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APPLICATION OF FLUORESCENCE MICROSCOPY FOR HISTOLOGICAL DISCRIMINATION OF GOLDEN CAMELLIAS LEAVES

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SUMMARY

Histological analysis, which aims to investigate the microscopic anatomy of biological tissues, has been a simple and powerful technique for plant taxonomy. Sectioning followed by staining methods is widely used in observing histological structures. However, the staining techniques often destroy tissue and provide low-quality images due to nonspecific reactions with the dyes making further analysis difficult. In this report, we propose an applicable non-staining histology protocol based on auto-fluorescence characteristics of plant tissues and its application in the anatomical discrimination of six similar-appearance species of golden camellias as a case study. We compared the images from the same tissue under a bright field with the staining step and under fluorescence directly without the staining step in the sample preparation. The images were taken from Eclipse Ni-U microscopy (Nikon, Japan) with a color DS-Ri2 camera (Nikon, Japan) and NIS-ELEMENTS Basic Research Imaging software. The non-staining method demonstrated significant advantages compared to the staining protocol. The fluorescent images showed the distinction between adjacent leaf tissues with their own naturally reflective colors. In addition, the anatomical parameters, including the xylem area, phloem area, bundle sheath area, and palisade/spongy width ratio, were easily measured in good-quality images. These parameters were used in discriminative analysis by the Principal Component Analysis (PCA). The PCA diagram demonstrated the separation of six species, thus suggesting that these anatomical parameters can be used for taxonomy. In conclusion, our study showed a helpful technique in histological analysis that significantly contributes to the taxonomy of golden camellias species and can be applied in other plant varieties.

Keywords*:* fluorescence, microscopy, golden camellias, leaf anatomy, bundle sheath, xylem, phloem, palisade, spongy

INTRODUCTION

Histological analysis, which aims to investigate the microscopic anatomy of biological tissues, has been a simple and powerful technique for plant taxonomy. This approach consists of two main stages: embedding the samples into the paraffin and collecting images using microscopy. For the former step, the samples must be fixed in the formalin solution and then dehydrated, infiltrated, and mounted in melt paraffin to obtain the paraffin block (Zelko *et al*., 2012). For the latter, the sections must be stained with dyes to visualize different cell wall components in the visible spectrum (Yuanyuan *et al*., 2019). For instance, Toluidine blue has been used to detect the lignin and pectin (Till *et al*., 2008), while Wiesner

(phloroglucinol) stain can specify lignin in the xylem, fiber, and vascular tissues (Serge *et al*., 2011). However, this conventional approach has some disadvantages. Firstly, due to the lengthy fixation, dehydration, and infiltration procedure, the plant tissues become hardened; thus, staining agents cannot interact appropriately with the cell wall. In addition, the residual paraffin may also stay on the surface of plant samples after clearing, interfering with the staining solution and obstructing the collection of high-resolution images (Karel *et al*., 2008). Therefore, each plant tissue requires a long and laborious optimization time to be observed in a microscope.

In this study, we aim to develop a histology protocol based on auto-fluorescent characteristics of different chemical classes in plant tissues without a staining step. Indeed, plants produce abundant auto-fluorescent molecules that can be used for imaging studies. Pigments (chlorophyll with orange/red fluorescence, anthocyanins, flavonoids with green/yellow fluorescence) and secretory compounds or structural components of cell walls (alkaloids, lignin with blue/green fluorescence) are usually present in plant leaves. These compounds absorb the light emitted by fluorescence sources, thus, can be observed under fluorescent microscopy. Auto-fluorescence can be considered a non-invasive method for detecting specific molecules in plant tissues which can bring advantages for the phenotyping of genetic variants (García-Plazaola *et al*., 2015).

We also showed a case study of applying this protocol on six closed species of golden camellias. In general, golden camellia is a group of species in the *Camellia* genus (Theaceae family) that flower in yellow. They are traditional herbal medicines distributed mainly in China and Vietnam. Fifty-two species have been discovered, and twenty-two of these were explicitly described based on their morphology of flower, fruit, and leaf (Manh *et al*., 2019). The morphology of golden camellias leaves was highly similar, making it challenging to classify between species (Gao *et al*., 2005). On the other hand, the flowers, which were reported to be unique to species and thus can provide more reliable identification, are limited as it appears in only one reproductive season per year.

