BIODIVERSITAS
Volume 21, Number 11, November 2020
Pages: 5531-5539

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d211162

Morphoanatomy of three *Indigofera* species (Leguminosae-Papilionoideae) in Java, Indonesia

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Manuscript received: 14 July 2020. Revision accepted: 31 October 2020.

Abstract. *Fugarasti H, Muzzazinah, Ramli M. 2020. Morphoanatomy of three Indigofera species (Leguminosae-Papilionoideae) in Java Indonesia. Biodiversitas 21: 5531-5539.* This study aimed to explore the morphological and detailed anatomical features of the stems, leaves, and roots from three Indonesian *Indigofera* species**.** Morphological-anatomical studies of three Indonesian *Indigofera* species were carried out using embedded microscopic preparations. The anatomical characters of the specimens were observed using a compound optical microscope with magnification 40x, 100x, and 400x. The observation showed the anatomical cross-section of *I. tinctoria* stem was rectangular, *I. suffruticosa* was hexagonal, and *I. arrecta* was rounded. The tissue structures of *Indigofera* species, from the outside layer, were the epidermis, thin cortex, secondary phloem (narrow or wide), thick secondary xylem, and conspicuous pith in the middle. Whilst, the corner of *I. suffruticosa* stem contained thick collenchyma. The vascular bundles were the open collateral. The leaves of *Indigofera* species are made up of the upper epidermis, mesophyll (palisade parenchyma, spongy parenchyma), and the lower epidermis. The vascular bundles were located in the middle, with five or six segments of the xylem elements and small groups of phloem elements, all in the parallel lines. The primary stele type of the roots is actinostele, cambium activity pushed him aside. The vascular bundle of the roots consisted of a dense and tight secondary xylem composed of thick-walled circular vessels (mostly tightly arranged). Data about the morphoanatomy structure of three Indonesian *Indigofera* species could complement the novelty of the morpho-anatomy information records obtained by previous researchers.

Keywords: Anatomy, *Indigofera*, Indonesia, morphology

INTRODUCTION

Indigofera is a genus member of the family Leguminosae-Papilionoideae and is highly diverse, with 700-750 species, and ranked third as the most diverse genus among Leguminosae (Tamilselvi et al. 2011). There are commonly found in various tropical and subtropical regions (Schrire et al. 2009; Soladoye et al. 2010; Zhao and Gao 2015), are annuals or perennials, and habit varies (Zhao et al. 2016). They can be found as low-growing herbs, shrubs, or small trees up to 1.5-3.0 m in height (Rzedowski and Grether 2018; Marquiafável et al. 2009), and with small flowers in axillary racemes (Adema 2011), compound leaves (imparipinnate), and cylindrical pods. This plant has great potency for economic and ecological purposes. It has a variety of uses, such as livestock fodder, ornamental plants, medicine, and textile dyes (Tamilselvi et al. 2011).

The highest *Indigofera* diversity is found in Africa and Madagascar (550 species), Asia (150 species), and Australia (50 species) (Schrire et al. 2009). Among species of Asia, 18 species have been found in Indonesia (de Kort and Thijsse 1984). The latest study describes well-known nine *Indigofera* species in Java and Madura, four species of which were used as sources of indigo dyes, i.e.: *Indigofera. arrecta, I. longiracemosa, I. suffruticosa*, and *I. tinctoria* (Muzzazinah et al. 2016). Indigo dye is famous for the natural blue colors obtained from the leaflets and branches (Soladoye et al. 2010). The dye which is among the most widely used natural dye in the world is obtained mainly from the leaves extraction through a process of fermentation (Leite et al. 2009; Pawade1 and Chinchamalatpure 2020). It is also found, that as the coloring agent for traditional textiles, *I. tinctoria* found in Java, Madura, and Flores showed different morphological characters that affected the hue and saturation of indigo color on the fabrics (Muzzazinah et al. 2018).

Some *Indigofera* species are strikingly similar morphologically, thus making it difficult to classify them if relying only on their morphological features. In this context, anatomical studies are related to the structure, contents, and development of cells and tissues (Tamilselvi et al. 2011). These studies can be used to identify and provide additional data for two or more taxa that have high morphological similarities. Moreover, Simpson (2006) stated that anatomical studies support other research in plant science, such as morphogenesis, physiology, ecology, taxonomy, evolution, genetics, and reproduction. Anatomical studies are also used in taxonomy and systematics to assist with species identification by placing the anomalous groups in the right taxonomic levels and show the patterns of relationships been observed in superficial convergence in morphological studies (Nwachukwu et al. 2016).

