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TAXONOMIC SIGNIFICANCE OF SOME LEAF ANATOMICAL FEATURES OF THE SPECIES OF *ANNONA* L. (ANNONACEAE) FROM NIGERIA

[1*]Adeniran, S. A., [2]Kadiri, A. B. and [3]Olowokudejo, J. D.

[1]Department Plant Biology, University of Ilorin, Ilorin Nigeria

[2, 3]Department Botany, University of Lagos, Akoka Lagos State

[*]Corresponding Author's E-mail: debunm11@gmail.com

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ABSTRACT

A comparative study of the some leaf anatomical features of four species of *Annona* occurring in Nigerian was undertaken with the aid of light microscope. The four foliar structures (epidermis, petiole, midrib and lamina architecture) studied revealed useful characters which support recognition of the species. A combination of these features has been used to prepare an artificial indented dichotomous key for identifying the species. The generic constant features encountered included hypostomata, paracytic stomatal type, linear nerves endings, uneven midrib outline, and centrally located vascular bundles in the petiole and midrib. However, the most reliable distinguishing characters found across the species included presence of brachyparacytic stomata in *A. reticulata*, presence of trichomes on the midrib in *A. senegalensis*, absence of druses on the abaxial surface in *A. muricata* and *A. squamosa*, a thick pitted anticlinal walls on the surfaces of *A. muricata* and consistent polygonal areola shape in *A. squamosa*. The overlapping characters which also justify the closeness of the species and their grouping in a genus were recorded in both the qualitative and quantitative features. Prominent among them are the mean stomatal width which is about 1.0 μm in all species, nerve endings within the areole which varies between 1-2, U- or V-shaped midrib on the adaxial surface and straight to curved anticlinal wall pattern. The significance of these observations is discussed in updating the existing data in the genus.

Keywords: Epidermis, Microscopy, Midrib, Petiole, Systematic

INTRODUCTION

Annona L. is a genus in the family Annonaceae having *ca.* 120 species worldwide (Geurt, 1981). The genus is made up of shrubs and small trees with simple exstipulate leaves which are alternately arranged. The flowers are hermaphrodite, usually somewhat fragrant, solitary or in fascicles with three green sepals and six petals arranged into two verticals (Leon, 1987; Michael, 2006).

The current distribution of these species covers almost all continents, with *A. muricata* and *A. squamosa* showing the widest distribution, mainly in the tropical regions. *A. squamosa* has been widely spread around the tropics and has become a prized backyard plant in many parts of Africa. *Annona senegalensis* is restricted to Africa, being widespread in sub-Sahelian tropical Africa (Pinto *et al*., 2005). The species have food, medicinal and economic values; and the presence of high amount of important secondary metabolites in them have been reported to underlie these uses (Pinto *et al*., 2005).

Wrong identities among some *Annonas* are relatively frequent. For instance, atemoya (a hybrid between cherimoya and sugar apple) was mistakenly called custard apple for many years (Morton, 1987), when this name more properly relates to *A. reticulata*. Custard apple is sometimes confused with *A. glabra*, and *A. montana* has been confused by some Brazilian growers with *A. muricata* (Pinto *et al*, 2005). Molecular phylogenetic studies on the Annonaceae family have also been found somewhat helpful (Doyle and Le Thomas, 1996; Mols *et al.*, 2004; Pirie *et al.*, 2006; Couvreur *et al.*, 2008; Erkens, *et al*., 2009; Chatrou *et al.*, 2012). In order to resolve this, Olowokudejo (1990) provided useful data on epidermal morphology where it was reported that only one type of stomata is present together with some other characteristics which have been updated in the present investigation. A follow-up study on the foliar epidermal characters and petiole anatomy of the genus was considered by Folorunso (2014), this revealed more anatomical characters which were of taxonomic values. Hutchinson and Dalziel (1958) opined that the identification

criteria for the species recognition were inadequate. Therefore, the gross morphological characters studied were insufficient to allow for clear identification of all the species. In all these studies, the anatomical characteristics were sparingly examined and reported. In view of this, some important leaf anatomical features of epidermis, lamina architecture, midrib and petiole which have not been fully studied were considered in this study with the sole aim of updating the existing knowledge of the epidermal features, leaf architecture and also to provide data on both the petiole and midrib that will aid species' distinction.

Materials and Methods

Sample Collection

Four *Annona* species collected from fields were used for the study. Table 1 shows the list of the species used for the study.

