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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2018 15(3):2721-2732. **OPEN ACCESS**

Botanical features and genetic profiling of *Bauhinia retusa* Roxb. growing in Egypt

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Bauhinia retusa Roxb. (Caesalpinioideae) is a large semi-deciduous tree cultivated in Egypt. In this work, for the first time, botanical features including micro- and macro-morphological studies of different organs of *B.retusa* Roxb. As well as, DNA fingerprinting were brought to light. This study presents an examination of the botanical features of the leaves, petioles, stems and flower buds through microscopical observation of the prepared entire, transverse sections and isolated elements of these organs using light microscope. Also, Extraction of the Deoxyribonucleic acid (DNA) of *B. retusa* Roxb. from leaf samples and Random Amplification of Polymorphic DNA (RAPD) analysis was done using ten primers of arbitrary sequences. Resulting in 22 fragments of amplified product, which were generated by ten primers; the primer Operon C-19 produced the highest number of rapid amplified polymorphic (RAPD) fragments (4 fragments), while the least number of fragments was produced by Operon A-18, Operon B-2 and Operon B-9 (1 fragment). Micro- and macro-morphological characters in contribution to DNA fingerprinting can be considered as the identifying parameters to authenticate and recognize *B. retusa* Roxb.at the anatomical and genetic level.

Keywords: Botany; DNA fingerprinting; Fabaceae; Plant anatomy; RAPD.

INTRODUCTION

The Leguminosae (Also, known as Fabaceae, legume, pea, or bean family) is a big and economically important family of blooming plants. This comprises 730 genera and over 19,500 species. Therefore, it considered the third-largest plant family in the number of species. Botanists divided Leguminosae family into three sub-families (Caesalpinioideae, Mimosoideae, and Papilionoidea) and 35 tribes (Colville et al., 2015). Sub-family Caesalpinioideae are mainly trees, shrubs and rarely herbs. It comprises 4 tribes (Colville et al., 2015), 56 genera and 650 species (Sharma and Kumar, 2013). One of the most large and important genus of family Caesalpinioideae are *Bauhinia* which is named by Linnaeus (Sharma and Kumar, 2013). *Bauhinia L*. was named after two Swiss botanists, the Bauhin brothers. Also, this genus known as “Cow’s hoof” due to the shape of their leaves (El-Sayed et al., 2015). It includes 300–350 species of trees and shrubs distributed mostly in tropical regions (Wunderlin, 2010). According to new phylogenetic studies, and depending on the difference in anatomical characters , *Bauhinia L*. is sub-classified into 9 genera (Farag et al., 2015). Various species, belonging to genus *Bauhinia* L., are able to decrease the oxidative stress resulted from various agents and diseases. So, it’s used mainly as an anti-diabetic remedy (Silva et al., 2011). *Bauhinia retusa* (Caesalpiniaceae) is a deciduous tree distributed in warmer parts of world (Semwal and Sharma, 2011) and was cultivated in Egypt for its warm weather. Since,

Anatomical characters of plants are greatly affected by the environmental conditions. Therefore, it's undependable separately for the recognition of the plant species. So, In recent studies genetic profiling is widely used in addition to botanical studies for authentication of different and closely related plant species as its not affected by environmental diversity (Shinde and Dhalwal, 2010). Genetic profiling refers to the use of techniques to appear the specific DNA profile for a specific plant species which is considered as a fingerprint, using PCR system for the amplification of DNA (El-hawary et al., 2016).
Random Amplified Polymorphic DNA (RAPD) results in the amplification of several DNA products using a single 'arbitrary' primer in a polymerase chain reaction (PCR) (Shinde et al., 2007).

Since, there's no genetic and botanical study has been reported on *B. retusa* Roxb. Therefore, this study aims to reveal the DNA fingerprinting in addition to the macro- and micro-morphological identification and characterization of the plant.

MATERIALS AND METHODS

Plant material

Fresh leaves, petiole, stem, flowers, pericarp and seeds of *B. retusa* Roxb. were collected from the botanical garden in Aswan, Egypt in the winter of 2016. The plant was authenticated by Miss. Therese Labib, head specialist for plant identification, El-Orman Public Garden, Cairo, Egypt. Voucher specimen kept in the Botanical garden in Aswan, Aswan, Egypt with number 23A.

Botanical studies

Specimens of leaves, petiole, stem and flowers of *B. retusa* Roxb. were preserved in 10 ml formalin, 5 ml glacial acetic acid and 85 ml ethanol 70% (FAA) for 48 hours to avoid the loss of the specimen tissues. Washing of the selected specimens in 50% ethyl alcohol is carried out, then dehydrated in a normal butyl alcohol series, after that, Specimens were immersed in paraffin wax of melting point 56^{o}C and Cut off to a thickness of 20 micron. Safranin-light green stain is used twice for staining of the obtained sections ,then Clarified in xylene and fixed in Canada balsam (Nassar and El-Sahhar, 1998). Slides were analyzed microscopically using light microscope and photographed using digital camera.

Other specimens of leaves, petiole, stem and flowers were air-dried and powdered separately then boiled with 5% KOH aqueous solution for preparation of the isolated elements. Then examined microscopically using light microscope and photographed using digital camera.
Surface preparation of the leaves was taken using razor and forceps, examined under light microscope and photographed using digital camera. Photos were taken for *B. retusa* Roxb. tree and the entire samples of leaves, petiole, stem, seeds, pericarp and flowers by digital camera.