Information about the anatomy of *Indigofera*s is still uncommon worldwide (Lievens 1992; Nwachukwu and Mbagwu 2006; Nwachukwu and Mbagwu 2007; Tamilselvi et al. 2011; Paulino et al. 2011; Ghosh et al. 2016; Nwachukwu et al. 2017). There are no significant studies

on the anatomy of *Indigofera* species in Indonesia. Three species (*I. tinctoria, I. suffruticosa*, and *I. arrecta*) were selected in this research because of their availability. These three have potency as natural dyes, and *I. tinctoria* has been widely used to produce blue dyes for batik-a traditional clothes in Java and other traditional textiles of Madura and Flores (Muzzazinah et al. 2016).

The stem and leaf anatomy can uncover many characteristics of the species and proved to be important in classification (Lu et al. 2008) and has been widely used in taxonomic research and systematic studies (Noman et al. 2014). Tamilselvi et al. (2011) stated that anatomical systematics is aimed at structures related to vegetative organs (stems, leaves, and roots) for plant classification.

Considering their morphological similarities and the lack of comparative anatomy studies that have been conducted on Indonesian *Indigofera* species, this study aimed to explore the morphological and detailed anatomical features of the stems, leaves, and roots from three Indonesian *Indigofera* species, especially the Javanese *Indigofera.*

MATERIALS AND METHODS

Study area

The seeds of three *Indigofera* species (*I. tinctoria, I. arrecta, I. suffruticosa*) were prepared and planted in Klaten District, Central Java, Indonesia, which has a tropical climate. Planted at 130 mdpl (meter above sea level) used to grow the *Indigofera* samples. The above three planting species were treated similarly.

Procedures

Three *Indigofera* species (*I. tinctoria, I. suffruticosa*, and *I. arrecta*) were germinated from seed, and grown until mature fruit had formed. Thirty (30) *Indigofera* plants were used in this experiment. Samples were taken from 4 sides: north, south, west, and east, then randomized the samples. Root, stem, and leaf samples of each species were harvested from live plants. Stem samples were taken about 30cm from the tip of the flowering plant, leaf samples were taken from twigs between 2-5 numbers of the plant, while the root samples used were taken from plant roots that were around 2-3 months old, and taken to the Central Laboratory Unit of Sebelas Maret University (UNS), Surakarta, Indonesia to observe their anatomical structure. Observations on the anatomical structures were preceded by the production of embedded microscopic preparations using the Paraffin method adopted from Johansen (1940).

The cleaned specimens were cut transversely to the length of 2 cm. The specimens were submerged into flacons containing FAA solution (1:1:18 formaldehyde 70%, glacial acetic acid 40%, 70% alcohol) for 24 hours. Then the specimens were rinsed with a series of alcohols (70%, 80%, 95%, and 100%) for 30 minutes each, then dealcoholized using a series of alcohol/xylol solution (3:1, 1:1, and 1:3), pure xylol I, and pure xylol II, each for 30 minutes. The pure xylol solution was replaced with a mixture of xylol/paraffin (1:9) and heated to 57 °C for 24 hours. Next, the xylol/paraffin mixture (1:9) was replaced with pure paraffin and heated to 57 °C for 24 hours. Pure paraffin was replaced with new paraffin and soaked for an hour. Specimens were inserted into the paraffin and hardened into blocks. The hardened paraffin blocks were sliced using a rotary microtome with 8-12 micrometers thickness. The sliced paraffin ribbons were glued to the object-glass smeared with a mixture of glycerine and albumin. The object-glass was placed on a hot plate and heated to 45 °C until the paraffin tape was stretched.

Staining was done by dipping the object-glass into the succession of solutions safranin 1% in 70% alcohol (24 hours), and fast green 1% in ethanol 95% (30 seconds). The last step was mounting in the deg-glass with Canada balsam (Johansen 1940). The dyed specimen was then labeled and observed with a compound optical microscope.

Data analysis

The morphological and anatomical data were described, photographed, tabulated, and presented in table 1-4 and figure 1-5. The images were analyzed using *Image Raster 3.0*, including the thickness of epidermal tissue, cortex, palisade, sponge, phloem, xylem, and pith. All of these data were then descriptively analyzed.

RESULTS AND DISCUSSION

Indigofera species is a dicotyledonous plant. It has a taproot with white nodules on its roots and has fruit with the pod type which characterizes the Leguminosae. They can be found as herbs, shrubs, or trees with a height of approximately 1-4 meters (Figure 1). The stems of *I. arrecta* are erect (Figure 1C), while in *I. tinctoria*, and *I. suffruticosa* the branches are more spreading (Figures 1.A, 1.B.). *I. suffruticosa* has a greenish-colored stem when young Figure 1.B).

The leaves are oval, compound, alternating, having odd leaflet, with each leaflet sits in a line arranged into the odd-pinnate position. *I. tinctoria* leaves have a darker green hue (Figure 1A) than *I. suffruticosa* and *I. arrecta* (Figure 1B., 1C.). The lamina of *I. suffruticosa* leaves was thinner and wider (Figure 1B, 2B), while the leaf blade of *I. arrecta* was thicker and narrower (Figures 1.C, 2.C).