MATERIALS AND METHODS

Sample collection

The leaves of golden camellia species: *Camellia phanii* (PHA, specimen code PHA-12-2021), *Camellia tamdaoensis* (TAM, specimen code TAM-12-2021), *Camellia tienii* (TIE, specimen code TIE-12-2021), *Camellia flava* (FLA, specimen code FLA-12-2021), and *Camellia petelotii* (PET, specimen code PET-12-2021) were all gathered in December 2021 at Hop Chau ward, Tam Dao district, Vinh Phuc province. *Camellia euphlebia* was assembled in the Quang Minh ward of Hai Ha district, Quang Ninh province (EUP, specimen code EUP-04-2022) in April 2022. For each golden camellia variety, the mature leaves were collected from 3 plants. Dr. Nguyen The Cuong, from the Institute of Ecology and Biological Resources, identified all plant materials. The specimen dossiers were stored and used for further experiments at the Department of Life Sciences at the University of Science and Technology of Hanoi.

Staining, fixation, dehydration, clearing, infiltration, embedding and sectioning

To determine the effect of the staining step, the samples were divided into two batches, with or without staining. In the staining samples, a square of 1 cm x 1 cm was cut from the midvein and blade area and immediately stained in staining solution (blue toluidine 0.1% (w/v)). After 1 hour, samples were washed with BPS solution (Phosphate Buffered Saline) once before transferring to the FAA solution (formaldehyde 3.7% (v/v), acetic acid 5% (v/v) and ethanol 50% (v/v)). In the non-staining protocol, samples were fixed directly in the FAA solution. These steps have been done on the collection site. At the laboratory, all samples were put in vacuum for 1 hour for better infiltration of chemicals into the tissues, followed by the overnight incubation in fresh FAA at 4^{o}C. The dehydration, clearing,

infiltration, embedding and sectioning steps were processed, as previously reported to produce paraffin blocks (Erxu *et al*., 2009, Le *et al*., 2022).

A vibrating HM 340E Rotary Microtome (Thermo Scientific, USA) was used to get a cross-sectional slice 10 µm thick, which was then spread out on glass slides and heated to dry at 50°C for a few hours. After that, the plant tissue-containing slices were then deparaffinized in a 100% xylene solution (Merck, USA), dried for one hour, and finally covered with glass coverslips.

Image collecting and data analysis

The images were captured by Eclipse Ni-U microscopy (Nikon, Japan) with a color DS-Ri2 camera (Nikon, Japan) and NIS-ELEMENTS Basic Research Imaging software. All samples were observed under Nikon Plan Fluor 4X/0.13 objective. The sections stained with blue toluidine were observed using a halogen lamp light source. Meanwhile, the sections without being stained were observed using the C-HGFI Intensilight Epi-fluorescence Illuminator (Nikon, Japan) with a long-pass V-2A filter cube (excitation wavelength at 380-420 nm; emission wavelength higher than 440 nm. NIS-ELEMENTS was used to take all measurements.

Each parameter was measured by 9 observations for each species. The obtained data matrix was analyzed by the Principal Component Analysis (PCA) implemented in R version 3.5.3 (http://www.R-project.org/) by using FactoMineR package (Le *et al*., 2008).

RESULTS AND DISCUSSION

Comparison of histological techniques

First, the effect of the staining step in the histological procedure was evaluated by comparing the images of the same tissue obtained from the staining-contributed protocol (observed in bright field) and the non-staining method (fluorescence). Since histochemical dyes usually present low specificity and a high signal-to-noise ratio caused by using the fixed tissues, we performed the staining step with the fresh plant tissue before the fixation and embedding in paraffin. The cross-sections were then captured using white light microscopy. The results in Figure 1A showed the tissue in blue toluidine staining, in which the blue color only appeared in palisade (abundant in polyphenol) and was less intense in the xylem. In contrast, pink was observed mainly in the xylem, whose cells contain the secondary cell wall (abundant in lignin). However, the picture did not clearly contrast different leave tissues. Moreover, the dye caused smears on the background and tissues, making it hard to observe.