DNA extraction

B. retusa Roxb. leaf samples that are free from any pathogenic symptoms were ground under liquid nitrogen to a fine powder, then DNA extracted using DNeasy plant Mini Kit (QIAGEN) as described by Williams (Williams et al.,1990).

2.3.1 Polymerase Chain Reaction (PCR)

PCR amplification was performed using 10 random arbitrary primers synthesized by (Operon biotechnologies, Inc. Germany). Names and sequences of the primers are shown in Table 1**.** Amplification was conducted in 25µl reaction volume containing the following reagents: 2.5µl of dNTPs (2.5mM), 2.5µl MgCl2 (2.5mM), 2.5µl of 10 x buffer, 3.0 µl of primer (10 pmol), 3.0µl of template DNA (25 ng/µl), 1µl of *Taq* polymerase (10/µl) and 10.5µl of sterile double distilled H2O. The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94^{o}C for 4 min followed by 45 cycles of 1 min at 94^{o}C, 1 min at 36^{o}C and 2 min at 72^{o}C. The reaction was finally stored at 72^{o}C for 10 min.

Gel Electrophoresis and Staining

Amplified products were size-fractioned using ladder marker (100 bp) Fermentas.co by electrophoresis in 1.5 % agarose gels in TBE buffer at 120 V for 1 h. The bands were visualized by ethidium bromide under UV fluorescence and photographed.

Data analysis

The similarity matrices were done using Gel works ID advanced software UVP-England Program. DICE computer package was used to calculate the pairwise difference matrix and plot the phenogram among species (Dice, 1945, Yang and Quiros, 1993).

RESULTS AND DISCUSSION

Macro-morphological studies of different organs of *B. retusa* Roxb.

In (Figure 1, 2 and 3) *B. retusa* Roxb. is a large semi-deciduous tree (Figure 1a), cultivated in Egypt. It grows up to 10-15 m in height with branches spread 3–6 m outwards which droop at the ends (Figure 1b), has a huge leaning trunk and large lobed dark green leaves. It also shows white small flowers that bloom in winter from the end of November till the end of January. After the flowering period, flowers wither and most of them produce large woody, flat and elongated brown dehiscent legumes that enclose rounded rough brown seeds. The main stem (trunk) is huge, very hard, thick and cylindrical with rough surface covered by a thick brown cork showing irregular longitudinal fissures and cracks (Figure 1a). The trunk measures about 100-150 cm in diameter at the base and grows obliquely for about 3-5 m, the trunk then continues growing by dividing into small branches and the branches grows to smaller branches. Branching is monopodial and the branches hold pairs of alternate leaves and/or inflorescences.

The young branches (Figure 1b) are green, flexible, glabrous or slightly hairy and cylindrical with about 0.5-1cm diameter. While, the old branches are brown, woody, rough and cylindrical with diameter 1-1.5 cm.

Leaves (Figure 2a, b, c) are simple, petiolate, exstipulate, and alternate. The mature leaves measures 17-18 cm length and about 10-15 cm width, slightly broader than long. While, the young leaves have a length of about 14-15cm length and 8-9 cm width with lobed margin at the base and cordate shape.

The lamina is subcoriaceous, glabrous measures 13-14cm length and 15cm width. With entire margin, apex entire or notched, base cordate or truncate. The lower surface is green hairy compared to the dark green glabrous upper surface. The midrib is more prominent in the lower surface and the venation is palmate reticulate usually 8-10 nerved. The petiole is green, cylindrical, 6-7 cm length, swollen at both ends (pulvinate) (Figure 2a, c) Inflorescence is compound raceme (panicle) composed of many florets most are in the bud form with or more developed flowers. (Figure 3 a, b and c)

Each Floret (Figure 3a, b) is very small 3 cm length, 2cm width, pedicellate, and off-white in color with dark spots, small bracts, hermaphrodite, zygomorphic and covered with soft hairs. Calyx 5-7.5 mm is concave, silky pubescent and with white color. Petals 1cm long, bented, obovate, hairy, white spotted with purple. Androecium consists of 3 stamens. Gynoecium consists of an ovary that is densely hairy on the margins. The style is elongated and sticky stigma with red color.

The fruits are twisted woody, very hard legumes 10-17cm long, 4-5 cm width, oblong and dehiscent. Each legume encloses 5-7 Seed. (Figure 3 d, f)

The seeds are flat, orbicular, dark-brown, hard and smooth measures 1.5-2 cm long and 1.5-1.8 cm width. (Figure 3 e)

Micro-morphological studies of different organs of *B. retusa* Roxb.