The flowers are complete, zygomorphic, irregular, bisexual, hypogynous, and pedicellate, sepals 5, gamosepalous in all the three species. The flowers were arranged in axillary racemes, and the sepals were five-jagged bell in shape. The petals are butterfly-shaped (Figure 2. D-F). They have pod-type fruit that is relatively thin- to thick-walled, ribbon-shaped, straight, or curved. The pods of *I. tinctoria* were straight, long, and slender, brown colored with a smooth surface, and slightly curved at the tip. *I. arrecta* has straight, short, and stubby pods with dark brown colored, whilst *I. suffruticosa* is the most different type, with short and strongly curved pods, deep brown colored with the densely hairy surface, dehiscent in the suture (Figure 2. B, G). The current morphological features on length and curvature of the pod were also similar to others (Khan et al. 2008; Sanjappa 1985).

Figure 1. Habit of: A. *Indigofera tinctoria*; B. *I. suffruticosa*; and C. *I. arrecta*

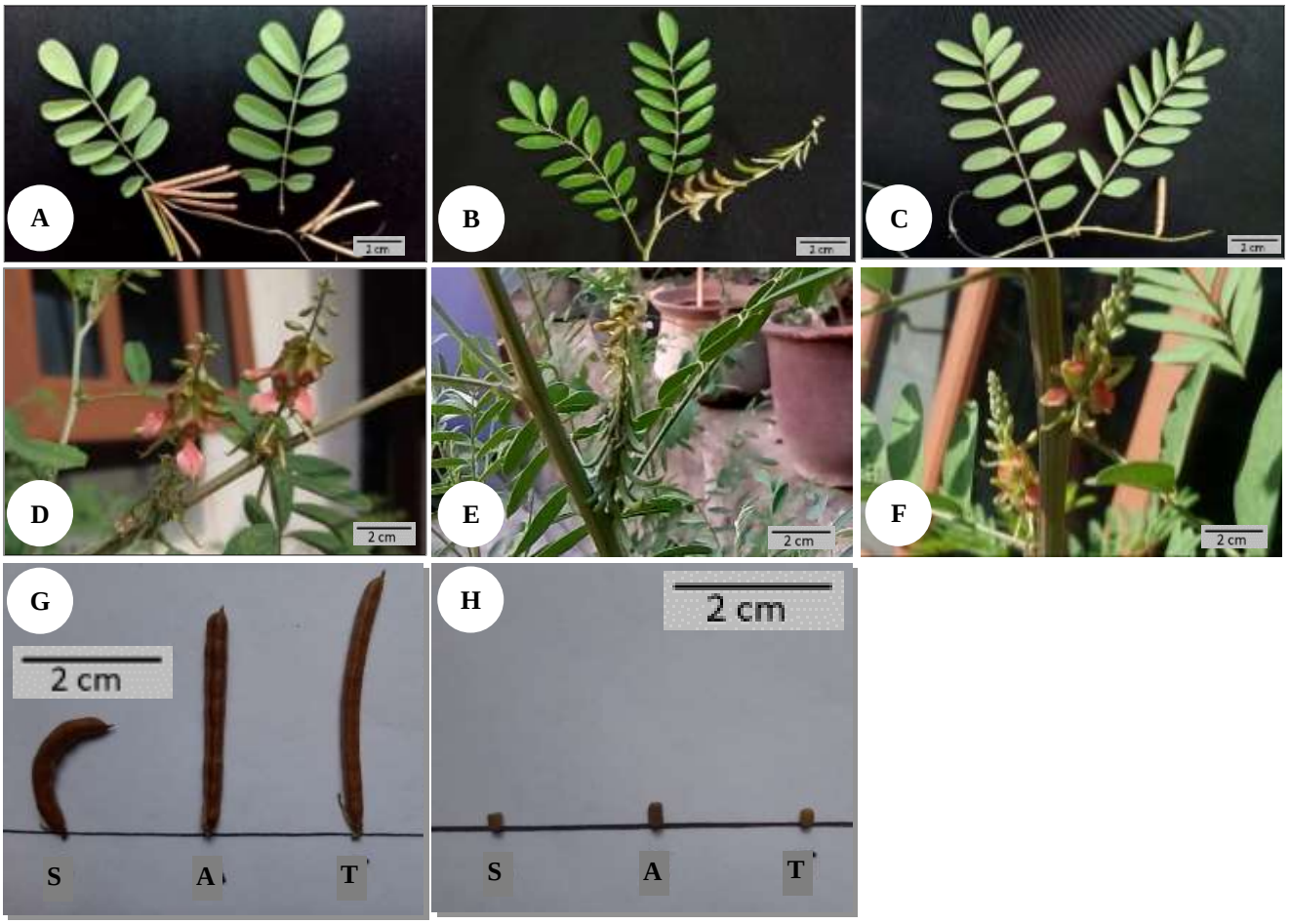

Figure 2. Leaves of: A. *Indigofera tinctoria*; B. *I. suffruticosa*; and C. *I. arrecta.* Flowers of: D. *I. tinctoria*; E. *I. suffruticosa*; and F. *I. arrecta*, G. pod, H. Seeds (S: suffruticosa, A: arrecta, T: tinctoria)

