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Analysis of the Anatomical Structure of Annual Branches of Female and Male *Diospyros Lotus* L. Plants

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Abstract:

Diospyros lotus L. (Ebenaceae) is commonly utilized as persimmon rootstock in agricultural production. But *D. lotus* make it challenging to distinguish between male and female plants at the seedling stage. Thus, studying the anatomical structure of both genders can facilitate early gender identification, which is useful for resource protection and utilization. The annual branches of female and male *D. lotus* plants were utilized as test materials, with paraffin sectioning technology employed for specimen. Microscope observation was then used to analyze the anatomical structures of the tissues in the annual branches. The test results indicated no significant differences in panel length, branching rate, xylem area, conduit area, conduit density, ratio of conduit area to xylem area, sieve tube area, sieve tube density and the ratio of xylem, phloem and medulla. However, slight differences in the xylem and phloem structure were observed between male and female plants. Specifically, female plants had a higher conduits density and conduits area compared to male plants, while male plants exhibited more obvious phloem rays in their annual branches. In this study, we compared the anatomical structures of annual branches of both female and male *D. lotus* plants. Overall, we found that both conduits and phloem rays can serve as indicators for early gender identification in *D. lotus*. These results lay the groundwork for the selection and utilization of superior individual plants for practical cultivation.

Key words: *Diospyros lotus* L.; Dioecious plants; Annual branches; Anatomical structure

Introduction

Diospyros lotus L. (Ebenaceae) is a deciduous tree commonly referred to as soft jujube, black jujube, and clove jujube[1,2], date-plum and caucasian persimmon in the West. *D. lotus* is indigenous to China and is highly prized for its economic benefits and nutritional properties. These trees develop extensive root systems, prefer full sun, are resistant to cold, and tolerate poor soils and barren conditions. In fact, *D. lotus* is well-known to be highly stress-resistant, robust, and long-lived. In agricultural production, *D. lotus* is commonly utilized as a rootstock for persimmon scions[3,4]. *D. lotus* is dioecious, with male and female plants utilized for different purposes. The

fruits of the female plant are utilized medicinally. Meanwhile, male plants have various flower colors, large flowers, and late flowering period, and have high ornamental value. Additionally, male plants are cold and drought tolerant and less susceptible to pests and diseases, and can be used as greening and street tree species[5]. The appropriate rootstock is selected according to production purpose.

Dioecious plants are widespread in nature. During the process of plant evolution, dioecious plants exhibit significant differences in reproduction, growth, resource utilization, physiological biochemistry, and gene expression, as well as in

their responses to adverse stress[6-8]. For instance, during early production, the presence of male *D. lotus* plants can increase workload and incur higher production costs. Conducting research on the differences between male and female plants not only aids in early gender identification and shortening the breeding cycle, but also sheds light on the adaptive implications of morphological traits during the evolution of dioecious plants[9].

To date, most research on dioeciousness has focused on differences in appearance, tissue morphology, and physiological and biochemical indicators between genders. In addition, progress has been made in exploring methods and approaches for the early identification of plant gender. For example, Gong Wenlou[10] compared seedlings and mature populations of *Populus tomentosa* and found that male and female plants could be distinguished based on bark and leaf characteristics in both summer and winter. Liu Yong et al[11] identified significant differences in morphology and distribution between male and female *Hippophae rhamnoides* L. plants, with females exhibiting narrower leaves and a higher length-to-width ratio. Xu Dongsheng et al[12] successfully differentiated between male and female kiwi fruit plants using multivariate statistical analysis. Zhang Liping et al[13] microscopically observed male and female grape plants and discovered distinct differences in pollen morphology. Specifically, female plants exhibited small, shriveled, and irregularly sunken pollen with a mesh-patterned outer wall, while male plants exhibited large, oval pollen with a hole-patterned outer wall.

