Customs Laboratories
European Network

ILIADe 580:2022 | CLEN Method

Identification of tobacco by transmitted light microscopy

Version 10 February 2022

Identification of tobacco by transmitted light microscopy method

1. Scope

This method is suitable for tobacco leaves (processed or unprocessed) and any products made or derived from tobacco leaves, in particular, products intended for human use and products that are used in a manner similar to smoking or smokeless products. The purpose of this method is verification of fulfilment of the legislative requirements applicable for the Common Customs Tariff classification, the Tobacco Products Directive 2014/40/EU, and for EU legislation on excise duties on tobacco.

2. Principle

Qualitative test method for identification of tobacco based on the microscopic determination of the distinctive botanical features of tobacco plant.

3. Reagents and materials

Reagents

3.1. Distilled water
3.2. Potassium hydroxide (aqueous, 5 % w/v) (CAS number: 1310-58-3)
3.3. (or) Chloral hydrate (aqueous, 60-80 % w/v) (CAS number: 302-17-0)

All reagents should be handled and stored according to their safety instructions.

Materials

3.4. Standard microscope slides
3.5. Standard glass coverslips
3.6. Watch glasses
3.7. Petri dishes
3.8. Scissors
3.9. Forceps and tweezers
3.10. Razor blade or scalpel
3.11. Pasteur pipettes
3.12. Paper towel
3.13. Chemically resistant laboratory gloves

4. Apparatus

4.1. Transmitted light microscope with ocular magnification 10x and objectives with a magnification of 4x, 10x, and 40x, equipped with a digital camera (if documentation is necessary) and a polarizer (for better observations of crystal structures; non-obligatory).

5. Procedure

5.1. Sampling

Samples for the test shall be taken in accordance with the sampling manual for customs and taxation authorities - SAMANCTA (SAM-119 Tobacco Sampling), which contains information and instructions on how to take and handle samples for laboratory testing.

5.2. Analytical sample preparation

5.2.1. Select a representative sample of the material by visual inspection e.g. whole, shredded or cut leaves. Select a few fragments of the above mentioned that should include a midrib (= the main vein, stem). No precise weights of sample need to be used in this step.

5.2.2. In case of herbal material in dried and fresh form it should be placed in a moist atmosphere or soak in distilled water in a watch glass (~ 2 minutes).

5.2.3. In case of products like water pipe tobacco, homogenized or powdered tobacco, it should be soak in distilled water or potassium hydroxide solution in a watch glass (~ 5 minutes).

5.3. Making microscopic preparations

General rules of microscopic slide preparations and examination of plant material are included in the literature on the subject, for example in the handbooks of pharmacognosy (e.g. 6 - Annex B)

5.3.1. Prepare glass coverslips and slides which should be thoroughly cleaned, degreased and dry.

5.3.2. Use razor blade or scalpel and small forceps or tweezers to strip off small pieces of leaf epidermis from the marginal of the upper or lower surfaces of the leaf (Fig. 1 - Annex A).

5.3.3. Make several transversal slices of leaves through the stem or vein, to produce material that is as thin as possible; choose the most transparent, thinnest and intact slices of the plant material.

5.3.4. In case of products mentioned in section 5.2.3. use a small, representative portion of sample.

5.3.5. Place a drop of distilled water (alternatively the solutions of potassium hydroxide or chloral hydrate)* in the center of the slide (*potassium hydroxide or chloral hydrate preparation are used in the case of thick and dark plant material to clear material in order to improve visualization of elements incl. calcium oxalate crystals, trichomes and tissues; chloral hydrate does not dissolve calcium oxalate, so it can be used effectively for detecting these crystals),

5.3.6. Place a small portion of sample on liquid, using tweezers (*leave to sit at room temperature for ca. 3 minutes to allow for digestion of intracellular components).

5.3.7. At an angle, place one side of the coverslip against the slide making contact with outer edge of the liquid drop (Fig. 1 - Annex A); lower the coverslip slowly, avoiding air bubbles; remove excess liquid with the paper towel.

5.3.8. Sample is analyzed in duplicate (= two separate subsamples taken from the same source and analyzed independently by the same method and laboratory).

5.4. Microscopic observation

5.4.1. Place slide on the microscope stage and start with a low-power magnification (4x objective) examination to determine the presence of plant structures.

5.4.2. Use the 10x and 40x objectives to investigate further for the distinctive features of tobacco.

5.4.3. When no plant material can be found under 4x magnification then the analysis can be ended.

5.5. Microscopic observation

The examination involves the analysis of the leaf anatomical characteristics which are of special importance in taxonomic consideration and are well documented in botanical literature (22,23,26,30,31 - Annex B). This taxonomically significant structures of tobacco are trichomes, stomata, vascular bundles and crystal structures which provide the primary diagnostic characters important to the identification of plant material.

5.5.1. Trichomes

Trichomes are uni- or multicellular structures that originate from epidermal cells of above-ground plant tissues (Fig. 1 - Annex C). In general, they protect plant from excess transpiration, high temperature, radiation, ultraviolet (UV) light, and herbivore attack (29 - Annex B). Trichomes can be classified morphologically as either non-glandular or glandular. Glandular trichomes are specialized hairs found on the surface of about 20-30% of all vascular plants, they are represented by over 300 different morphological forms and are responsible for a significant portion of a plant's secondary chemistry (7,8,28 - Annex B). Glandular trichomes on tobacco leaves secret high amounts of sucrose esters, fatty acids, microelements, phylloplanins and diterpenoids which are related to plant defense, since they have antifungal and insecticidal activity (10,13,32 - Annex B). The size, form, density, and location of hairs on tobacco are very variable within the genus *Nicotiana*. The trichome density is affected by environmental conditions such as illumination, temperature, fertilization, and irrigation (10 - Annex B).