The pods varied 1-3.5 cm in length, with 3-11 round or oblong seeds (Figure 2. G-H), conspicuous hilum. The seeds color is oily/dark green in *I. tinctoria*, dark brown in *I. suffruticosa*, and black color in *I. arrecta* (Figure 2. H). The seeds are small around 1-3,5 mm, in shape cube, cuboid, or cylindrical. The color of the seeds is dark green to brownish-black. This is supported by research Teixeira and Correa (2007), which shows that *Indigofera* species. seeds are mostly rhomboid (rhomboid) but sometimes cube or cylindrical, bright green to brownish-black. The seed color is also used to distinguish species from other taxa investigated where there are more than one species for each color, including *I. arrecta*, and *I. tinctoria* (Al-Ghamdi 2011). The pod-type fruit and seed morphological characters helped distinguish various *Indigofera* species. Chauhan and Pandey (2014), said that pod morphology in *Indigofera* is variable, and the character has little diagnostic value at the generic level. The macroscopic characters are useful in the quick identification of plant material and also serve as an essential criterion (Aguoru and Okoli 2012)(Geetha et al. 2016).

Stem anatomy

The stem of the studied *Indigofera* species was composed of epidermis, a thin cortex (parenchyma and collenchyma), narrow or wide secondary phloem, thick secondary xylem, and prominent pith in the middle. *I. suffruticosa* has thick collenchyma at the corners of the trunk (Figure 3.B1, 3.B2, 3.B3). Secondary xylem consisted of 2-8 thin lines of vessel elements, each row was separated by a wide gap. The vessels are small to medium-sized around 14-40 μm, thin-walled, round, oval to elliptical (Figure 3.A3-D3).

The cross-sectional structures showed the variation between the three investigated species. The stem of *I. tinctoria* was square or rectangular (Figure 3. A1), *I. suffruticosa* was hexagonal (Figure 3.B1), while *I. arrecta* has a rounded stem (Figure 3.C1).

The stem epidermis was multiseriate, consisted of small thick-walled spindle-shaped cells (Nwachukwu et al. 2017). Under the epidermis, one or two layers of collenchyma were present, followed by several layers of parenchyma, each with a width of three or four cells. According to Nwachukwu et al. (2017), the hypodermal layer in *I. tinctoria* consisted of three to five layers of lignified cells. In *I. suffruticosa*, the corners of the stem contain thick collenchyma (Figure 3.B1-B3, Table 2), which distinguishes it markedly from other species. The anatomical appearance of the stem can be a distinguishing feature because there is marked variation in the thickness of the layers between different species (Aguoru and Okoli 2012).

Table 1. Morphological comparison of *Indigofera tinctoria, I. suffruticosa, I. arrecta*

Characters	***I. tinctoria***	***I. suffruticosa***	***I. arrecta***
Stem	Slender and branched	Slender, erect, and branched	Slender, erect, and branched
Color of steam	Deep green	Green	Green
Type of leaf	Imparipinnate	Imparipinnate	Imparipinnate
Number of leaf	5-11 leaflets per leaf	9-17 leaflets per leaf	5-15 leaflets per leaf
Shaped of leaf	Obovate	Obovate oblong	Obovate oblong
Color of leaf	Dark green	Light green	Dark green
Color of root	Brown	Brown	Brown
Flower	Axillary	Axillary	Axillary
Color of corola	Pink- orange	Pink-orange	Pink-orange
Shaped of pod	Straight, long, and slender	Short and strongly curved	Straight, short and stubby
Size of pod	2,2 -3,3 cm	1,1-1,4 cm	1,8 - 2,8cm
Number of seeds	6-11 seeds	3-8 seeds	4-7 seeds
Size of seed	1-1,5 mm	1,5 mm	1,5-3,5 mm
Shaped of seed	Cylindrical	4-angled or cube	4-angled or cuboid
Color of seed	Dark yellow - bright green - dark brown	Dark green - dark brown - brownish black	Reddish-brown- dark brown

Table 2. The stem characteristics and cell sizes in the *Indigofera* sp. stem tissues (μm)