Table 1: List of the Nigerian Species of *Annona* studied.

Taxa	Herbarium Number	Locality	Collectors	Date of Collection
A. senegalensis	LUH 7364	Old Gawu Mountain Niger State	Adeniran, Kadiri, Olowokudejo	23/05/2013
A. reticulata	LUH	Department of Botany, Obafemi Awolowo University	Adeniran, Kadiri, Olowokudejo	08/08/2014
A. muricata	LUH 5222	Botanical Garden University of Lagos	Adeniran, Kadiri, Olowokudejo,Oyebanji	29/7/2012
A. squamosa	LUH 5233	NACGRAB, Ibadan	Adeniran, Kadiri, Olowokudejo,Oyebanji	1/4/2012

Leaf Epidermal Preparation

Four dried herbarium specimens per species were used for the study. For leaf epidermal study, 1-5 cm^2 portions were obtained from the standard median portion of the leaf lamina near the mid-rib following the approaches of Olowokudejo, (1990), Olowokudejo (1993), Kadiri (2003), Kadiri and Ayodele (2003). Leaves were hydrated by boiling in water and later soaked in concentrated trioxonitrate(v) acid (HNO_3) in glass bottles for about eight to twenty-four hours to macerate the mesophyll while the recalcitrant samples were irrigated in sodium hypochlorite solution (commercial bleach) for thirty minutes to two hours to bleach the leaf portions. Tissue disintegration was indicated by bubbles and the epidermides were transferred into Petri dishes containing water for cleansing and then, epidermides were separated with forceps. Tissue debris was cleared off the epidermides with fine-hair brush and wash in several changes of water. Drops of different grades of ethanol: 50%, 70%, 75% up to 100% added in turn to harden the cells.

Preparations were later stained with safranin O in 50% alcohol for about five minutes before mounting in glycerine on the glass slide. The epidermides were mounted with the uppermost surface facing up, covered with cover-slips and ringed with nail varnish to prevent dehydration. Then the slides were appropriately labelled and 20 microscope fields were examined per specimen at x100, and x400. Photomicrographs were taken using Toupview 3.2 Image Camera attached to the microscope.

For quantitative analysis of epidermal characters, 20 epidermal cells and 10 stomata were randomly selected for measurement per microscope field. Stomata index was calculated using the formula reported by Stace (1965).

Leaf Architecture

The areolation patterns and higher order venation in the leaf lamina were studied. Dried leaf samples were boiled in water for about five minutes and later transferred into commercial bleach solution for diaphanization (approximately 5% sodium hypochlorite), for 30 to 120 minutes. Thereafter, the samples were transferred into Petri dishes containing water for cleansing and then, drops of different grades of ethanol were added in turn to harden the cells after mounting on the slides. The adopted method follows Foster (1952), Hickey

(1973) and Dilcher (1974), with some modifications. Slides were examined with light microscope at x100 only while photomicrographs were taken using Toupview 3.2 Image Camera attached to a computer.

Petiole and Midrib Anatomy

Dried specimens were used through free hand sectioning. The sections were obtained transversely and boiled for about five minutes then drop of sodium hypochlorite was added; washed in several changes of water and then stained with few drops of aqueous Safranin. Samples were mounted in glycerin, covered with cover-slips and ringed with nail polish to prevent dehydration. Specimens were observed at x40 magnification and hand drawings were taken.

RESULTS

Summary of the findings are shown in figures 1-4 and tables 1-5. The leaves were hypostomatic with paracytic stomata common to all the species. Brachyparacytic (a derivative of paracytic stomata) was also found in *A. reticulata* in addition to paracytic stomata. In the genus, mean stomatal number varied from 20 in *A. reticulata* and *A. squamosa* to 35 in *A. muricata* while stomata index ranges from 13% in *A. senegalensis* to 22% in *A. squamosa* (Table 3). The anticlinal walls were largely straight on the adaxial surfaces but curved to wavy on the abaxial surfaces in all the species studied. *A. muricata* revealed thick straight pitted anticlinal wall on both surfaces (Figure 1). The leaves were mostly glabrous, where hairs were present; they are usually unicellular with tapering ends.

The mean cell size on the adaxial surface ranges from 17.9 ± 0.3 μm x 16.1 ± 0.2 μm in *A. senegalensis* to 23.0 ± 0.6 μm x 19.2 ± 0.5 μm in *A. squamosa*; similarly, values obtained on the abaxial surface follows the same pattern (Table 3). Crystals as druses were present on both surfaces of the cells of all species except *A. muricata* and *A. squamosa* where they were restricted to the adaxial surface (Table 2).