Most recently, the use of molecular methods for plant gender identification has been explored. Gender-specific molecular markers have been successfully developed in various plants, including *Actinidia*[14-18], *Ginkgo biloba* L.[19,20], *Vitis vinifera* L.[21-23], and others. In *D. lotus*, the persimmon male sex-linked marker DISx-AF4S has been successfully used to distinguish between male and female plants[24]. However, distinguishing between male and female plants through physiological and biochemical indicators, chromosomal analysis, molecular markers, and other specialized methods can be challenging and costly. Conversely, while appearance and morphology lend themselves to

simple and fast analysis, each can be significantly influenced by growth stage, development period, and external environmental factors, resulting in inaccurate results. Luckily, plants generally exhibit stable anatomical structures which can be reliably utilized to determine gender at an early stage through the use of sectioning techniques.

Recent Chinese research on *D. lotus* has focused on germplasm resource exploration[4,25], cold resistance[26], rootstock grafting[27,28], high-yield cultivation techniques[29], and genetic stability[30]. Meanwhile, research conducted in other countries has focused on evolutionary history[31], stress resistance[32], genetic transformation[33], and vascular meristem morphology[34]. Despite this robust and growing body of research, we still lack a conclusive method for the early identification of female and male *D. lotus* plants. In a previous study, we utilized the persimmon male sex-linked marker DISx-AF4S to accurately determine the sex of *D. lotus* offspring[24]. As a follow up to that work, here we analyzed anatomical differences between the annual branches of female and male *D. lotus* plants using paraffin sectioning. These findings will enable male and female *D. lotus* plants to be distinguished at an early age, aiding in the identification and classification of individual plants and the selection of superior lines. In addition, these findings will be useful for research into differences in the environmental adaptability of male and female *D. lotus* plants.

2 Materials and Methods

2.1 Experimental Materials

This research was conducted on 3-year-old *D. lotus* plants from the *Diospyros lotus* L. planting area located at the eastern district experimental base of the Henan Institute of Science and Technology, China. In November 2016, annual branches were collected on a sunny morning from each of four male and four female *D. lotus* trees of similar age and growth potential. At least five vigorous, strong, and full annual branches were selected from each tree and transported to the laboratory for future use.

2.2 Experimental Methods

Each 0.5-1 cm branch segment was cut from the same position on an annual branch and promptly fixed with Formal-Acetic-Alcohol (FAA) fixative

consisting of 90 mL of 70% alcohol, 5 mL of 30% formalin, and 5 mL of glacial acetic acid. Permanent paraffin transverse and longitudinal sections were prepared for microscopic observation. To minimize error, a stage micrometer was utilized to calibrate the eyepiece micrometer.

2.3 Measurement Methods

Fruit tree pruners were used to cut annual branches according to panel length, and each cut branch was measured using a vernier caliper following the cross-shaped method. This process was repeated eight times for each plant to calculate the average panel length.

The branching rate was determined following the cross-shaped method, wherein a vernier caliper was utilized to measure the branch diameter both before and after cortex removal. The measurement position remained consistent before and after cortex removal to avoid errors resulting from branch sharpness. This process was repeated eight times for each plant to calculate the average branching rate.

$$n = \left(1 - \frac{ab}{AB}\right) \times 100\%$$

Note: n is the branching rate; a and b are the vertical and horizontal diameters of the branches after peeling, respectively; and A and B are the vertical and horizontal diameters of the branches before peeling, respectively.

A micrometer under a microscope was used to determine conduit density, total conduit area, xylem area, the ratio of total conduit area to xylem area, and the ratios between xylem, phloem, and medulla of annual branches. All data were recorded separately to ensure analytical accuracy.

2.4 Data Processing and Analysis

Data was processed using Microsoft Excel and analyzed using DPS.