Characters	***I. tinctoria***	***I. suffruticosa***	***I. arrecta***
Shapes	Rectangular	Hexagonal	Rounded
Epidermis	16.21	23.33	17.32
Cortex (collenchyma)	31.35	60.47, The corner: 134.62	35.59
Phloem	39.49	88.59; 65.27	54.14
Xylem	132.25	224.04	188.669
Pith	479.90	1020.52	864.754

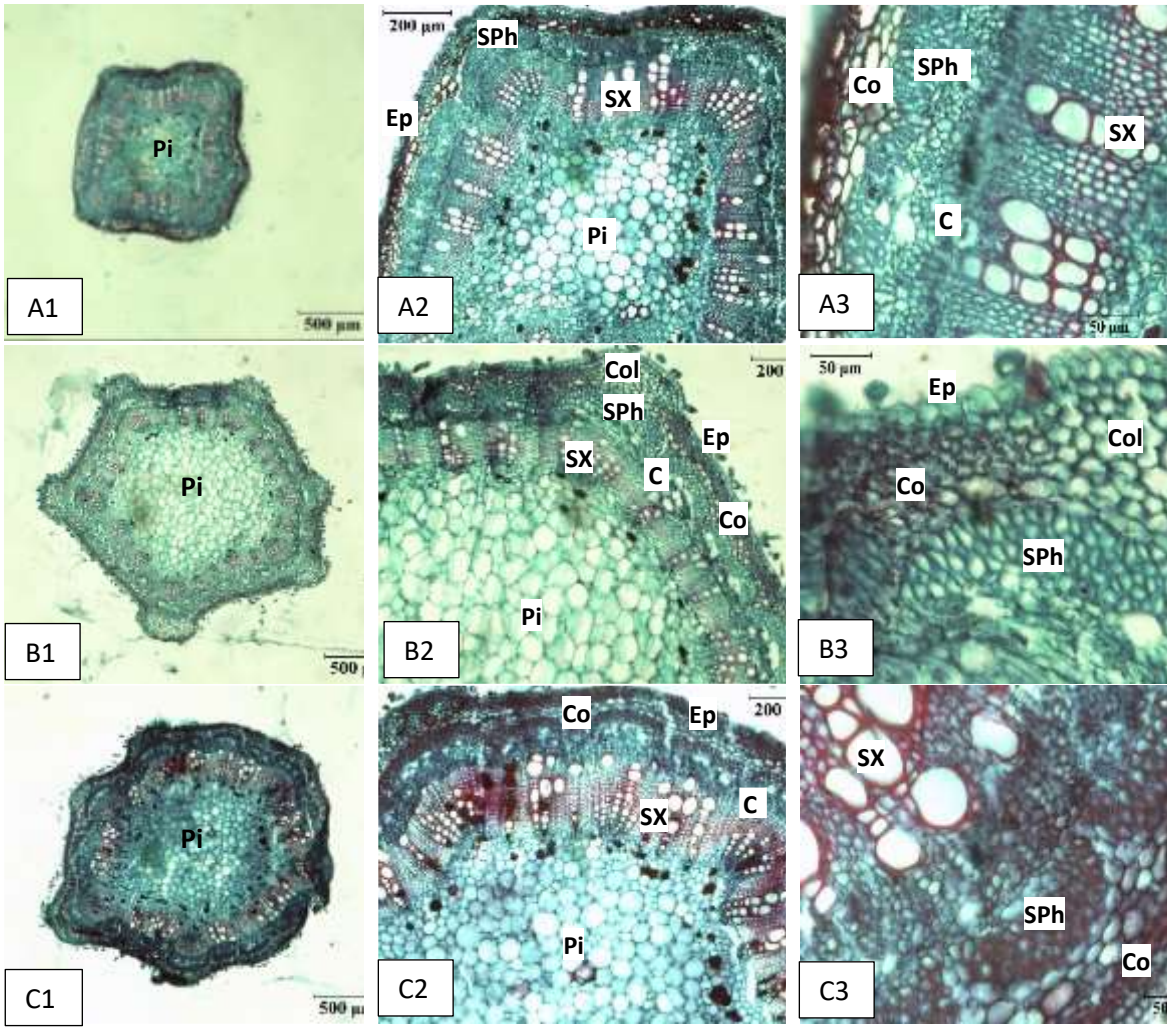

Figure 3. Stem anatomy of: A. *Indigofera tinctoria*; B. *I. suffruticosa*; and C. *I. arrecta* (A1-C1 Magnification: 40x), (A2-C2 M: 100x), (A3-C3 M: 400x). Ep: Epidermis; Co: Cortex; Col: Collenchyma; SPh: Secondary Phloem; SX: Secondary Xylem; Pi: Pith.

The vascular bundles in *Indigofera* species were arranged in open collateral. The vascular bundles consisted of the narrow outer part of the phloem and followed by a thick xylem cylinder. The phloem elements were arranged in short thin compact rows, while the xylem elements were composed of thick-walled xylem fibers. Xylem in *I. suffruticosa* was thickest compared to the three other species.

