 mm	2.8-3.3 mm
Diameter of central cylinder	2.0-2.2 mm	2.5-3.0 mm
Diameter of pity cavity	0.8-0.9 mm	1.0-1.2 mm
Xylem vessels diameter	10-100 µm	10-150 µm
Number of xylem vessels larger as 50 µm	25	34
Epidermis cells cuticle	6-7 µm thickness	4-5 µm thickness

Table 3. Quantitative changes observed in the *Passiflora caerulea* plants treated with *Trichoderma* consortium bioproduct as compared to control plants

The organ	Quantitative changes of treated plants with *Trichoderma* consortium bioproduct
The leaf lamina	- Lamina with 10-20% thicker - Palisadic cells with 10-20% longer - Larger stomata - Stomatal index, 15% increase
The leaf petiole	- The diameter with ~30% larger - Conducting bundles with 20% more developed - The adaxial conducting bundles with ~30% bigger
The stem	- The diameter with 15-20% larger - Central cylinder 15-20% bigger - Xylem vessels of more than 50 µm diameter, 20% more developed

The data from tables 1-3, revealed that: (i) the mesophyll is more voluminous, palisadic cells and stomata are larger and the stomatic index is higher; (ii) the petiole has a larger diameter, has more conductive tissue and higher bundles and (iii) the stem is thick, and the conducting tissue from its secondary structure is better represented.

Morpho-anatomical data are important for the autentification of these features in different plants as *Passiflora caerulea* [VANDERPLANK, 2000; ULMAN & MacDOUGAL, 2004; ŞESAN & al. 2016], *Panax quinquefolius* [LI & al. 2014], *Buttia* [SANT'ANA-SANTOS & al. 2018] a.o.

Conclusions

Basic morpho-anatomical features of *Passiflora caerulea* plants, treated with *Trichoderma*, have not changed, compared with the untreated plants. In this respect, the following structural aspects are common both types of plants: (i) the leaf lamina has a dorsi-ventral structure, is hipostomatal and with lack of trichoms; (ii) the leaf petiole has a mono-symmetrical structure, with distinct conducting bundles of open collateral type; (iii) the stem has a circular outline, irregular ribbed and a secondary structure, with concentric rings of secondary xylem and phloem; (iv) the mechanical tissue consists of collenchyma in leaf and of collenchyma and sclerenchyma in stem; (v) calcium oxalate crystals of different size are present in both studied organs.

The treatment determined significant quantitative changes on morpho-anatomical features, on the leaf lamina (lamina with 10-20% thicker, palisadic cells with 10-20% longer, larger stomata and stomatal index increased by 15%), on the leaf petiole (the diameter ~30% larger, conducting bundles, 20% more developed, the adaxial conducting bundles, ~30% increase) and on the stem (the diameter with 15-20% larger, central cylinder with 15-20% bigger, xylem vessels of more than 50 µm diameter, with 20% more present). These morpho-anatomical features demonstrate the plant biostimulants effects of *Trichoderma* consortium.

Storage of phytochemical products is related to stomatal annexes cells and lacunose parenchyma cells from the leaf.

Morpho-anatomical data are important for the autentification of these features in different plants as *Passiflora caerulea* [VANDERPLANK, 2000; ULMAN & MacDOUGAL, 2004; ȘESAN & al. 2016], *Panax quinquefolius* [LI & al. 2014], *Buttia* [SANT'ANA-SANTOS & al. 2018] a.o.

To the best of our knowledge such approaches haven't been yet performed till now and represent the scientific novelty of this work, both at national and international level of plant morpho-anatomical domain.

Notes on contributors

Tatiana Eugenia ȘESAN – coordinated and monitorized the project 160/2014, performed designed experiments, collected samples from experimental field, gave general interpretation of the data, participate to the writing and finishing the manuscript. Anca SÂRBU and Monica Anca PARASCHIV - performed section of vegetal material and microscopic analysis of the slides, providing anatomical data, their interpretation in connection with the applied treatment in *Passiflora* crop, all the photos of optical microscopy, drafting and translation of the scientific work. Florin OANCEA - contributed and supervised the finalization of the manuscript.