In contrast, the image collected from the tissues without staining using fluorescence in Figure 1B showed significant advantages. The fluorescence images show different, bright, and vivid colors in multiple leaf tissues thanks to their self-reflective ability, allowing us to see the difference in the histological structures of leaves. These results suggested that fluorescence can increase the color contrast and specificity of the obtained signal, providing high-quality images for further analysis. This method also helped to avoid the limiting step in the histological procedure.

Leaf anatomical parameters are potential for taxonomy

The developed method was then applied for the taxonomic identification of variants. Six golden camellias species collected in Vinh Phuc and Quang Ninh provinces were subjected to histological analysis. Figure 2A shows the auto-fluorescence images of six representative samples' blades and midvein cross-section. Overall, excited under the same wavelength of 380-420 nm, fluorescent images of the leaf midvein clearly showed the differences in color between tissue types. Bundle sheath fiber gives the brightest color, mainly due to the high amount of lignin (a conjugated compound in the cell wall), which was significantly distinguished from other tissues. This result is consistent with the previous study on the self-reflection in the sapwood and heartwood of *Eucalyptus*

bosistoana bundle sheath fiber, explained by two sources of fluorophores: vacuoles and cell walls (Mishra *et al*., 2018). The phloem is the darkest component in the centre of midvein, while the xylem is more lustrous because of its high condensed lignin content. Our high-quality images perform a shiny bright green color at the abaxial epidermis monolayer, which has large cells with low chlorophyll content. It may be due to other auto-fluorescent molecules such as catechins (Chenxu *et. al*., 2020); tannins (Lloyd Donaldson, 2020) or flavonols (Emi *et al*., 2009). In the leaf blade, palisade layers give a light brown color, whereas spongy areas express pale intensity due to sparse cell distribution.

Regarding the histological structure, the midvein leaves of six species shared similar components, including parenchyma, collenchyma, bundle sheath fiber wrapping xylem and phloem. The midrib's anatomy appeared concave towards abaxial leaves in all the samples, except for EUP and TIE, whose midribs are nearly flat. The angles of protrusion of bundle sheath varied among highly convex species (TAM, PET), slightly convex (PHA, TIE), and nearly flat (FLA, EUP) (Le *et al*., 2022). For the leaf blade, all the samples showed a common histological structure, one layer of the upper and lower epidermis, two primary mesophyll tissues, including palisades and spongy. Except for PHA with two palisade cell layers, the other species possess only one cell layer of the palisade (Le *et al*., 2022).

Four parameters of midvein and blade, including xylem area, phloem area, bundle sheath area and palisade/spongy width ratio, were then measured by using an automatic imaging Nis application (Table S1, Supporting Information). The statistical analysis showed the significant differences among four parameters (Table S2, Supporting Information). These obtained data were subjected to Principal Component Analysis (PCA) to examine the diversity of six golden camellia species. The PCA results indicated that the first two components explained 90.3 % of the total variances, meaning that the histological parameters were strongly discriminated. This PCA analysis confirmed the histological diversity of six species. The result allows us to conclude that histological data provides valuable information for taxonomic identification.