Leaf anatomy

The leaf anatomical structures of the three species were almost identical. The tissues were arranged from outside to inside with the following order: the upper epidermis, mesophyll (palisade parenchyma, spongy parenchyma), and lower epidermis. The *Indigofera* vascular bundle is in the middle. The vascular bundle consists of three or four parallel lines of xylem elements and a small group of phloem elements.

The leaf epidermis of the three *Indigofera* species consists of a single layer of cells in the shape of a cuboid, rectangular or hexagonal. There were no trichomes in all three species. Anatomical leaf epidermis is beneficial for systematics, although environmental factors sometimes influence it and generally, does not show high differences in species within the same genus (Dickison 2000).

Mesophyll was divided into adaxial zones composed of circular palisade cells, and abaxial zones composed of spongy parenchyma cells. Nwachukwu and Mbagwu (2006) also found *I. tinctoria* mesophyll has four to five layers of cells. The palisade cells were narrow, cylindrical, and neatly arranged into two rows. The spongy parenchyma cells were rounded and loosely arranged into three rows. In *I. tinctoria*, the thickest sponge parenchyma was 33.87 μms (Figure 4. A1; Table 3). *I. suffruticosa* has a denser palisade parenchymal arrangement (Figure 4.B1). And *I. arrecta* has an identical structure as *I. tinctoria*.