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PLATE I

***Passiflora caerulea* – LAMINA**

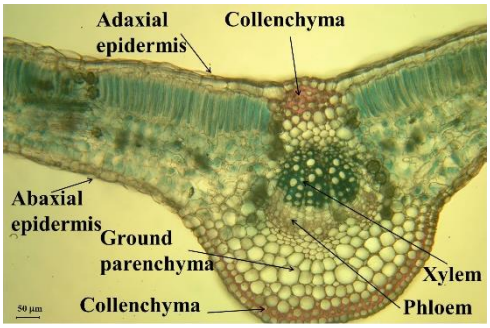
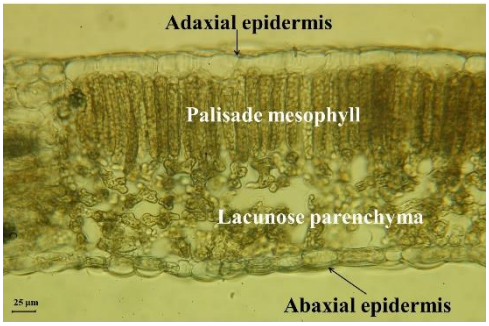


Figure 1



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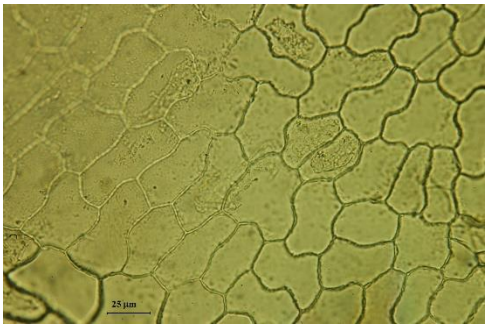

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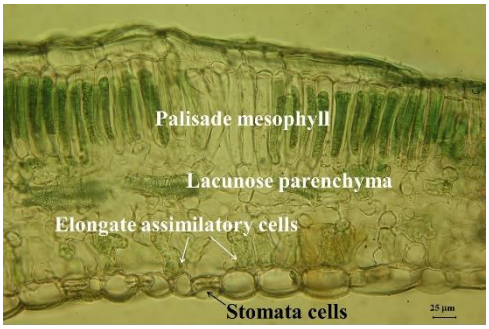

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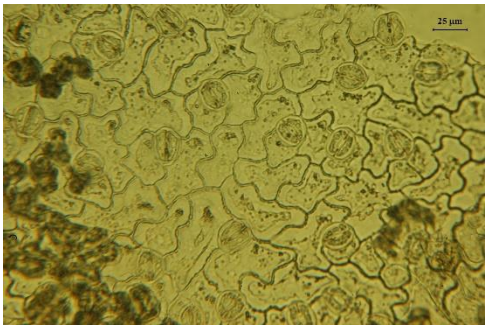