Based on scientific literature (1,2,3,10,11,14,27 - Annex B) trichomes of tobacco can be described as follows:

I. non-glandular trichome type is characterized by an acute or rounded terminal tip that is never spherical or swollen,

(a) simple (unbranched) trichome which is slender, uniseriate (= single-row), unicellular or multicellular (Fig. 2.1; 2.3 - Annex C),

(b) branched, multicellular trichome,

II. glandular trichome type is characterized by having terminal cell that is modified to secrete or store various substances,

(a) unbranched trichome with slender, uniseriate, multicellular (generally consisting of 2-6 cells) long stalk terminating in multiseriate (= multi-row) and multicellular gland that form an ellipsoid head; this is the most distinctive trichome type of tobacco (Fig. 1; Fig. 2.5; 2.8; 2.9 - Annex C),

(b) unbranched trichome with uniseriate, multicellular stalk terminating in simple, unicellular gland cell (Fig. 2.2; 2.4 - Annex C),

(c) hydathode - small trichome with a one- or two-celled stalk and a peltate disk of cells (consisting of 4-8 cells) at the tip; occasionally, there are two or three layers of cells in the peltate head (Fig. 2.6; 2.7 - Annex C),

(d) unbranched trichome with stalk which consist one or two very large, swollen, spherical or hemispherical basal cells and a few slender cells at the tip, terminating in a small, unicellular glandular head (Fig. 2.10; 2.11 - Annex C),

(e) unbranched trichome with uniseriate, multicellular stalk composed of a number of specific dark cells and unicellular gland cell at the tip (Fig. 2.12; 2.13 - Annex C),

(f) branched, multicellular trichome with small, unicellular glandular heads (Fig. 2.14; 2.15 - Annex C).

5.5.2. Stomata

The stomata are apertures in the epidermis, bounded by guard cells, which regulate the opening and closing of the stomatal pore (5 - Annex B). The epidermal cells directly adjacent to stomata constitute the subsidiary cells, which provide support for the functioning of guard cells. The guard cells contain chloroplasts, while subsidiary cells and other epidermal cells do not. The stomata play a critical role in photosynthesis and transpiration by regulating gas and water exchange in plants (9 - Annex B). The stomata are most common on green aerial parts of plants, particularly the leaves and less commonly on stems. Stomata vary in size, density, and distribution among different species. The shape of the epidermal cells around stomata in tobacco varies from polygonal to irregular (Fig. 3A,B,D - Annex C). According to stomata distribution on leaf surface, tobacco leaves represent the amphistomatous type where stomata occur equally on both adaxial (upper) and abaxial (lower) surfaces of leaves (e.g. 1,11,24 - Annex B). Tobacco provides, basically, two types of stomata: anomocytic and anisocytic (1,20,24 - Annex B).

Description of the stomata types in tobacco:

(a) anomocytic type; stomata are surrounded by a variable number of subsidiary cells which do not differ from other epidermal cells so far as size and shape are concerned (Fig. 3C, Fig. 4B - Annex C),

(b) anisocytic type; stomata are surrounded by three unequally sized subsidiary cells of which one is distinctly smaller than the other two (Fig. 3A,B,D; Fig. 4A - Annex C).

5.5.3. Vascular bundle

The characteristic feature of vascular plants is the presence of the vascular tissue system composed of conducting tissues, xylem and phloem. According to Reddy and Chary (2003) xylem tissue is used mostly for transporting water from roots to stems and leaves but also transports other dissolved compounds. Phloem is responsible for transporting food produced from photosynthesis from leaves to non-photosynthesizing parts of a plant such as roots and stems. The xylem and phloem are arranged in distinct, various structural units called vascular

bundles. Tobacco belongs to the family *Solanaceae* which is characterized by having the bicollateral type of vascular bundles (11,20,24 - Annex B). Schematic micrographs showing structure of the bicollateral vascular bundle specific to tobacco, characterized by “U” shape, presents figures 5 and 6 in Annex C.

5.5.4. Crystal structures

Calcium oxalate crystal formation play a central role in a variety of important functions, including tissue calcium regulation, protection from herbivory, and metal detoxification (18 - Annex B). According to Mhinana et al. (2010) calcium oxalate crystals are composed of calcium (32.46%), oxygen (58.97%), sulphur (7.78%), potassium (0.99%) and chlorine (0.84%). They generally form within cells although extracellular crystals have been reported. Calcium oxalate crystals vary widely in size and appearance, upon which they can be grouped into classes (17 - Annex B). The presence or absence of the different crystal types represent useful taxonomic characters. In tobacco, calcium oxalate is frequently found in the form of solitary crystals, clusters and crystal-sand (mass of tiny individual crystals), and occur in the mesophyll tissue, in the leaf epidermis, and sometimes in the gland cells of trichomes (11,15,16,19,24 - Annex B); Fig. 7 - Annex C). Calcium oxalate crystals are best visible after applying a chloral hydrate solution followed by simple polarized light microscopy observations.

5.6. Quality control

5.6.1. Laboratory should have a policy for quality-control testing establishing the number and type of quality-control materials needed and the frequency of testing them.

5.6.3. The positive quality-control sample can be:

(a) a sample prepared from a reference tobacco specimen

(b) a sample prepared from tobacco-containing material, which had been tested by inter-laboratory proficiency test participants

5.6.5. The negative quality-control sample can be:

(a) a sample prepared from a reference non-tobacco specimen

(b) a sample prepared from tobacco-free material, which had been tested by inter-laboratory proficiency test participants

6. Calculation

6.1. The presence of tobacco in the sample determines the identification of four anatomical features.

6.2. Test outcomes are classified as "positive" when tobacco has been identified in the test sample or “negative” when no tobacco has been identified.