The leaf lamina

The upper epidermis appears as single layer in the transverse section and the surface preparation showed that it consists of polygonal isodiametric cells with straight anticlinal wall and smooth cuticle, and devoid of stomata. (*Figure 4* b) The lower epidermis consists of polygonal isodiametric cells larger than upper epidermis with straight anticlinal wall and smooth cuticle, and contains numerous Anomocytic stomata (*Figure 4* a). Numerous elongated unicellular non-glandular hairs are found in the epidermis. The mesophyll is dorsiventral, with 2 compact layers of palisade cells discontinuous over the midrib which are cylindrical, columnar, straight, closely packed, thin walled and contain green chloroplasts (Figure 4d). Several layers of the spongy tissue are present which are parenchymatous cells with large intracellular spaces. The midrib is more prominent in the lower surface and consists of 1-2 rows of polygonal collenchyma cells beneath the upper and lower epidermis; followed by two layers of parenchymatous cells beneath the upper epidermis and 8-10 rows of large polygonal parenchymatous cells in the lower part some of them contain prisms and clusters of calcium oxalate

(Figure 5a, b). The pericycle is formed of a continuous ring of lignified fibers 2-3 rows downwards and 10 rows upwards, the fibers are lignified with wide lumen and surrounded by parenchymatous cells contain prisms of calcium oxalate. (Figure 4d**Error! Reference source not found.** and 5c)

The vascular tissue consists of collateral arc-shaped vascular bundle divided into two uneven parts. The phloem located underneath consists of sieve elements and phloem parenchyma. The

xylem is endarch with spiral vessels wood fibers and wood parenchyma.

Figure 1: ***Macro morphology*** **of** ***Bauhinia retusa Roxb. Tree and branches: (*****A) The tree of** ***B. retusa*****, (B) The lateral branches**

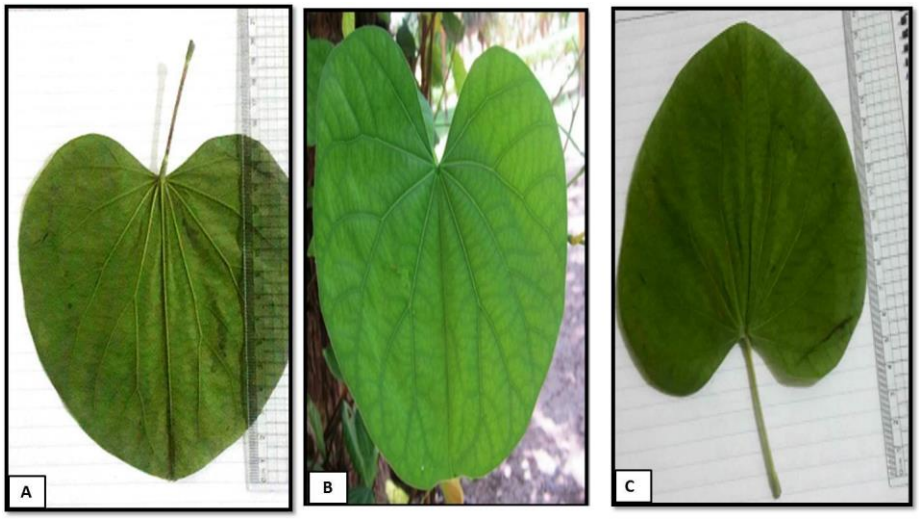

Figure 2: ***Macro morphology*** **of** ***Bauhinia retusa Roxb leaves*****: A: The lower surface of the leaf and the petiole, (B) the entire leaf, (C) The upper surface and the petiole.**

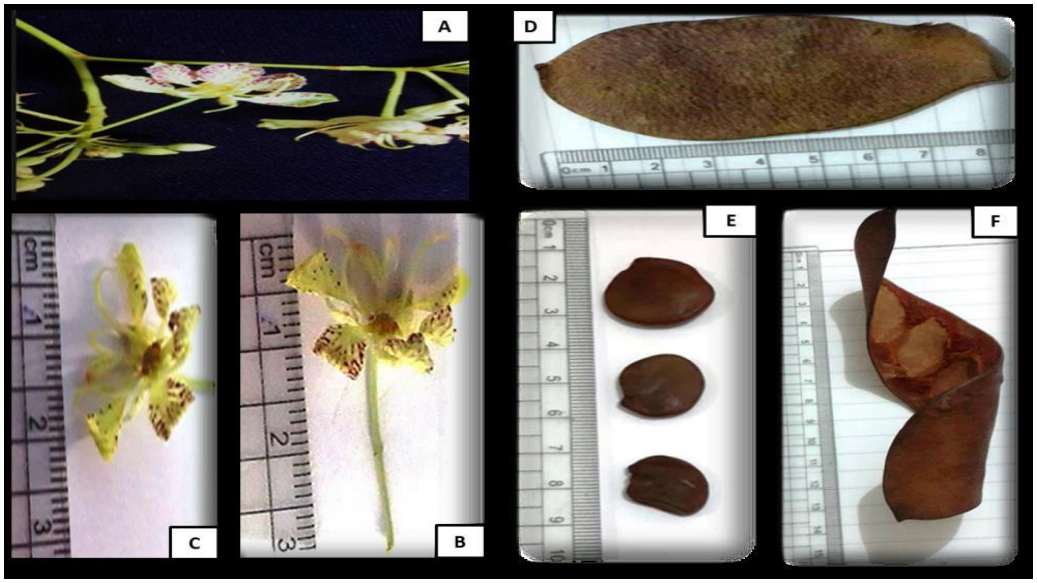

Figure 3: Macro-morphology of the flowers, fruit, seeds and pericarp. (A) The inflorescence, (B) The floret and the pedicel, (C) The floret of *B. retusa*, (D) the fruit of *B. retusa*, (E) The seeds of *B. retusa*, (F) The pericarp of *B. retusa* .