3 Results

3.1 The overall structure of annual branches of female and male *D. lotus* plants

Microscopic observation of paraffin sections revealed that the annual branches of female and male *D. lotus* plants were primarily made up of epidermis, cortex, and vascular cylinder (Figure 1A, Figure 2A), which is similar to the primary

structure of most dicotyledonous plant stems. Periderm (Figure 1A a, Figure 1C, Figure 1D, Figure 2A a, Figure 2C, Figure 2D) is a secondary protective tissue which replaces the epidermis and is composed of the cork layer (Figure 1C a, Figure 1D a, Figure 2C a, Figure 2D a), the cork cambium (Figure 1C b, Figure 1D b, Figure 2C b, Figure 2D b) and the phelloderm (Figure 1C c, Figure 1D c, Figure 2C c, Figure 2D c). When the stems and roots of dicotyledonous plants increase in thickness, the periderm replaces the epidermis to serve a protective function. In plant stems and roots, cortex (Figure 1A b, Figure 1E a, Figure 2A b, Figure 2E a) refers to the parenchyma located between the epidermis and vascular cylinder. The vascular system is composed of primary phloem, secondary phloem, cambium (Figure 1F a, Figure 2F a), primary xylem (Figure 1B b, Figure 2B b, Figure 2F c), and secondary xylem (Figure 1F c, Figure 2F d), as well as the rays which penetrate it[35,36]. Overall, we observed no obvious differences in the general annual branch structure between female and male *D. lotus* plants.

3.2 The organization of annual branches of female and male *D. lotus* plants

3.2.1 Periderm

Periderm is a secondary protective tissue which forms on the surface of thickened roots and stems and serves to replace the epidermis as plant roots and stems grow thicker. Periderm helps to control the rate of water loss, fend against pathogens and pests, and reduce mechanical damage to the internal tissues. Periderm typically develops on annual branches (including in both female and male *D. lotus* plants) and gradually increases in thickness. Specifically, the periderm is formed by the periclinal division of cambium cells in the secondary meristem cork. These cells then differentiate outward to become the cork layer and inward to form the phelloderm. Cork layer cells are typically arranged in neat radial rows, and are often flat, tangentially elongated, thin-walled, suberized or lignified, and lack intercellular spaces.

We observed between three and four layers of cells in the cork layer of the annual branches of both female and male *D. lotus* plants. The cells in the cork cambium (Figure 1C b, Figure 1D b, Figure 2C b, Figure 2D b) are generally flat, thin-walled, slightly tangentially-elongated, and

contained small intercellular spaces. The phelloderm (Figure 1C c, Figure 1D c, Figure 2C c, Figure 2D c) is located inside the cork cambium and resembles the cork cells. In addition, the radially-arranged rows of cells making up the phelloderm are typically not corkified and have large intercellular spaces. Overall, we observed no obvious differences in the periderm structure between female and male *D. lotus* plants.

3.2.2 Cortex

The cortex is comprised of the parenchyma located between the epidermis and vascular bundles in plant stems and roots. In the annual branches of male and female *D. lotus* plants, the cortex is positioned between the periderm and vascular bundles (Figure 1A b, Figure 2A b). The cortex primarily consists of longitudinal rows of thin-walled, slightly elongated cells with noticeable intercellular spaces, as well as several layers of collenchyma (Figure 1E b, Figure 2E b). The loosely-arranged cells making up the collenchyma are large and have unevenly-thickened cell walls. Overall, we observed no obvious differences in the cortex structure between female and male *D. lotus* plants.

3.2.3 Vascular Cylinder

The vascular cylinder, which includes vascular bundles, medulla, and medullary rays, originates from the procambium of the primary meristem[37] and is located within the endodermis. The vascular tissue in the annual branches of male and female *D. lotus* plants is organized into bundles, and the vascular bundles are composed of xylem and phloem. The procambium (Figure 1F a, Figure 2F a) develops inwardly into primary xylem (Figure 2F c) and outwardly into primary phloem, while also producing phloem rays (Figure 2F h). Overall, we observed no obvious differences in the vascular cylinder structure between female and male *D. lotus* plants.