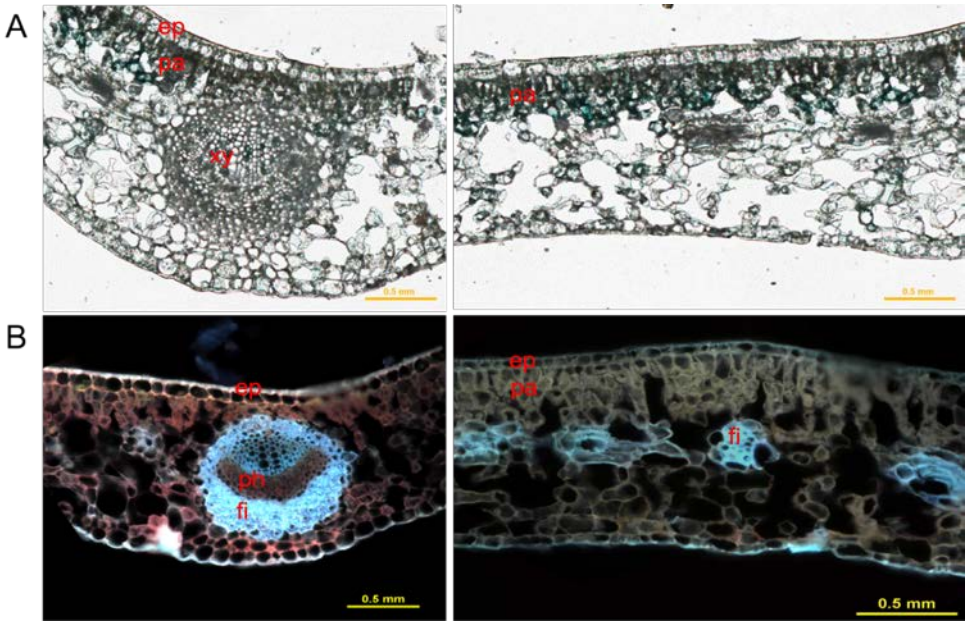
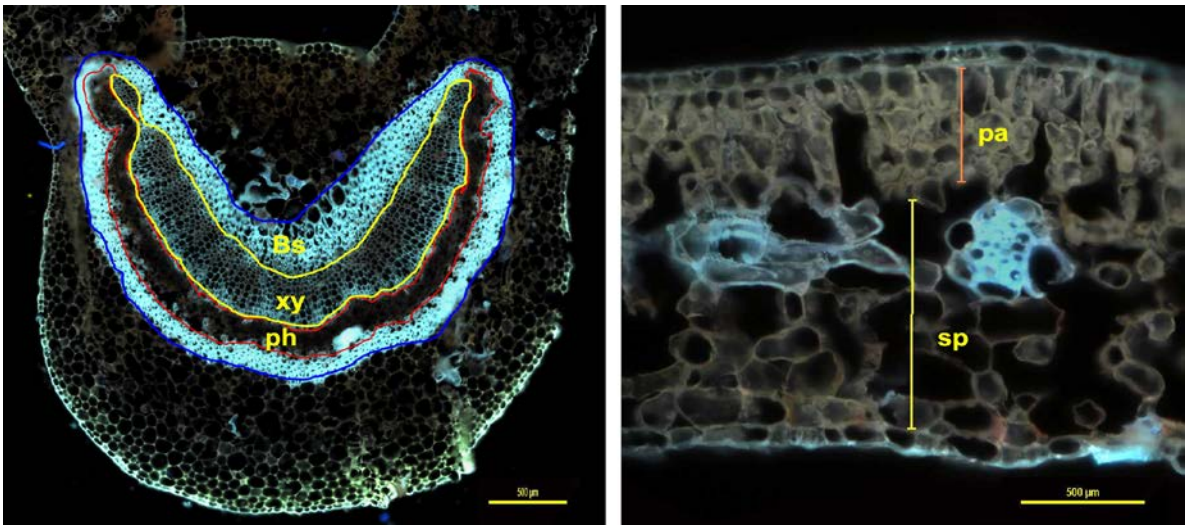


Figure 1. Cross section of secondary vein and leaf blade. A: section stained by blue toluidine in bright field light; B: section with auto-fluorescence. p: epidermis, pa: palisade, xy: xylem, ph: phloem, fi: bundle sheath fiber.