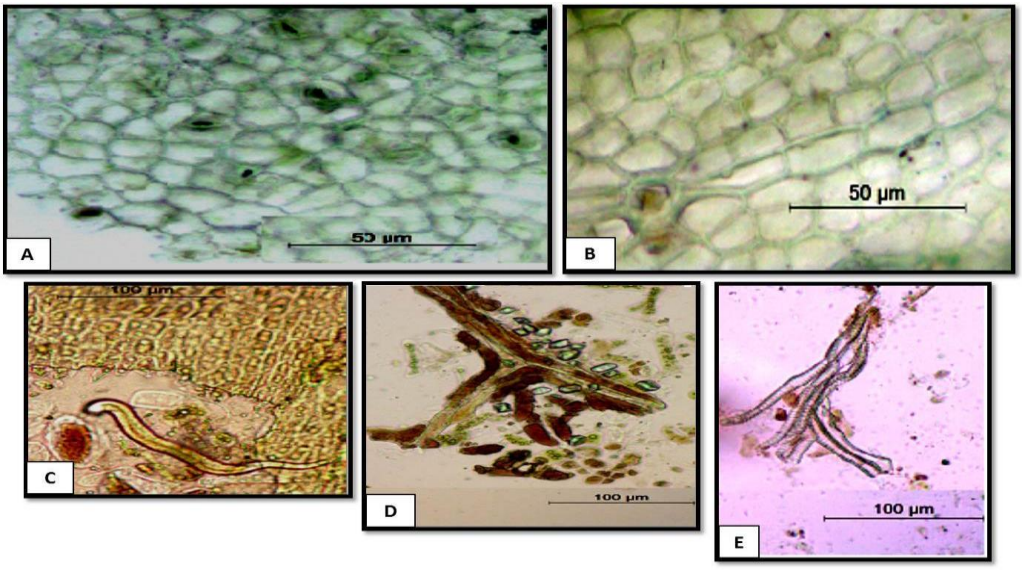

Figure 4: The isolated elements of the leaf lamina: (A) lower epidermis showing the stomata, (B) Upper epidermis, (C) wavy unicellular non-glandular hair, (D) Pericyclic fibers and prisms of calcium oxalate (crystal sheath), (E) Spiral xylem vessels.

The leaf petiole

A transverse section in the leaf petiole shows that it has a circular outline with slight projection from one side (Figure 6a). The section showed that the petiole consists of the epidermis consists of one layer of tangential elongated cells. The cortex consists of 1-2 rows of rounded collenchymatous cells followed by 5-6 small parenchymatous cells contain prisms of calcium oxalate. The pericycle consists of a continuous ring of lignified with narrow lumen pericyclic fibers. The fibers are surrounded by parenchymatous cells contains prisms of calcium oxalate forming crystal sheath (Figure 7a). The vascular tissue consists of a collateral vascular bundle divided to 2 unequal parts. The phloem consists of sieve elements, fibers and phloem parenchyma.

The xylem is endarc located underneath the phloem and the isolated elements showed lignified vessels some of them are narrow with spiral thickening and some with wider with annular thickening. The pith consists of 5-8 rows of rounded parenchymatous cells some of them contain prisms and clusters of calcium oxalate. Also, contain patches of non-lignified fibers with wide lumen and acute apices. A small central vascular bundle appears in the center of the pith and contains an outer layer of inner xylem followed by inner phloem layer and enclosing narrow central pith. (Figure 6a, b)

The stem

The transverse section shows that the outline is four armed or quadrilateral shaped and consists of a layer of epidermis of tangential elongated cells followed by the cortex layer that shows 6-9 rows of c Figure 8a). Next to cortex the pericyclic layer takes place and is formed of a continuous ring of 6-7 rows of lignified fibers with narrow lumen. The vascular tissue consists of a continuous layer of open collateral vascular bundle separated by cambium and transversed by medullary rays. The phloem forms a continuous ring and consists of phloem elements formed of sieve tubes, companion cells, phloem fibers. The phloem fibers (Figure 9f) are with slightly lignified walls, narrow lumens, acute tapering apices surrounded by a sheath of parenchymatous cells containing prisms of calcium oxalate. The xylem is endarch appears as scattered patches and is formed of radially arranged xylem vessels. The xylem vessels are wide, lignified with spiral thickening. The pith takes a large portion of the total stem diameter. Consists of large, polygonal, thin walled, with wide intracellular spaces parenchymatous cells.

The flowers

A transverse section in the flower bud was taken and showed that it's with nearly more or less circular outline and consists of consecutive layers of calyx, corolla, androecium and gynoecium respectively (Figure10a). The calyx consists of 3 sepals. Each sepal comprising upper and lower epidermis and mesophyll in between transversed by small vascular bundles.

The upper and lower epidermis is formed of single row of narrow polygonal isodiametric cells with smooth cuticle and straight anti-clinal wall showing various non-glandular trichomes. The mesophyll is formed of 1-2 rows of collenchymatous cells followed by 8-10 rows of slightly wide, polygonal isodiametric parenchymatous cells. Some vascular bundles may appear in between the parenchymatous cells due to the small veins present in the calyx. The trichomes (Figure 11b, c) are numerous, unicellular and non-glandular with 1 or 2 basal cells. The corolla is plano convex consists of five petals. Each petal comprising upper and lower epidermis and mesophyll in between, transversed by small vascular bundles. The upper and lower epidermis is formed of single row of slightly elongated cells with smooth cuticle and straight anticlinal wall. Stomata and hairs are absent. The mesophyll is homogenous containing 6-10 rows of densely packed narrow parenchymatous cells contain scattered clusters of calcium oxalate. The androecium consists of three stamens. Each stamen is contains a filament and anther. The anther shows two lobes attached by a connective tissue. Each anther lobe has 2 pollen sacs containing numerous smooth, spherical pollen grains with three germ pores. The anther wall consists of epidermis, fibrous layer and tapetum (Figure 10a, c and Figure 11f, g). The ovary in transverse section appears oval in outline, unilocular with ovule attached to a marginal placenta. The ovary wall consists of an epidermis which is densely hairy, and enclosing parenchymatous cells containing scattered clusters of calcium oxalate and traversed by several vascular bundles (Figure 10b).