The xylem is a composite tissue which serves both conductive and mechanical functions. The xylem consists of conduits, tracheids, wood fibers, and wood parenchyma cells. Typically, xylem is combined with phloem to form vascular bundles which are distributed throughout the plant, creating a complex vascular system. In the annual branches of both female and male *D. lotus* plants, the xylem (Figure 1A e, Figure 2A e) is situated

between the cambium (Figure 1A d, Figure 2A d) and the medulla (Figure 1A f, Figure 2A f). In vascular plants, the primary xylem (Figure 1B b, Figure 2B b) is divided during growth and differentiation into protoxylem and metaxylem, according to the timing of differentiation. Protoxylem is initially formed from the inner region of the procambium and subsequently develops centrifugally, gradually differentiating to give rise to epixylem. This developmental order is known as endarch. The protoxylem consists of rings, threaded conduits, and wood parenchyma cells, and the metaxylem is comprised of larger-diameter reticular patterns, pore patterns, ladder-like conduits, wood parenchyma cells, and occasionally wood fibers. In the annual branches of both male and female *D. lotus* plants, the xylem contains both primary xylem (Figure 2F c) and secondary xylem (Figure 1F c, Figure 2F d).

Both conduits (Figure 1G a, Figure 2G a) and tracheids transport water and inorganic salts. Conduits are tubular structures formed by longitudinally connecting multiple elongated dead cells, with each cell referred to as a conduit element. Female *D. lotus* plants contain a higher density of conduits than male plants, as shown in Figures 1G and 2G. Tracheids consist of elongated cells with pointed ends which lack protoplasts at maturity. These cells have lignified secondary walls, making them challenging to observe microscopically. Wood fibers are typically found in the secondary xylem of woody plants, and are characterized by thick cell walls, narrow cell cavities, elongated shapes, and grouped or bundled distribution. At maturity, wood parenchyma cells typically develop thickened, lignified secondary walls and are situated within the secondary xylem. Wood rays are vascular rays located in the secondary xylem of the cambium, and are primarily responsible for lateral transport and storage. Overall, we observed slight differences in the xylem structure between female and male *D. lotus* plants. Specifically, female *D. lotus* plants contain a higher density of conduits than male plants.

The phloem is a composite tissue with transport, storage, and support functions. As a conductive tissue, the phloem forms the vascular system together with the xylem. In the annual branches of both female and male *D. lotus* plants, the phloem (Figure 1A c, Figure 2A c) is situated between the

cortex (Figure 1A b, Figure 2b d) and the cambium (Figure 1A d, Figure 2A d). The phloem is composed of sieve tubes, companion cells, phloem parenchyma cells, and phloem fibers. Depending on the source, the phloem (Figure 1F b, Figure 2F b) can be divided into primary phloem or secondary phloem. According to the timing of differentiation, primary phloem can be categorized as either protophloem or metaphloem. The primary phloem originates from the outer layer of the procambium and progresses centripetally towards the inner layers. The protophloem is initially established on the outer side, followed by the formation of the metaphloem on the inner side. This developmental order is known as exarch. The annual branches of both female and male *D. lotus* plants contain primary phloem (Figure 1F b, Figure 2F b). Secondary phloem is formed during the thickening of plant stems. Similarly to secondary xylem, secondary phloem is produced by the vascular cambium, albeit in smaller quantities compared to xylem. Sieve tubes (Figure 1F e and Figure 2F f) are fundamental phloem structures which consist of a chain of sieve tube elements linked at the upper end, with sieve pores facilitating communication between neighboring elements and sieve plates located at the terminal walls, plays the role of transporting nutrients.

Companion cells (Figure 1F f, Figure 2F g) accompany sieve tube elements and play a role in transporting nutrients and providing energy.

Phloem parenchyma cells are dispersed throughout the phloem and are responsible for the storage of starch and other substances. The procambium, which originates from the stem apical meristem, can undergo further differentiation to form primary phloem. Phloem rays are tough, elastic, and supportive, and are often distributed in bundles on the outermost side of the primary phloem. The annual branches of male *D. lotus* plants exhibit prominent phloem rays (Figure 2F h). These phloem rays are comprised of thick-walled cells with round, oblong, or polygonal cross sections. Overall, we observed slight differences in the phloem structure between female and male *D. lotus* plants. Specifically, male *D. lotus* plants exhibit prominent phloem rays.

The medulla (Figure 1B, Figure 2B) of the annual branches of the male and female *D. lotus* plants consists of a central section made up of parenchyma tissue containing intercellular spaces. These cells typically store nutrients. Overall, we observed no obvious differences in the medulla structure between female and male *D. lotus* plants.