A



B

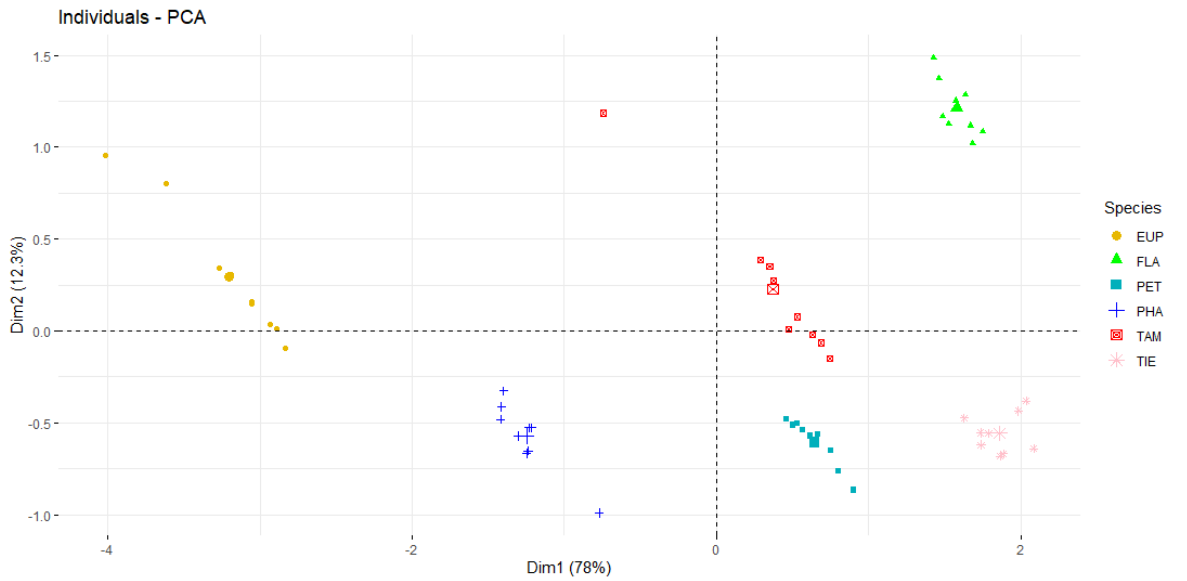


Figure 2. The measurement manner of xylem area, phloem area, bundle sheath area in midvein and palisade/spongy width ratio in blade of leaf sample (A). PCA analysis of four parameters in six golden camellias species (B). Bs: bundle sheath, xy: xylem, ph: phloem, pa: palisade, sp: spongy

CONCLUSION

The present study successfully conducted fluorescence imaging to investigate leaf anatomy without staining and applied for discrimination of six golden camellias species. Our study points out the benefit of the fluorescence technique to unravel the specific properties of leaf anatomical structure, which can be considered a potential criterion for taxonomic identification. The findings suggested that this protocol effectively demonstrated the distinguished anatomy of species in the Camellia genus and can be applied for the discrimination of these species.

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Supporting Information

Table S1. The parameters used for histological analysis and the anatomical diversity of investigated golden camellia species.

Sample	Species name	Bundlsheat area (⌈m²)	Xylem area (⌈m²)	Phoem area (⌈m²)	Palisade/spongy
PHA1	*C. phanii*	2992697	1261592	826077	0.298969
PHA2	*C. phanii*	2999627	1226774	788732	0.278846
PHA3	*C. phanii*	2945649	1246169	790198	0.281553
PHA4	*C. phanii*	2987693	1253030	824244	0.185185
PHA5	*C. phanii*	2934221	1265667	803726	0.30303
PHA6	*C. phanii*	2987710	1200938	810521	0.319588
PHA7	*C. phanii*	2999016	1213111	836824	0.329545
PHA8	*C. phanii*	2953754	1253811	793646	0.326316
PHA9	*C. phanii*	3045344	1198527	822745	0.282609
TAM1	*C. tamdaoensis*	4335982	1205126	1257704	0.157303
TAM2	*C. tamdaoensis*	4373584	119287.3	1241658	0.152941
TAM3	*C. tamdaoensis*	4244654	1195745	1239699	0.151515
TAM4	*C. tamdaoensis*	4358663	1202774	1274536	0.222222
TAM5	*C. tamdaoensis*	4379551	1204455	1277085	0.211765
TAM6	*C. tamdaoensis*	4302670	1205290	1275599	0.197368
TAM7	*C. tamdaoensis*	4337105	1215695	1258124	0.135922
TAM8	*C. tamdaoensis*	4421992	1199146	1247639	0.123894
TAM9	*C. tamdaoensis*	4369129	1191526	1248610	0.102362
TIE1	*C. tienii*	5791296	1812171	1219517	0.110169
TIE2	*C. tienii*	5539340	1773720	1190640	0.114035
TIE3	*C. tienii*	5409233	1836260	1193763	0.148148
TIE4	*C. tienii*	5876536	1799338	1234345	0.145833
TIE5	*C. tienii*	5494426	1781006	1198550	0.144444
TIE6	*C. tienii*	5580457	1859554	1195389	0.162791
TIE7	*C. tienii*	5823013	1798653	1273521	0.140187
TIE8	*C. tienii*	5325138	1772732	1220921	0.155963
TIE9	*C. tienii*	5484168	1879241	1207345	0.137931
FLA1	*C. flava*	5493288	1519552	1675373	0.224719
FLA2	*C. flava*	5206399	1492076	1748764	0.285714
FLA3	*C. flava*	5276942	1553588	1713091	0.290698
FLA4	*C. flava*	5339253	1516337	1664770	0.218391
FLA5	*C. flava*	5233736	1487742	1670441	0.247191
FLA6	*C. flava*	5430461	1496233	1710836	0.25