DNA fingerprint

Polymerase chain reaction (PCR) with ten random primers produced a total of 22 fragments as shown in (Figure 12) and (Table 2)**.**

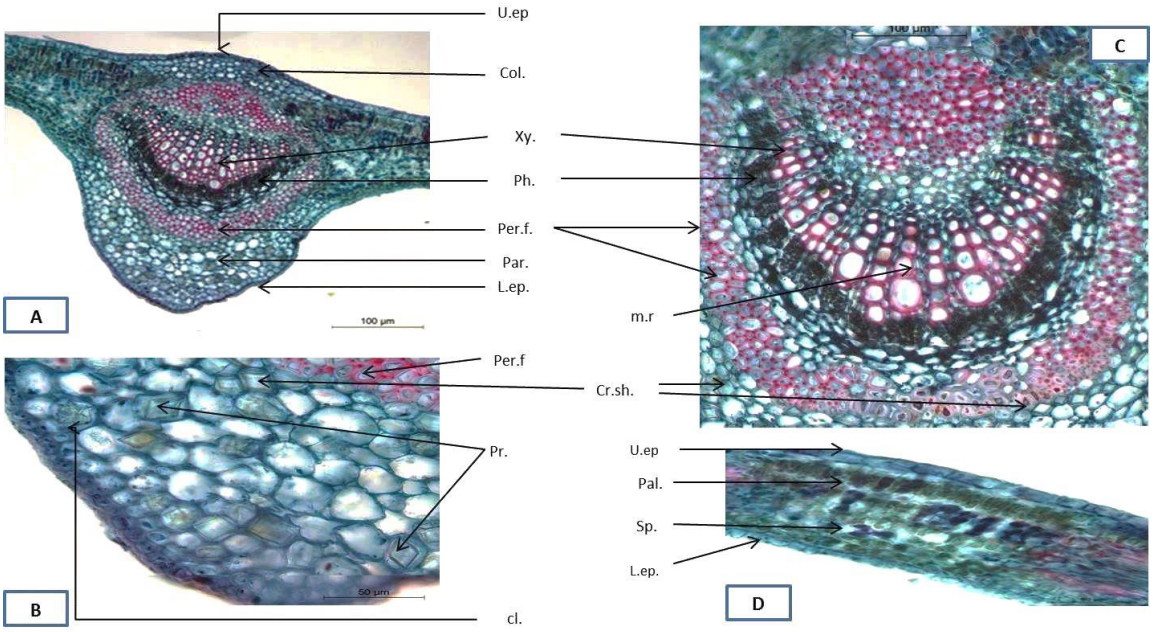

Figure 5: A transverse section in the leaf lamina of *B.retusa Roxb*.and magnified regions. (A) Entire T.S, (B) A magnified section of the epidermis and hypodermis region, (C)A magnified section of the vascular bundle, (D)A magnified section of the lamina

Where; U.ep, Upper epidermis; col., collenchyma; Xy, xylem; ph., phloem; Per.f., pericyclic fiber; par, parenchyma; L.ep., lower epidermis; m.r., medullary rays; cr.sh., crystal sheath; pr., prisms of calcium oxalate; cl., clusters of calcium oxalate; pal., palisade cells; sp., spongy cells

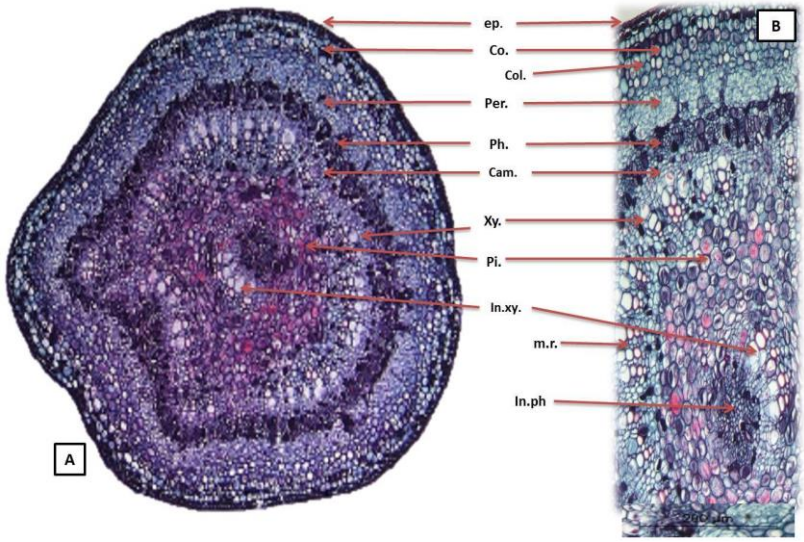

Figure 6: Transverse section in the leaf petiole and magnified region: (A) Entire T.S., (B) A magnified section. Where; ep., epidermis; co., cortex; col., collenchyma; per., pericycle; ph., phloem; cam., cambium; Xy., xylem; pi., pith; In.xy., internal xylem; m.r., medullary rays; in.ph., internal phloem.