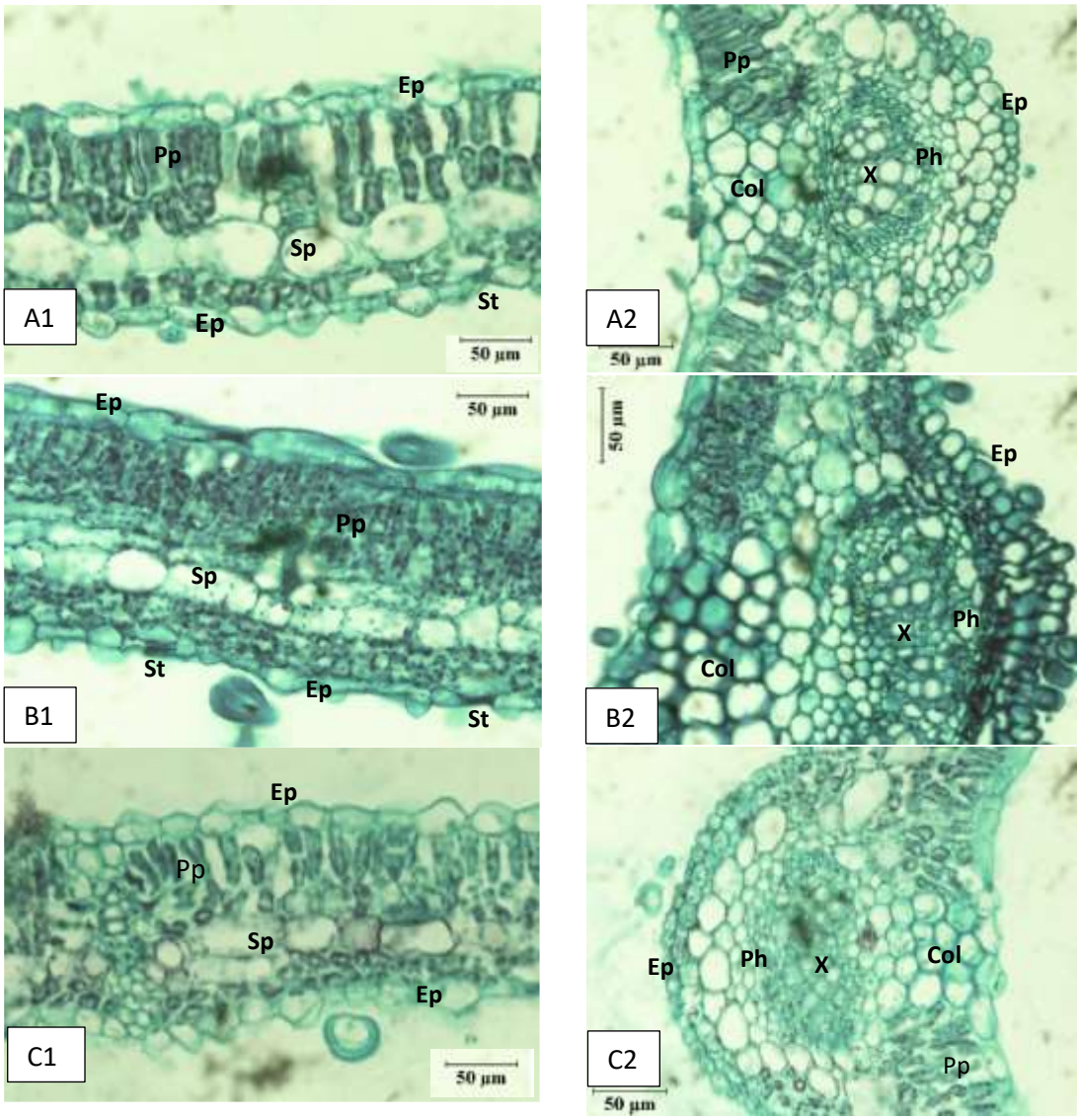

Figure 4. Leaf anatomy of: A. *Indigofera tinctoria*; B. *I. suffruticosa*; and C. *I. arrecta* (A1-C1 Cross-section of the leaf); (A2-C2 Cross-section of the leaf vein). Ep: Epidermis; Pp: Palisade Parenchyma; Col: Collenchyma; Sp: Sponge Parenchyma; X: Xylem; Ph: Phloem

Table 3. The characteristics of cells in the *Indigofera* sp. leaves tissues (µm)

Tissues	*I. tinctoria*	*I. suffruticosa*	*I. arrecta*
Upper epidermis	13.37	14.76	12.85
Palisade	42.48	50.78	32.43
Spongy mesophyll	33.87	33.68	29.55
Xylem	54.55	48.61	55.56
Phloem	17.85	33.09	20.42
Lower epidermis	11.15	11.12	10.36

Table 4. The characteristics of cells in the *Indigofera* sp. root tissues (µm)

Tissues	*I. tinctoria*	*I. suffruticosa*	*I. arrecta*
Epidermis	13.20	10.77	11.51
Cortex	81.25	99.52	86.44
Endodermis	64.78	38.67	51.39
Xylem	265.24	264.48	300.25
Phloem	Divergent	Divergent	Divergent

Indigofera has a bond of vessels consisting of xylem and phloem to form a collateral (Amphicribal) in the form of a small circle located in the middle of the leaf. The vascular bundle consists of five or six segments, with parallel lines of the xylem elements and small groups of phloem elements. The abaxial epidermis is composed of papillae cells. The arrangement of blood vessels of the three species is almost the same (Figures 4.A2-C2).

Root anatomy

The root anatomical structures of the three species were identical. The root structure showed the dense, very compact secondary xylem comprised by the thick-walled circular vessel (tightly arranged) in connective tissue (Figure 5).

The tissues were arranged from the outside-in the following order: epidermis, cortex (sclerenchyma, parenchyma), endodermis, phloem, and xylem. Periderm was present to substitute the damaged epidermis due to secondary growth. The periderm was wide with narrow gaps. Periderm was followed by sclerenchyma cells accompanied by thick parenchyma. The secondary phloem was wide and arranged continuously. The secondary xylem was tight and dense. The vascular bundle walls were thick and composed of sclerenchyma. Periderm was arranged in discontinued radial. The primary stele type is usually actinostele. Cambium activity pushed him aside. Radial-type vascular bundles were arranged into fan-shape with 1.5 mm xylem elements and nine radial bands in each xylem fiber. The xylem is further composed of small units with lignified broad and thick fibers. Lateral xylem was accompanied by small fibers around 20-60 μms.

The differences in the root anatomy structure of the three *Indigofera* species can be seen in the endodermic tissue. In *I. arrecta*, the endodermic tissue is hexagonal (Figure 5. C1), whereas in *I. tinctoria* and *I. suffruticosa* the endodermic tissue is spherical (Figure 5. A1, B1). The systematic value of root anatomical characters can also be seen in the contribution of (Nwachukwu et al. 2016) to the roots of several *Indigofera* species.