FLA7	*C. flava*	5430247	1517085	1672076	0.235294
FLA8	*C. flava*	5256840	1458711	1666652	0.230769
FLA9	*C. flava*	5431260	1503298	1684605	0.258065
PET1	*C. petelotii*	4735396	1595739	1003378	0.235294
PET2	*C. petelotii*	4724423	1558622	1037378	0.195652
PET3	*C. petelotii*	4620487	1558650	1034501	0.206667
PET4	*C. petelotii*	4704232	1570344	1021182	0.159509
PET5	*C. petelotii*	4700501	1559161	1039509	0.174419
PET6	*C. petelotii*	4588270	1588232	1038919	0.133721
PET7	*C. petelotii*	4665743	1581673	1025392	0.224638
PET8	*C. petelotii*	4620889	1566057	1039138	0.198582
PET9	*C. petelotii*	4645379	1549277	1016390	0.22695
EUP1	*C. euphlebia*	2578175	566800.2	605527	0.416667
EUP2	*C. euphlebia*	2413129	603130.4	589084	0.633333
EUP3	*C. euphlebia*	2582386	607396.7	626526	0.571429
EUP4	*C. euphlebia*	2550040	578037.3	613855	0.452381
EUP5	*C. euphlebia*	2455008	631007.4	614240	0.371429
EUP6	*C. euphlebia*	2448502	669278	628669	0.4
EUP7	*C. euphlebia*	2496293	606015.2	613855	0.421687
EUP8	*C. euphlebia*	2378001	591972.5	631702	0.460674
EUP9	*C. euphlebia*	2393220	607591.9	633007	0.38835

Table S2. The area of bundle sheath, xylem, phloem, and the palisade/spongy ratio of 6 investigated golden camellia species

Sample	**Species name**	**Bundle sheath area (⌠m²)**	**Xylem area (⌠m²)**	**Phoem area (⌠m²)**	**Palisade/spongy**
PHA	*C. phanii*	2982857±33889 ^{e}	1235523±26187 ^{d}	810746±17642 ^{e}	0.29±0.04 ^{b}
TAM	*C. tamdaoensis*	4347037±50837 ^{d}	1082116±361125 ^{d}	257851±14768 ^{b}	0.16±0.04 ^{d}
TIE	*C. tienii*	5591512±194525 ^{a}	1821519±38202 ^{a}	1214888±26487 ^{c}	0.14±0.02 ^{d}
FLA	*C. flava*	5344270±104894 ^{b}	1504958±26302 ^{c}	1689623±28609 ^{a}	0.25±0.03 ^{b}
PET	*C. petelotii*	4667258±51769 ^{c}	1569750±15618 ^{b}	1028421±12741 ^{d}	0.20±0.03 ^{c}
EUP	*C. euphlebia*	2477195±78458 ^{f}	606803±29899 ^{e}	617385±14302 ^{f}	0.46±0.09 ^{a}

Different letters in the same column indicate a statistically significant difference of the sample mean with $p < 0.05$ (Duncan's test)