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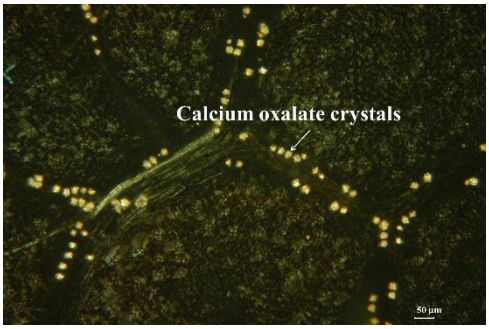
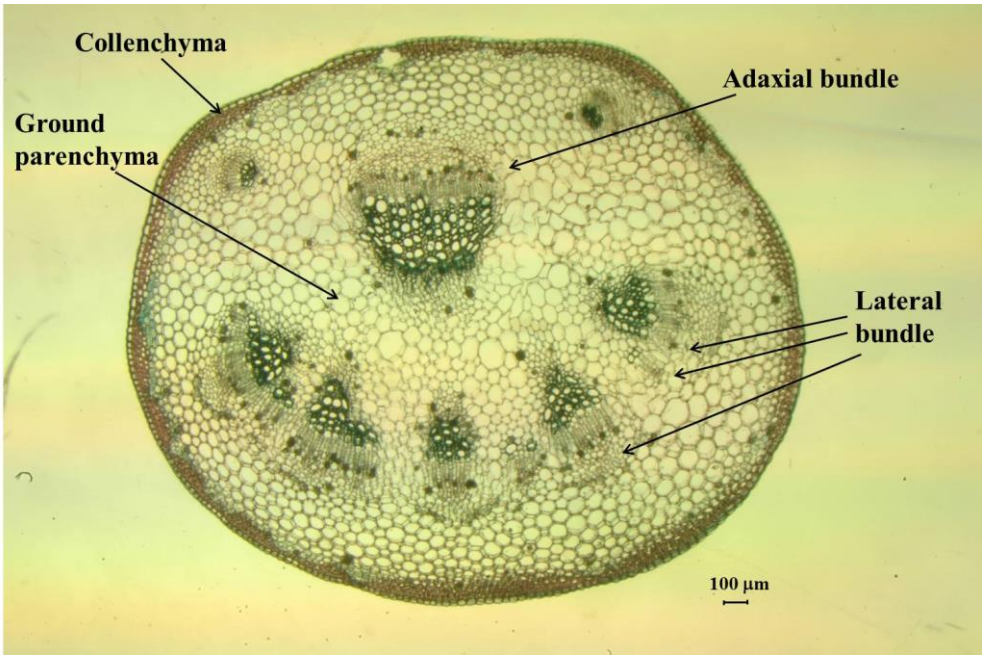


Figure 6

PLATE II

***Passiflora caerulea* – PETIOLE**



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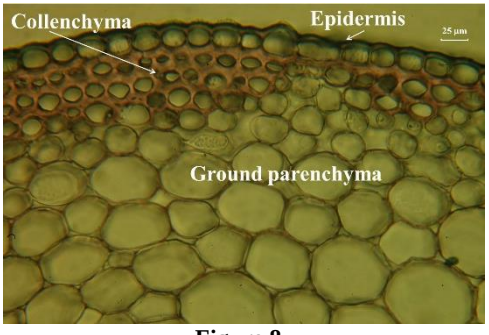

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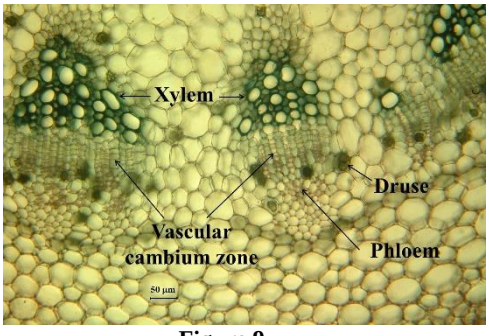