6.3. Sample is analyzed in duplicate.

6.4. Two “positive” results are required to correctly conclude that there is tobacco in the sample.

6.5. In the case of inconclusive, uncertain or negative results, the sample shall be re-tested.

6.6. The results of the sub-tests of the real sample as well as the quality-control samples shall be recorded on the dedicated forms

7. Expression of results

7.1. The final result concerning tobacco presence is expressed as “identified/not identified”, “present/absent” or “detected/not detected”

8. Precision

The method is qualitative, precision data are not relevant.

If need be, the identification can be confirmed by DNA analysis.

Annex A

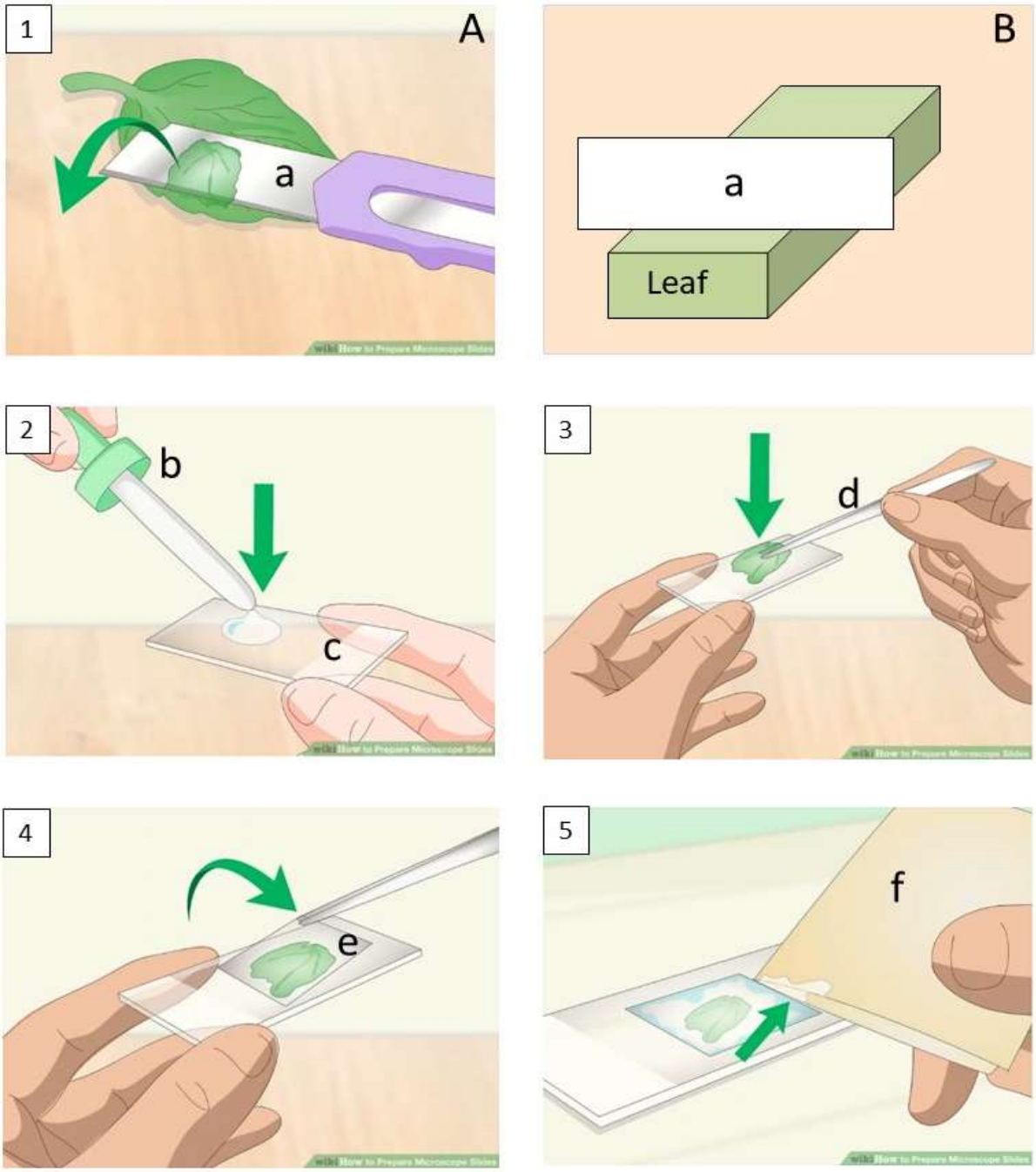

Fig. 1. Microscopic slide preparation. (1) schematic diagram showing: A - stripping off leaf epidermis; B - making a transversal slice; a - razor blade; (2) placing a drop of fluid in the center of the slide; b - pipette c – slide; (3) positioning a sample on liquid using tweezers (d); (4) placing, at an angle, one side of the coverslip (e) against the slide by making contact with outer edge of the liquid drop; (5) removing excess liquid with the paper towel (f); source: https://www.wikihow.com/, amended.