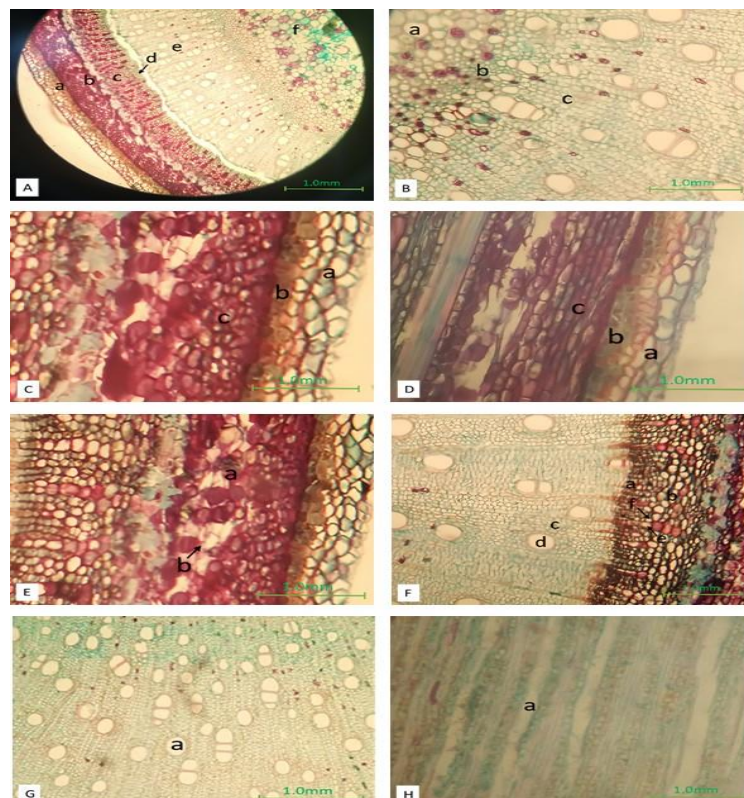


Figure 1: Anatomical structure of annual branches of female *Diospyros lotus* L. plants. A. General structure: a. periderm; b. cortex; c. phloem; d. cambium; e. xylem; f. medulla. B. Medulla cross section: a. medulla; b. primary xylem; c. secondary xylem. C. Transverse section of periderm: a. cork layer; b. cork cambium; c. phelloderm. D. Longitudinal section of periderm: a. cork layer; b. cork cambium; c. phelloderm. E. Transverse section of cortex: a. cortex; b. collenchyma. F. Transverse section of phloem: a. cambium; b. phloem; c. secondary xylem; d. conduit; e. sieve tube; f. companion cell. G. Transverse section of conduit: a. conduit. H. Longitudinal section of conduit: a. conduit.