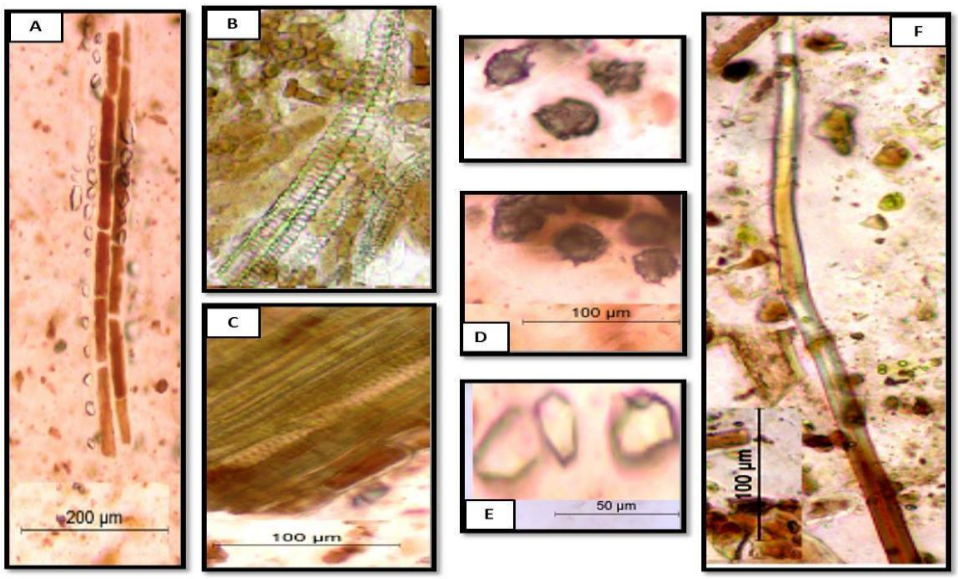

Figure 7: Isolated elements of the leaf petiole. (A) Crystal sheath (B) Spiral xylem vessel, (C) Annular xylem vessel, (D) Clusters of calcium oxalate , (E) Prisms of calcium oxalate , (F) Phloem fiber .

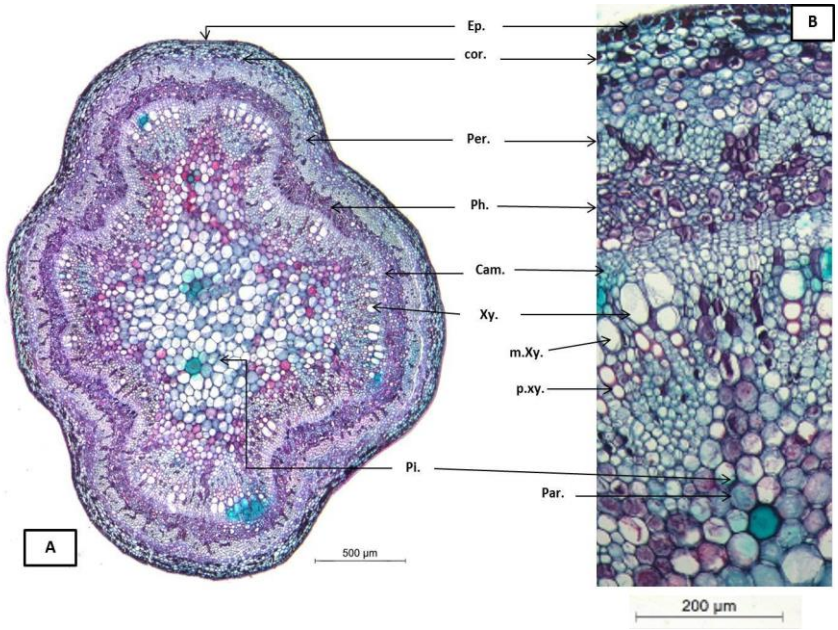

Figure 8: A transverse section in a young stem. (A) Entire T.S., (B) A magnified section Where, Ep., epidermis; cam, cambium; cor, cortex; per., pericycle; ph., phloem; xy., xylem; m.xy, meta xylem; p.xy, proto xylem; pi, pith; par, parenchyma.