Annex B

References

1. Adedeji O., Ajuwon O.Y., Babawale O.O. (2007). Foliar epidermal studies, organographic distribution and taxonomic importance of trichomes in the family *Solanaceae*. International Journal of Botany, 3(3):276–282.
2. Akers C.P., Weybrew J.A., Long R.C. (1978). Ultrastructure of glandular trichomes of leaves of *Nicotiana tabacum* L., cv Xanthi. American Journal of Botany, 65(3):282–292.
3. Dai X., Wang G., Yang D.S., Tang Y., Broun P., Marks M.D., (...), Zhao P.X. (2010). TrichOME: a comparative omics database for plant trichomes. Plant physiology, 152(1):44–54.
4. Eleftheriadou M., Tsimillis K.C. (eds). (2013). Eurachem guide: Accreditation for Microbiological Laboratories, 2nd ed.
5. Esau K. (1965). Plant Anatomy, 2nd ed., Wiley.
6. Evans, W.C. (2009). Trease and Evans Pharmacognosy, 16th ed., Saunders Elsevier.
7. Fahn A. (2000). Structure and function of secretory cells. Advances in botanical research, 31: 37–76.
8. Glas J.J., Schimmel B.C., Alba J.M., Escobar-Bravo R., Schuurink R.C., Kant M.R. (2012). Plant glandular trichomes as targets for breeding or engineering of resistance to herbivores. International journal of molecular sciences, 13(12):17077–17103.
9. Hetherington A.M., Woodward F.I. (2003). The role of stomata in sensing and driving environmental change. Nature, 424(6951):901–908.
10. Huang M., Zhang H., Wang Z., Niu D., Li Y., Cui H. (2018). Comparative studies of leaf surface chemical biosynthesis in different tobacco cultivars. *Acta Physiologiae Plantarum*, 40(4):67.
11. Jacquin-Dubreuil A., Breda C., Lescot-Layer M., Allorge-Boiteau L. (1989). Comparison of the effects of a microwave drying method to currently used methods on the retention of morphological and chemical leaf characters in *Nicotiana tabacum* L. cv Samsun. Taxon, 38(4):591–596
12. Johnson A.W., Severson R.F., Hudson J. (1985) Tobacco leaf trichomes and their exudates. Tobacco Control, 27:67–72.
13. Kennedy B.S., Nielsen M.T., Severson R.F. (1995) Biorationals from *Nicotiana* protect cucumbers against *Colletotrichum lagenarium* (Pass) ell and halst disease development. Journal of Chemical Ecology, 21:221–231.
14. Marks C.E., Newbigin E., Ladiges P.Y. (2011). Comparative morphology and phylogeny of *Nicotiana* section *Suaveolentes* (Solanaceae) in Australia and the South Pacific. Australian systematic botany, 24(2):61–86.
15. Meyberg M., Krohn S., Bruemmer B., Kristen, U. (1991). Ultrastructure and secretion of glandular trichomes of tobacco leaves. Flora, 185(5):357–363.
16. Mhinana Z., Mayekiso, B., Magwa, M. L. (2010). Anatomy and morphology of *Nicotiana glauca* with regard to its crystals characterization. African Journal of Plant Science, 4(6):172–178.
17. Mukherjee P.K. (2019). Quality Control and Evaluation of Herbal Drugs: Evaluating Natural Products and Traditional Medicine. Elsevier.
18. Nakata P.A. (2003). Advances in our understanding of calcium oxalate crystal formation and function in plants. Plant Science, 164(6):901–909.
19. Nielsen M.T., Akers C.P., Järlfors U.E., Wagner, G. J., Berger, S. (1991). Comparative ultrastructural features of secreting and nonsecreting glandular trichomes of two genotypes of *Nicotiana tabacum* L. Botanical Gazette, 152(1):13–22.
20. Pedroso A.N.V., Alves E.S. (2008). Anatomia foliar comparativa das cultivares de *Nicotiana tabacum* L. (Solanaceae) sensível e tolerante ao ozônio. *Acta Botanica Brasilica*, 22(1):21–28.
21. PM 7/98 (4) (2019) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. EPPO Bulletin, 49: 530–563.

22. Reddy S., Chary S. (2003). University botany: Gymnosperms, Plant anatomy, Genetics, Ecology, vol. II. New Age International Publisher.
23. Stace C.A. (1991). Plant taxonomy and biosystematics. 2nd ed., Cambridge University Press. Cambridge.
24. Tokumoto H., Shimomura H., Hakamatsuka T., Ozeki Y., Goda Y. (2016). Detection of *Nicotiana tabacum* leaf contamination in pharmaceutical products. Biological and Pharmaceutical Bulletin, 39:1263–1272.
25. Trullols, E., Ruisánchez, I., Rius, F. X. (2004). Validation of qualitative analytical methods. TrAC Trends in Analytical Chemistry, 23(2):137–145.
26. Upton R., Graff A., Jolliffe G., Länger R., Williamson E. 2011. American Herbal Pharmacopoeia: Botanical Pharmacognosy – microscopic characterization of botanical medicines. Boca Raton: CRC Press.
27. Uzelac B., Janošević D., Stojičić D., Budimir S. (2015). In vitro morphogenesis and secretion of secondary metabolites of *Nicotiana tabacum* tall glandular trichomes. Botanica Serbica, 39(2):103–110.
28. Wagner G.J. (1991) Secreting glandular trichomes: more than just hairs. Plant Physiology, 96(3):675–679.
29. Wagner G.J., Wang E., Shepherd R.W. 2004. New approaches for studying and exploiting an old protuberance, the plant trichome. Annals of Botany, 93:3–11.
30. World Health Organization. (1998). Quality control methods for medicinal plant materials. WHO.
31. World Health Organization. (2011). Quality control methods for herbal materials. WHO.
32. Yan N., Du Y., Liu X., Zhang H., Liu Y., Zhang Z. (2019). A review on bioactivities of tobacco cembranoid diterpenes. Biomolecules, 9(1):30.