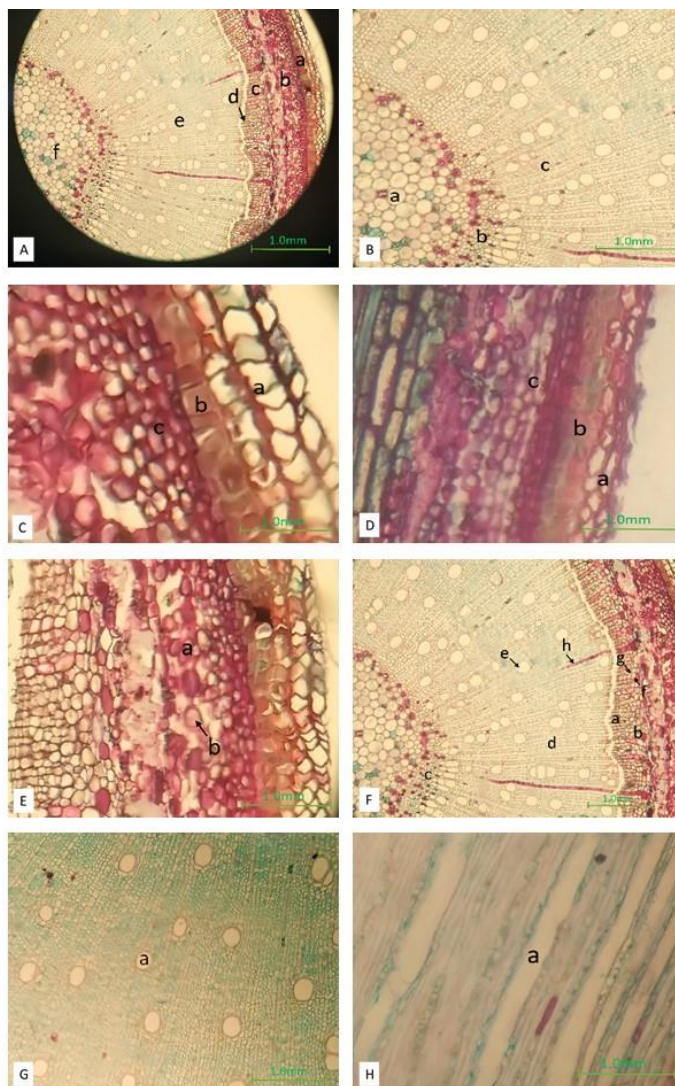


Figure 2: Anatomical structure of annual branches of male *Diospyros lotus* L. plants. A. General structure: a. periderm; b. cortex; c. phloem; d. cambium; e. xylem; f. medulla. B. Medulla cross section: a. medulla; b. primary xylem; c. secondary xylem. C. Transverse section of periderm: a. cork layer; b. cork cambium; c. phelloderm. D. Longitudinal section of periderm: a. cork layer; b. cork cambium; c. phelloderm. E. Transverse section of cortex: a. cortex; b. collenchyma. F. Transverse section of phloem: a. cambium; b. phloem; c. primary xylem; d. secondary xylem; e. conduit; f. sieve tube ; g. companion cell; h. phloem rays. G. Transverse section of conduit: a. conduit. H. Longitudinal section of conduit: a. conduit.

3.3 Overview of differences in the anatomical structure of annual branches between female and male *D. lotus* plants

No significant differences in the anatomical structure of annual branches were observed

between female and male *D. lotus* plants (Figure 1, Figure 2). Similarly, no significant differences in panel length, branching rate, or xylem area were observed between female and male *D. lotus* plants (Table 1).

Table 1: Comparison of annual branch anatomical structure between male and female *Diospyros lotus* L. plants

| | Panel length (mm) | Branching rate (%) | Xylem area (mm ²) |
|---------------|-------------------|--------------------|-------------------------------|
| Female | 23.6987±3.0351a | 16.1547±2.0202a | 4.0175±0.4674a |
| Male | 21.4625±4.7723a | 16.6372±2.6183a | 3.9575±1.4318a |

Note: Different letters in the same column indicate statistically significant differences at the $P < 0.05$ level.

No significant differences in the cross-cutting structure of annual branches were observed between female and male *D. lotus* plants (Table 2). Specifically, no significant differences in conduit area, conduit density, or the ratio of

conduit to xylem area were observed between female and male *D. lotus* plants. However, male plants exhibited a slightly lower conduit density than female plants.

Table 2: Comparison of annual branch cross-cutting structure between male and female *Diospyros lotus* L. plants

| | Conduit area (mm ²) | Conduit density (PCS/mm ²) | Conduit/xylem area ratio (%) |
|---------------|---------------------------------|--|------------------------------|
| Female | 0.5600±0.1023a | 80.00±7.3485a | 14.164±2.4938a |
| Male | 0.4275±0.0998a | 74.25±10.2429a | 11.420±2.6211a |

Note: Different letters in the same column indicate statistically significant differences at the $P < 0.05$ level.

No significant differences in the longitudinal structure of annual branches were observed between female and male *D. lotus* plants (Table 3). Specifically, no significant differences in

either conduit area or conduit density were observed between female and male *D. lotus* plants.