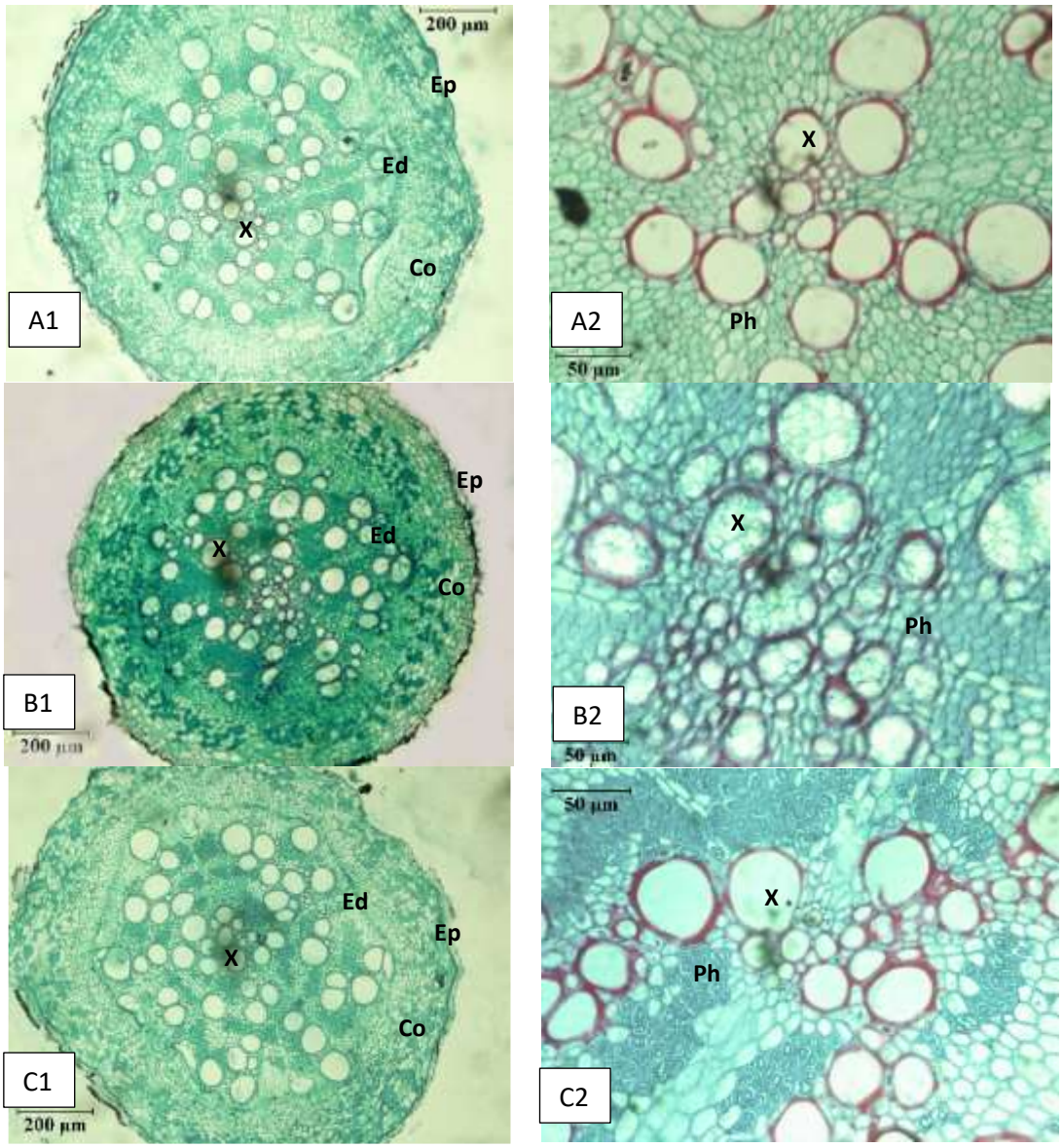

Figure 5. Root anatomy of: A. *Indigofera tinctoria*; B. *I. suffruticosa*; and C. *I. arrecta.* (A1-C1 Magnification: 100x), (A2-C2 M: 400x). Ep: Epidermis; Co: Cortex; Ed: Endodermis; Ph: Phloem, X: Xylem

In conclusion, morphological and anatomical study of the three *Indigofera* species showed variations in the tissue forms, colors, and sizes. Those three species have striking identical morphologies, only have minor differences in the leaf's colors and pod shapes. The pods of *I. tinctoria* were straight, long, and slender, *I. arrecta* has straight, short, and stubby pods, whilst *I. suffruticosa* is the most different type, with short and curved pods. The anatomical study revealed *I. tinctoria* has rectangular stem cross-section, *I. suffruticosa* was hexagonal, and *I. arrecta* has rounded one. The stems were composed of various tissues in the following succession: epidermis, thin cortex, thin or thick secondary phloem, thick secondary xylem, and prominent pith. *I. suffruticosa* has corners on it stems with thick collenchyma that differentiates it from other species. The leaf was composed of the upper epidermis, mesophyll (palisade parenchyma, sponge parenchyma), and lower epidermis. The vascular bundles were located in the middle, with five or six segments of the xylem elements and small groups of phloem elements, all in the parallel lines. The root anatomy showed dense secondary xylem. The secondary xylem was composed of thick-walled vessel elements, endodermis tissue in *I. arrecta* was hexagonal.

The results of this study provide additional information regarding the morphological and anatomical characteristics of *Indigofera* which can be used as additional data for identification, characteristics, and classification.tasks, and also could complement the novelty of the morfoanatomy information records obtained by previous researchers. Researchers also recommend conducting similar research not limited to the three *Indigofera* species but with other *Indigofera* species in greater numbers so that the information obtained is more complete.

ACKNOWLEDGEMENTS

This research was sponsored by the Grant for Postgraduate Research, Universitas Sebelas Maret, Indonesia, Year 2018-2019, No. 516/UN27.21/PP/2019.

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