Annex C

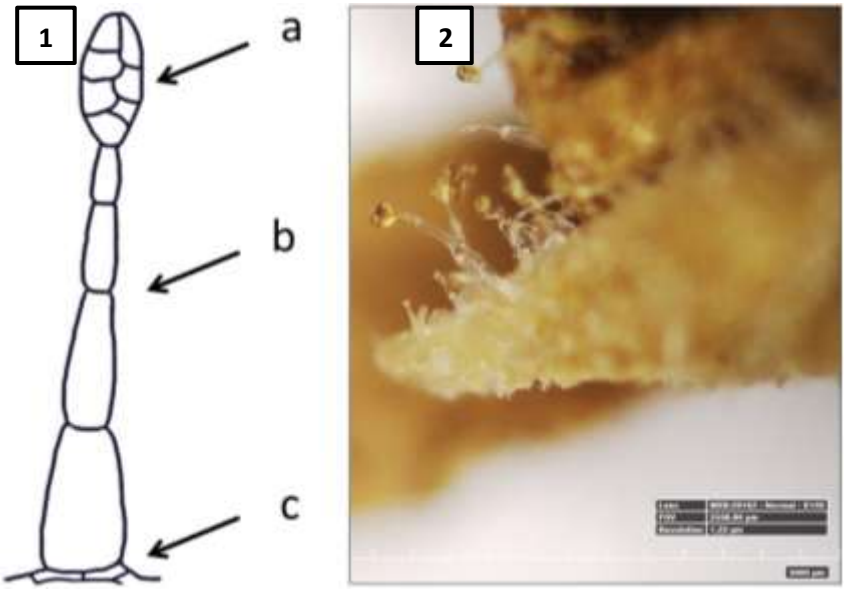

Fig. 1. Glandular trichome, type II(a). (1) schematic diagram showing structure of the trichome: a – multicellular gland head; b – multicellular stalk; c – basal cells; (2) microphotography; digital microscope Hirox RH-2000; source: own study.

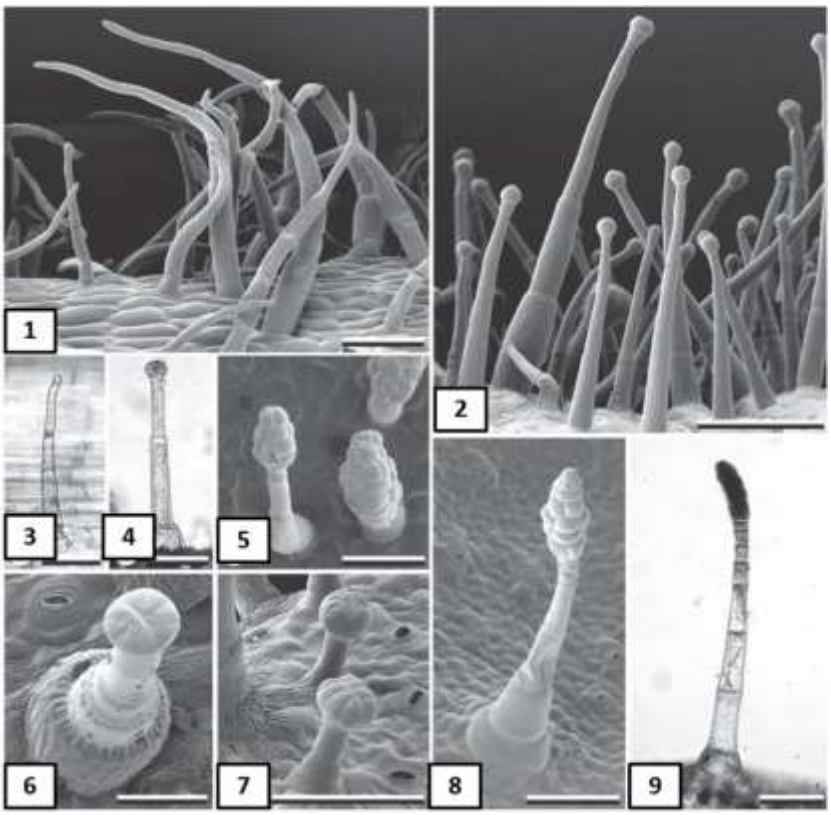

Fig. 2. Scanning electron micrographs and light micrographs of tobacco trichomes types acc. to Marks *et al*., 2011 (amended). Description of the trichomes types is included in point 7.1; scale bars: 200 µm (1,12,13), 100 µm (2,4,5,7,8,9,10,14,15), 50 µm (3,6,11).