Figure 9

PLATE III

***Passiflora caerulea* – STEM**

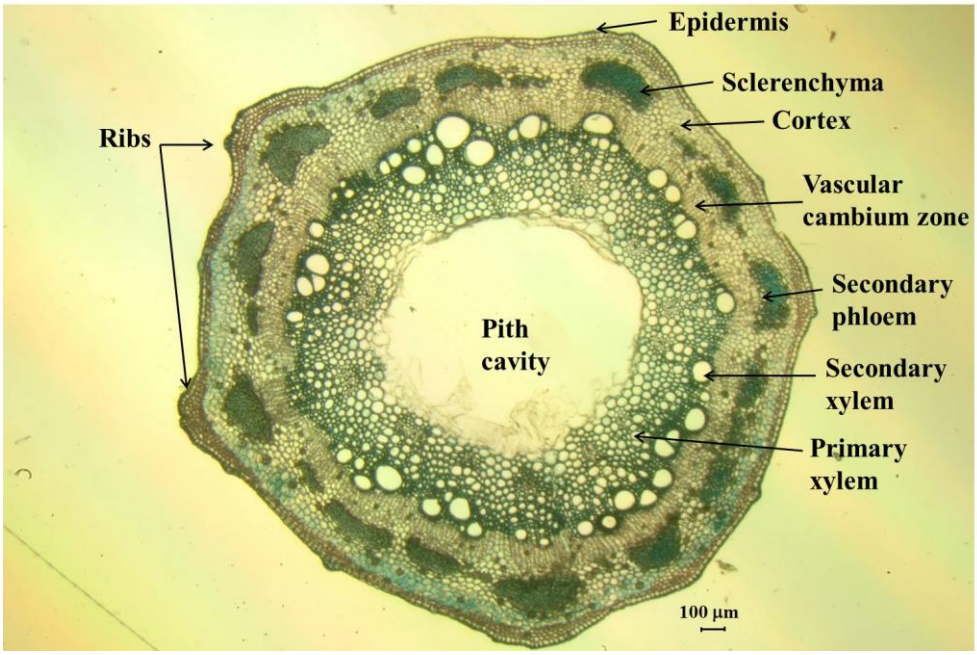

Figure 10

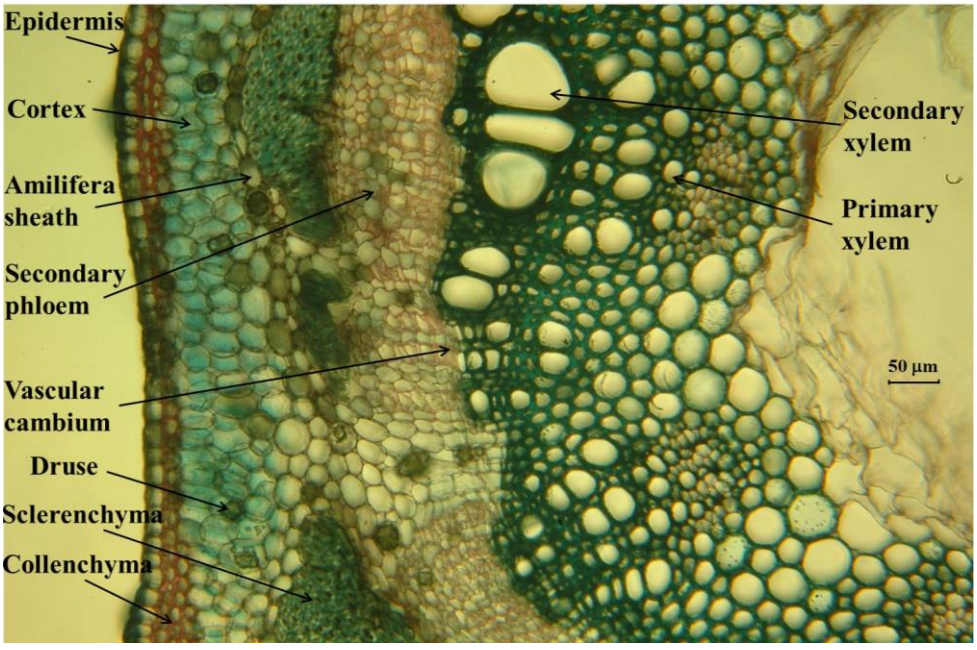

Figure 11

PLATE IV
***Passiflora caerulea* – STORAGE CELLS**

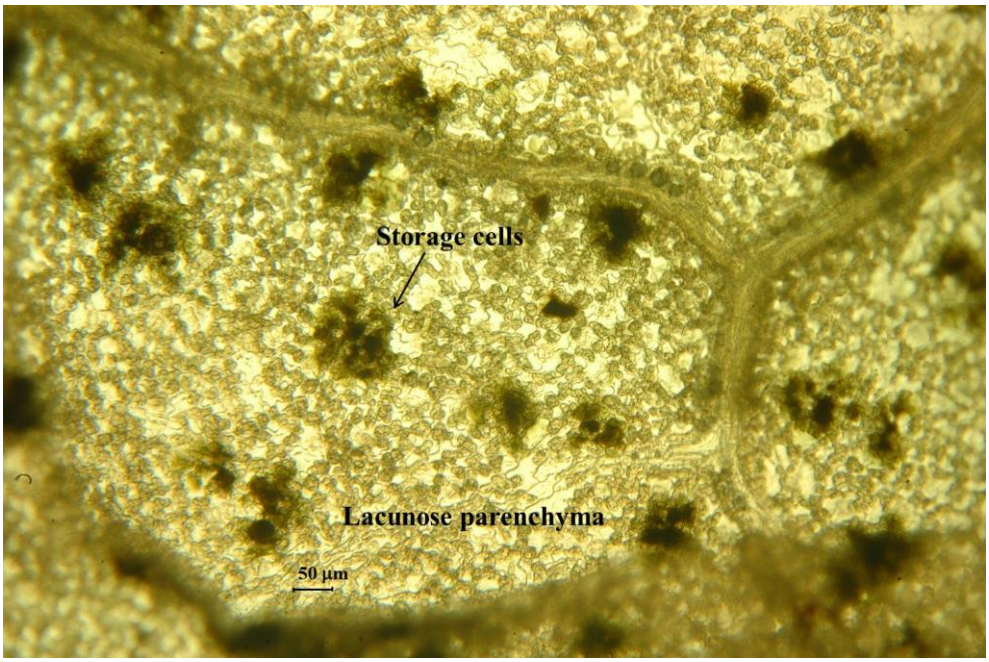

Figure 12

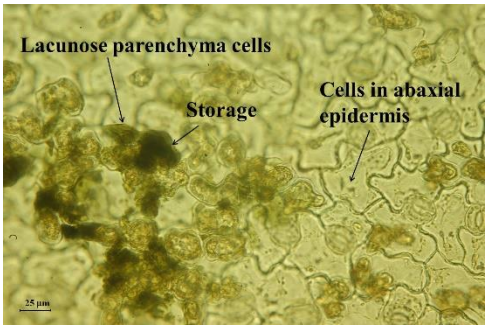

Figure 13

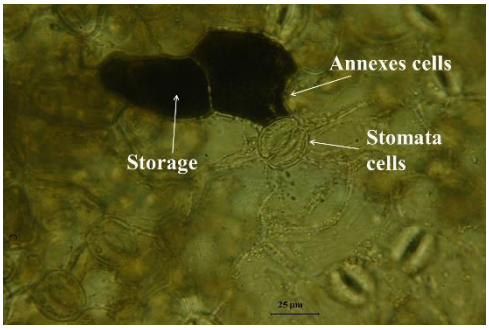

Figure 14

Explanation of plates and figures

PLATE I (photo: Anca Sârbu) – *Passiflora caerulea* – **LAMINA**
- Cross section through median zone of the leaf segment, with evidence of the median nervure, colorants Iodine green and Carmin Alum (Figure 1)
- Cross section through the median zone of leaf segment, with evidence of epidermis and mesophyll, colorants Iodine green and Carmin Alum (Figure 2)
- Adaxial epidermis (superior) in apical view (Figure 3)
- Cross section through the median zone of leaf segment with evidence of epidermis and assimilatory cells, colorants Iodine green and Carmin Alum (Figure 4)
- Abaxial epidermis (inferior) in apical view (Figure 5)
- Paradermal section through the leaf segment, with evidence of lacunose parenchyma, abaxial epidermis and calcium oxalate crystals in apical view, using polarized lights (Figure 6).

PLATE II (photo: Anca Sârbu) – *Passiflora caerulea* – **PETIOLE**
- Cross section through the petiole, colorants Iodine green and Carmin Alum (Figure 7)
- Cross section through the petiole, with evidence of epidermis, collenchyma and ground parenchyma, colorants Iodine green and Carmin Alum (Figure 8)
- Cross section through the petiole, with evidence of the lateral bundles, colorants Iodine green and Carmin Alum (Figure 9)

PLATE III (photo: Anca Sârbu) – *Passiflora caerulea* – **STEM**
- Cross section through the median zone of the stem, colorants Iodine green and Carmin Alum (Figure 10)
- Cross section through the median zone of the stem, with evidence of epidermis, cortex and of central cylinder elements, colorants Iodine green and Carmin Alum and IIK (Figure 11)

PLATE IV (photo: Anca Sârbu) – *Passiflora caerulea* – **STORAGE CELLS**
- Paradermal section through the leaf segment, with evidence of lacunose parenchyma and storage of phytochemicals products (Figure 12)
- Paradermal section through the leaf segment, with evidence of phytochemicals products accumulation in the cells (Figure 13)
- Paradermal section through the leaf segment, with evidence of the abaxial epidermis, the stomata cells and the accumulation of phytochemicals products in the adjacent cells (Figure 14)