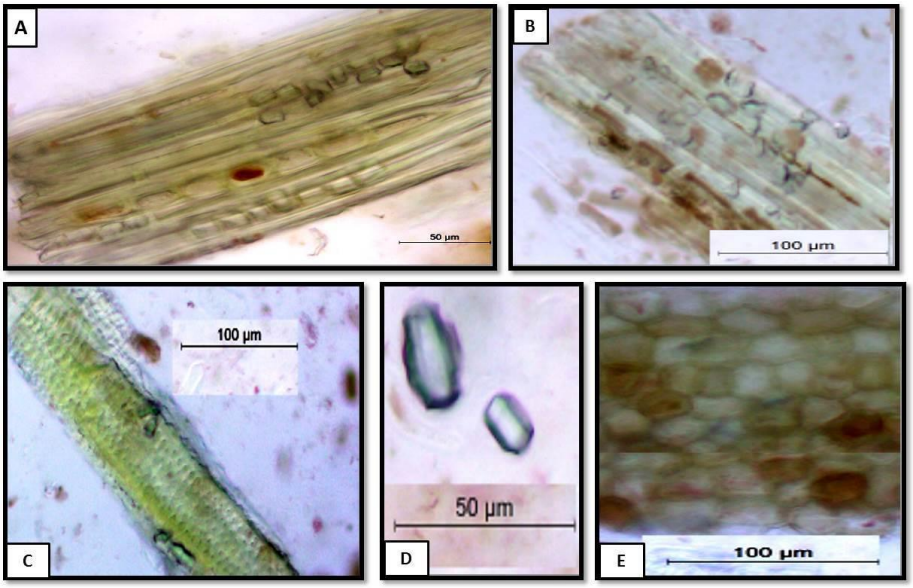

Figure 9: Isolated elements of the stem. (A) Crystal sheath, (B) Crystal sheath, (C) xylem vessels, (D) prism of calcium oxalate, (E) Epidermal cells.

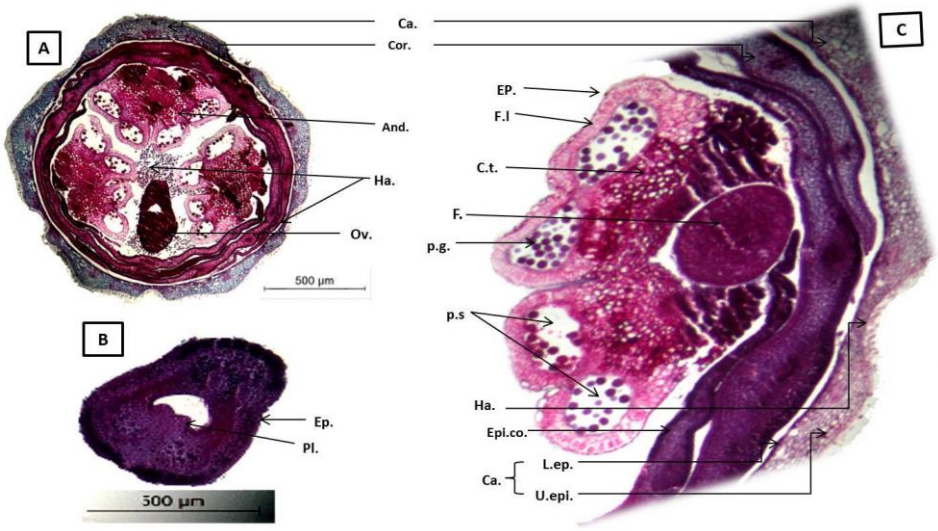

Figure 10: A transverse section in the flower bud. (A) Entire T.S, (B)A magnified region of the ovary, (C) A detailed section for versatile androecium.

Where; ca, calyx; cor., corolla ; And., androecium; Ha., hairs; Ov., ovary; Ep., epidermis; Pl., placenta; F.L., fibrous layer of anther; C.t., connective tissue; F, filament; p.g. , pollen grains; p.s., pollen sacs; Epi.co, epidermis of the corolla; U.ep., upper epidermis; L.ep., lower epidermis.

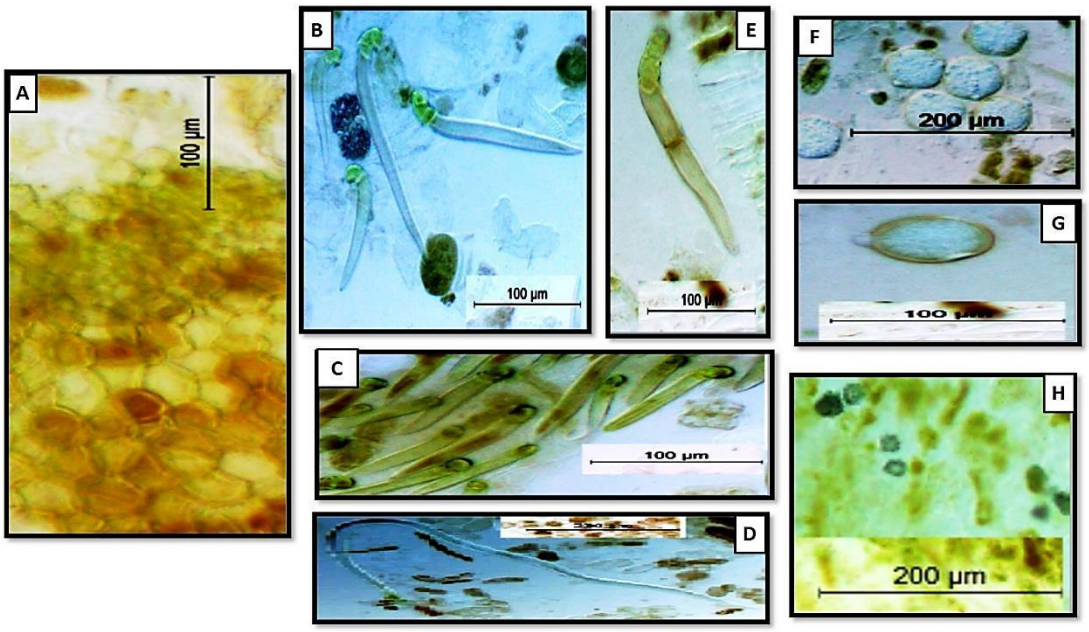

Figure 11: Isolated elements of the flower. (A) The Epidermis, (B) Non-glandular hairs, (C) Non-glandular hairs, (D) long wavy unicellular non-glandular hair, (E) bicellular, uniserriate non-glandular hair, (F) numerous smooth spherical pollen grains, (G) Smooth spherical pollen grain with three germ pores, (H) clusters of calcium oxalate.