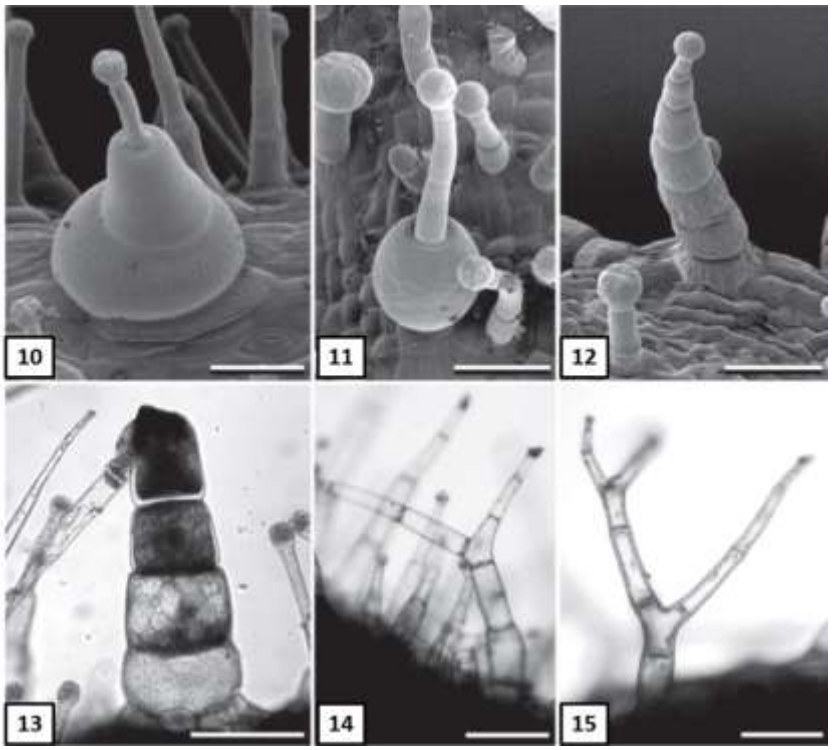

Fig. 2. Continued.

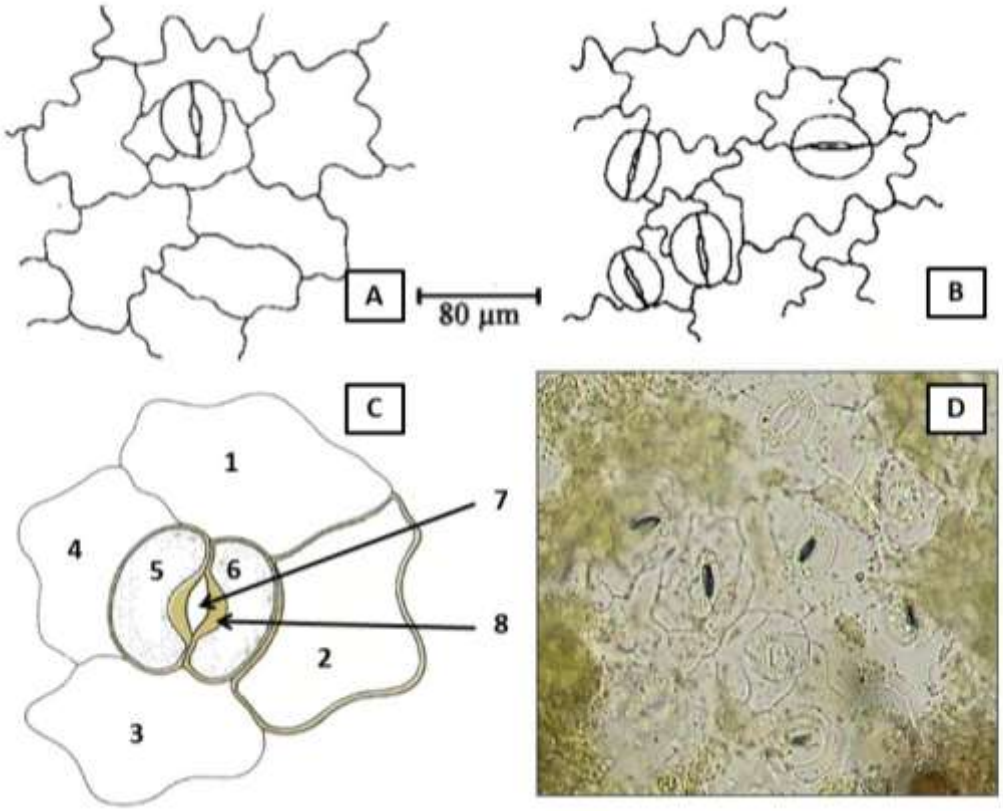

Fig. 3. Tobacco leaf epidermis showing stomata: (A) anisocytic stomata and epidermal cells in the adaxial surface of leaf; (B) anisocytic stomata and epidermal cells in the abaxial surface of leaf; (C) diagram showing structure of anomocytic stomata: 1-4 – subsidiary cells, 5,6 – guard cells, 7 – thickened inner wall of guard cell, 8 – stomatal pore; (D) microphotography, microscope Nikon Eclipse E400, 10x objective; source: (A, B) acc. to Adedeji *et al*., 2007, amended; (C) acc. to Reddy and Chary, 2003, amended; (D) own study.

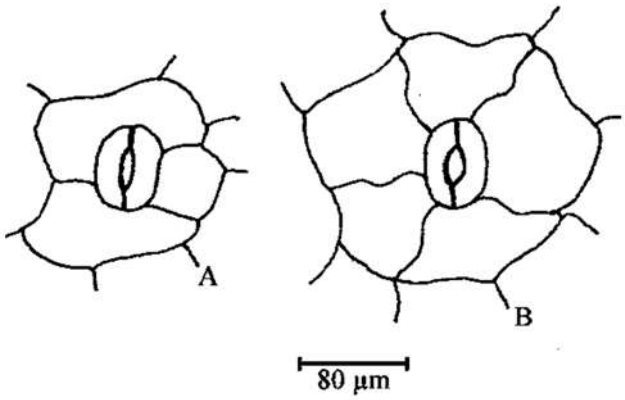

Fig. 4. Stomata in tobacco: (A) anisocytic stomata; (B) anomocytic stomata; source: acc. to Adedeji *et al*., 2007, amended.