Table 3: Comparison of annual branch longitudinal structure between male and female *Diospyros lotus* L. plants

| | Conduit area (mm ²) | Conduit density (PCS/mm ²) |
|---------------|---------------------------------|--|
| Female | 0.4060±0.0266a | 5.3100±0.8361a |
| Male | 0.3526±0.0319a | 5.8600±1.0331a |

Note: Different letters in the same column indicate statistically significant differences at the $P < 0.05$ level.

No significant differences in the longitudinal sieve tube structure of annual branches were observed between female and male *D. lotus* plants (Table 4). Specifically, no significant differences in

either sieve tube area or sieve tube density were observed between female and male *D. lotus* plants.

Table 4: Comparison of annual branch longitudinal sieve tube structure between male and female *Diospyros lotus* L. plants

| | Sieve tube area (mm ²) | Sieve tube density (PCS/mm ²) |
|---------------|------------------------------------|---|
| Female | 0.0400±0.0076a | 4.5400±0.5964a |
| Male | 0.0369±0.0007a | 5.0050±0.3396a |

Note: Different letters in the same column indicate statistically significant differences at the $P < 0.05$ level.

Non-significant differences in the longitudinal xylem, phloem, and medulla structures of annual branches were observed between female and male *D. lotus* plants (Table 5). Specifically, female

plants exhibited slightly longer medullas and xylem elements, and shorter phloem elements, than male plants.

Table 5: Comparison of annual branch xylem, phloem, and medulla longitudinal structures between male and female *Diospyros lotus* L. plants

| | Xylem | | Phloem | | Medulla | |
|---------------|----------------|-----------|----------------|-----------|----------------|-----------|
| | Length (mm) | Ratio (%) | Length (mm) | Ratio (%) | Length (mm) | Ratio (%) |
| Female | 2.5715±0.4842a | 64.75 | 0.3950±0.0480a | 10 | 1.0600±0.1304a | 25.25 |
| Male | 2.4922±1.0282a | 63.00 | 0.4052±0.0493a | 11 | 0.9346±0.2141a | 26.00 |

Note: Different letters in the same column indicate statistically significant differences at the $P < 0.05$ level.

Discussion

Overall, the anatomical structures of the annual branches of both female and male *D. lotus* plants are similar to those of other dicotyledonous plants. However, we found no statistically significant anatomical differences between female and male *D. lotus* plants. Xylem conduits transport water and inorganic salts to various organs in order to meet metabolic and transpirational demands. Increasing xylem conduit diameter can enhance water transport efficiency[38]. Moreover, conduit area and density are indicators of transport capacity, with greater values indicating increased transport capacity[39]. Higher transport capacity results in increased growth potential and growth rate because efficient transport allows the transpiration and metabolic needs of the plant to be met rapidly.

Notably, female *D. lotus* plants tended to exhibit higher conduit area and density than male plants, suggesting that females of this species have a better growth potential. These findings also suggest that differences in conduit elements may aid in differentiating between male and female plants, which aligns with the research of Guo Xuemin[40]. Phloem rays are radially-elongated tissues located in the secondary phloem. We observed that male *D. lotus* plants exhibited prominent phloem rays, while those of female plants were less obvious. In the structure of annual branches, the proportion of phloem in male plants is slightly higher than that in female plants.

In summary, although we observed no statistically significant anatomical differences between female and male *D. lotus* plants, the sexes can still be differentiated based on the structural characteristics of the conduits and phloem rays. Subsequent anatomical studies should be conducted on the young stems of other Ebenaceae species to draw more precise conclusions.

Conclusion

In this study, we compared the anatomical structures of annual branches of both female and male *D. lotus* plants. Overall, we found that both conduits and phloem rays can serve as indicators for early gender identification in *D. lotus*. These results lay the groundwork for the selection and utilization of superior individual plants for practical cultivation.

Declarations:

Data availability statement

Data can be made available on request.

Author contribution statement.

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Shuda Li, Zihao Yu, Peng Zhang and Xiaona Zhang. The first draft of the manuscript was written by Ruijin Zhou and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

Data can be made available on request.

Declaration of Competing Interest

Authors declare that they have no conflict of interest.

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