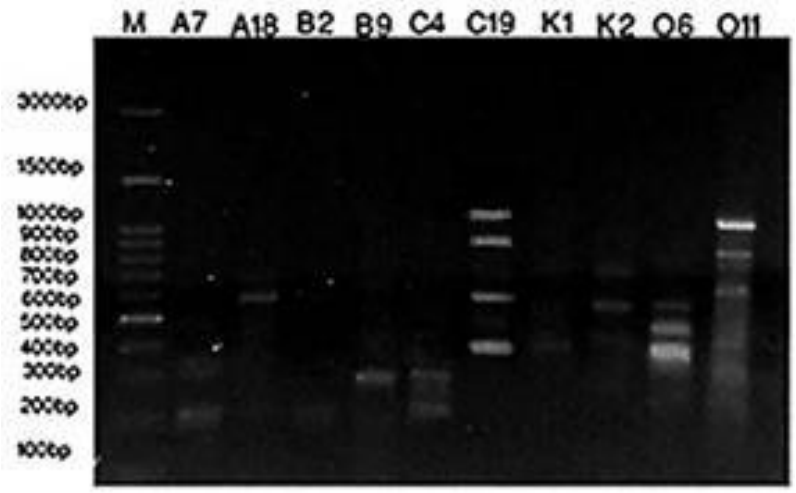

Figure 12: RAPD-PCR analysis of B.retusa with primers; M: marker, A7: OPA-07, A18: OPA-18, B2: OPB-02, B9: OPB-09, C4: OPC-04, C19: OPC-19, K1: OPK-01, K2: OPK-02, O6: OPO-06 and O11: OPO-11.

Table 1: List of the primer names and their nucleotide sequences used in the study

No.	Name	Sequence
1	OPA-07	5` GAAACGGGTG 3`
2	OPB-02	5` TGATCCCTGG 3`
3	OPA-18	5`-AGGTGACCGT-3`
4	OPC-04	5` CCGCATCTAC 3`
5	OPC-09	5` CTCACCGTCC 3`
6	OPC-19	5` GTTGCCAGCC 3`
7	OPK-01	5` CATTCGAGCC 3`
8	OPK-02	5` GTCTCCGCAA 3`
9	OPO-06	5` CACCTTTCCC 3`
10	OPO-11	5` GACAGGAGGT 3`

Table 2: RAPD polymorphic bands of *B.retusa* for primers: A7: OPA-07, A18: OPA-18, B2: OPB-02, B9: OPB-09, C4: OPC-04, C19: OPC-19, K1: OPK-01, K2: OPK-02, O6: OPO-06 and O11: OPO-11

Band Number	Molecular Weight (Base pairs)	Primer Name									
		A7	A18	B2	B9	C4	C19	K1	K2	O6	O11
1	1380	0	0	0	0	0	1	0	0	0	1
2	800	0	0	0	0	0	1	0	0	0	0
3	760	0	0	0	0	0	0	0	0	0	1
4	710	0	0	0	0	0	0	0	0	1	0
5	600	0	1	0	0	0	1	0	1	1	0
6	500	0	0	0	0	0	0	1	1	0	1
7	440	0	0	0	0	0	1	1	1	1	0
8	340	1	0	0	1	1	0	0	0	0	0
9	220	1	0	1	0	1	0	0	0	0	0
Total		2	1	1	1	2	4	2	3	3	3

The molecular size of the produced fragments revealed the presence of a wide range of sequences. The primer OPC-19 produced the highest number of rapid amplified polymorphic DNA (RAPD) fragments (4 fragments), while the lowest number of fragments was produced by OPA-18, OPB-2 and OPB-9 (1 fragment). The maximum size was 1380 base pairs after using primer OPC-19 and OPO-11 which produced RAPD fragments. However, the minimum molecular size was 220 base pairs when OPA-07, OPB-02 and OPC-04 primers were used.

CONCLUSION

The botanical features of *B.retusa* Roxb. different organs presented in this study can be considered as a helping data for correct authentication and recognition of this species; Anomocytic stomata are the type of stomata in *B.retusa* leaves. Numerous non-glandular hairs present in the organs with absence of glandular hairs. Pollen grains present in the flower puds are spherical and smooth. Furthermore, in genetic profiling, the ten oligonucleotide primers induced successive amplifications with a wide range of molecular sizes. The analysis of the amplified fragments generated by RAPD reactions revealed that the primers OPC-19, OPK-02, OPO-06 and OPO-11 can be used for the identification of *B.retusa*.

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CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The authors are thankful to Prof. Dr. Ramadan Mohammed and Dr. Hafeez Rofaeel in the botanical garden of Aswan for authorizing the collecting of the species and for their help and kindness. Also, Ph/ Ahmed Omar for his valuable advices and continuous help during this work.

AUTHOR CONTRIBUTIONS

HBS collected the plant material, carried out the laboratory work and wrote the manuscript. MAS supervised the laboratory work and provided

critical reading and perceptive recommendations of the manuscript. ESS and ARM designed the study, contributed to critical reading and final editing of the manuscript. All the authors have read the final manuscript and approved the submission.

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