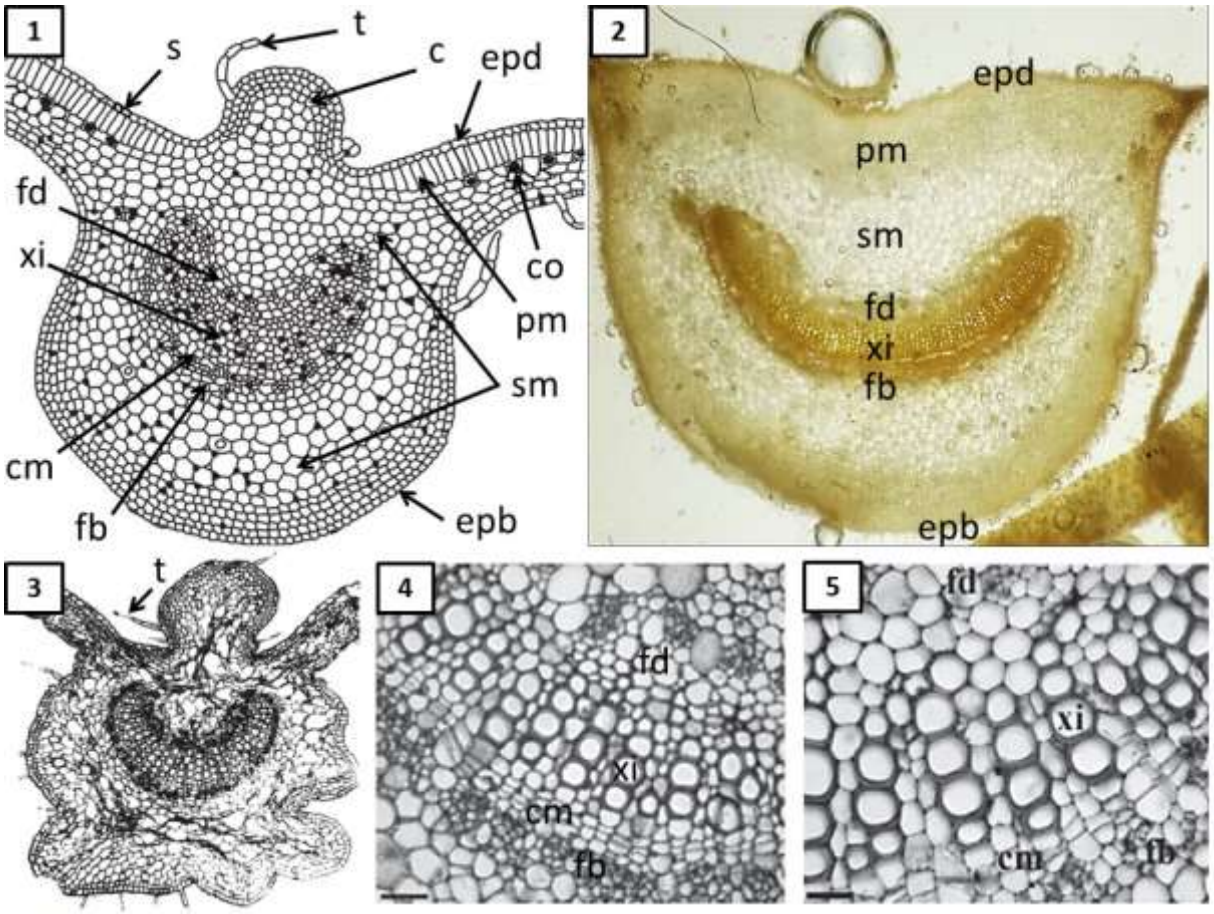

Fig. 5. Diagram showing the structure of the bicollateral vascular bundle of tobacco - transverse section of leaf through the minor vein; t – trichome; epd – epidermal cells of adaxial leaf surface; c – collenchyma; s – stomata; pm – palisade mesophyll; sm – spongy mesophyll; fd – adaxial phloem; xi – xylem; cm – cambium; fb – abaxial phloem; co – calcium oxalate crystal; epb – epidermal cells of abaxial leaf surface; scale bars: 100 µm (3), 50 µm (4,5); source: (1) http://www.pharmacy180.com/, amended; (2) microphotography, own study; (3) acc. to Jacquin-Dubreuil *et al*., 1989; (4,5) acc. to Pedroso and Alves, 2008, amended.

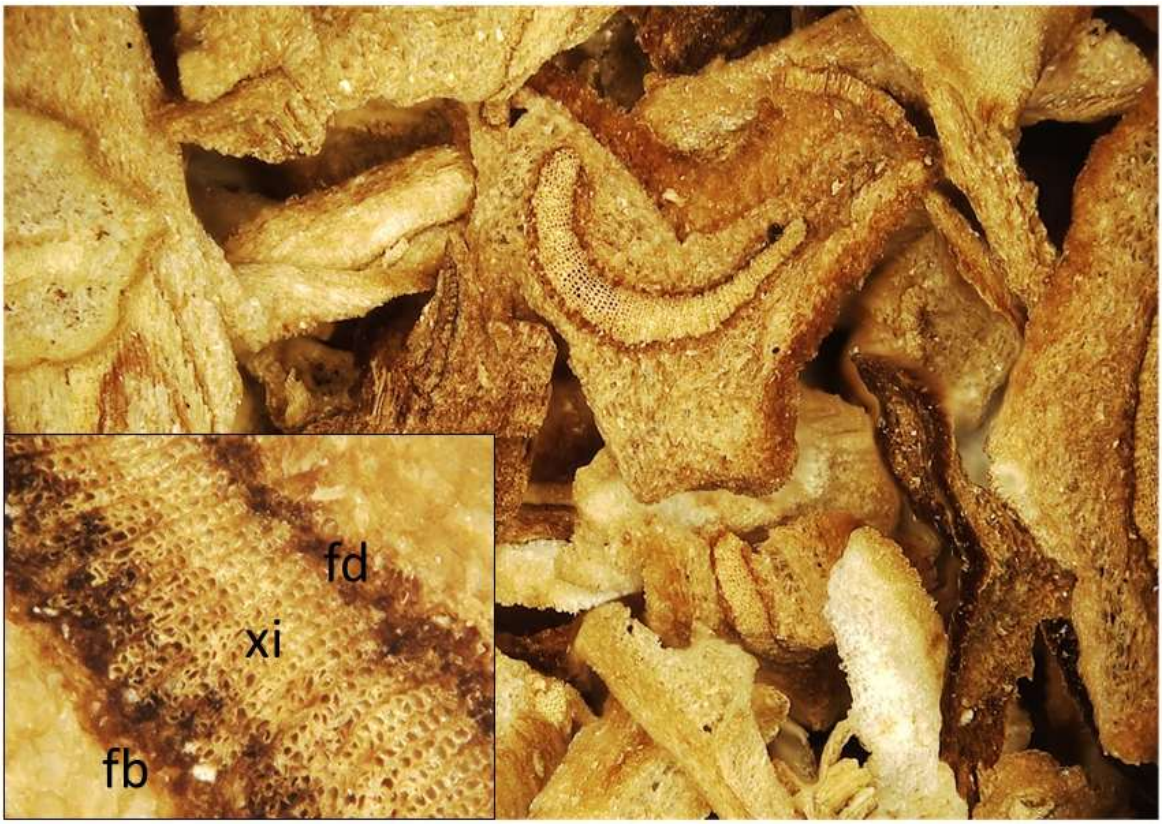

Fig. 6. Transverse section of the midrib (= the main vein, stem) of tobacco leaf; fd – adaxial phloem; xi – xylem; fb – abaxial phloem; source: own study (microphotography; digital microscope Hirox RH-2000).

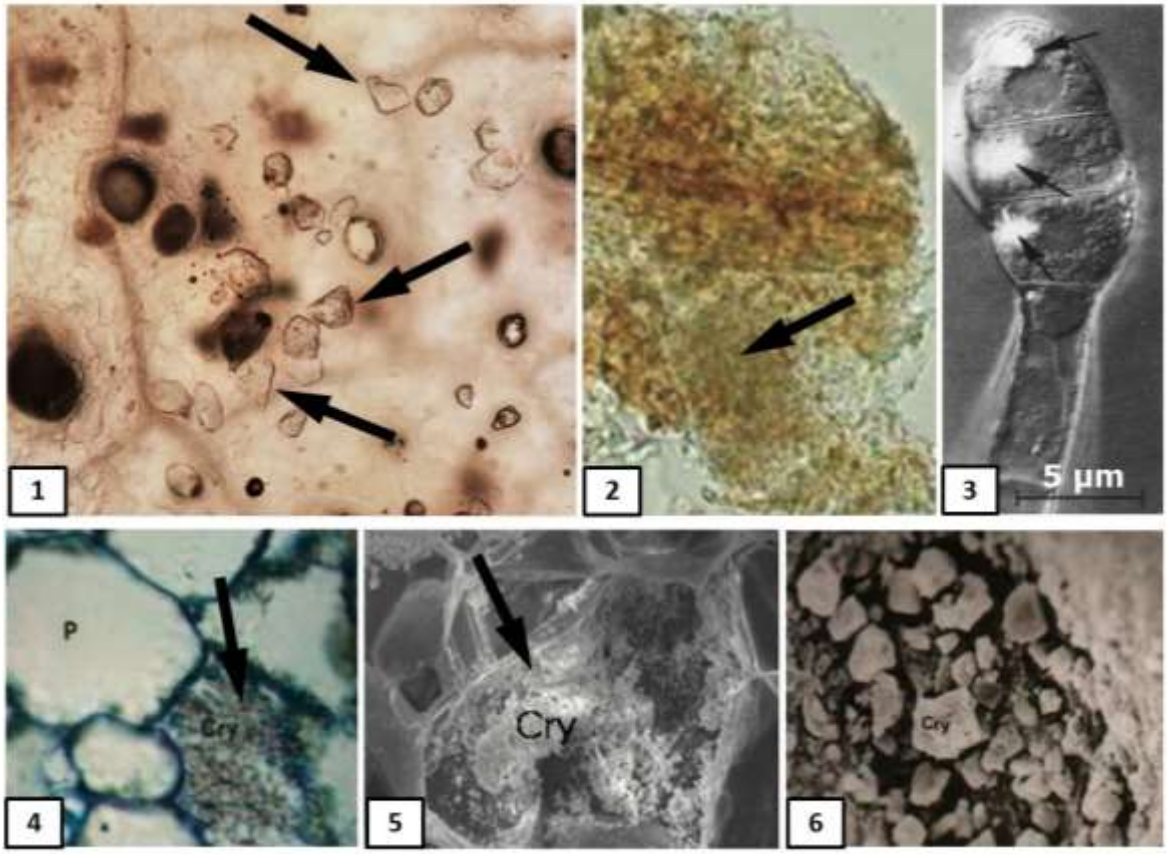

Fig. 7. Calcium oxalate crystals in tobacco (arrows; Cry): (1,2,4,5) in the form of crystal-sand; (3) in the form of small solitary crystals in the gland cells of trichome; (6) cluster of crystals in the epidermal cell; P – parenchyma; source: (1) own study, microscope Nikon Eclipse E400; 10x objective; (2) acc. to Tokumoto *et al.,* 2016, amended; (3) acc. to Nielsen *et al.,* 1991, amended; (4,5,6) acc. to Mhinana *et al.,* 2010, amended.