The three regions of the midribs of all the species have uneven surface which may be glabrous or pubescent. The vascular bundles in both midribs and petioles were centrally located and collateral. The petiolar vasculature shows little or no variation within the species from proximal to distal region. The vascular bundle is discontinuous except in proximal of *A. senegalensis*. Petiole outer surface may be even, uneven or slightly uneven in the species. These structures have almost identical shape both adaxially and abaxially especially from the proximal region to distal region of the petiole anatomy in all species. Venation pattern may be eucamptrodromous, craspedodromous or bronchidodromous while the vein angle of divergence generally varies from 73-138 across the species studied (Table 5).

DISCUSSION

The taxonomic relevance of leaf anatomical features for species identification is substantiated with four Nigerian species of *Annona*. The genus is generally hypostomatic and paracytic stomatal type is generic constant. This observation corroborates the report of Olowokudejo (1990) and Folorunso (2014) on the genus and Metcalfe and Chalk (1979), who documented paracytic stomata as basic for the family Annonaceae. Brachyparacytic stomatal type found in *A. reticulata* is a new entry and diagnostic for the species. Irregular cell shape was reported on the surfaces of *A. muricata* and *A. squamosa* according to Olowokudejo (1990) while Folorunso (2014) reported polygonal cell shape: our present result is in agreement with the findings of Folorunso (2014). A combination of these shapes may occur on the same leaves among the species. This observation corroborates the unstable nature of the cell shape which can be controlled by the prevailing environmental conditions, especially insolation. In certain plant taxa, the cell shape may be stable and good for species' distinction as revealed by Davis and Heywood (1963) and Stace (1965). Thick straight pitted anticlinal wall in *A. muricata* is diagnostic in this genus. Anticlinal cell wall pattern may be straight on the adaxial surface in *A. senegalensis*, *A. muricata* and *A. squamosa*. Straight-curved or curved were also encountered across the species on both abaxial and adaxial surfaces though there were inconsistencies when compared with the report of Olowokudejo (1990) and Folorunso (2014). Measured characters usually have

overlapping values; however, this does not reduce their taxonomic usefulness (Davis and Heywood, 1963; Stace, 1965). However, this present account re-affirms this assertion. Olowokudejo (1990) mostly recorded sparse distribution of trichomes in the genus. The presence and absence of trichomes on the adaxial and abaxial surfaces respectively in *A. muricata* distinguishes it from other species which can either be glabrous or pubescence on both surfaces. This confirms the opinion that trichomes can be influenced by environmental factors (Stace, 1965); and under strict influence of the genes (Davis and Heywood, 1963; Stace, 1965; Carpenter *et al*., 2005). Though its taxonomic relevance has been shown by researchers (Olowokudejo, 1990; Carpenter *et al*., 2005). Another important character useful for species distinction was the cell inclusion which occurs as druses deposited in the cell lumen (Table 2). Olowokudejo (1990) and Folorunso (2014) reported wax of various sizes, shapes and patterns. Crystals were identified as druses in the genus studied and were in varying quantities. All these epidermal features reported have been used for taxa delimitation and grouping in many angiosperms (Olowokudejo, 1990; Olowokudejo, 1993; Kadiri, 2003; Kadiri and Ayodele, 2003; Carpenter *et al*., 2005, Folorunso, 2014), and they have high potential in providing additional identification criteria for the species.

Midribs of the species seem identical except in shape and surface hairiness. Midrib is an important structure which offers features that can be diagnostic and reflect affinity among taxa (Radford *et al*., 1976, Wilkinson, 1989). The shape varies from U- to V-shape with varying degrees of wideness on the adaxial surface whereas on the abaxial surface, it may be hollow or crescentiform (Figure 3, Table 4). Only *A. senegalensis* has trichomes. This is also a supporting identification criterion for this species. Radford *et al*., (1976) reported the usefulness of petiolar anatomy in taxa description and distinction. The diagnostic relevance of the petioles can be found in the outline which is even only in *A. reticulata*, and gland duct which recorded only in *A. muricata*.

Doyle and Le Thomas (1996) as well as Klucking, (1986) pointed out the vein angle of divergence as an important taxonomic feature of the lamina architecture in the family Annonaceae. Doyle and Le Thomas (1996) further revealed that many members of Annonaceae have higher secondary angles. The angle divergence of the *Annona* species studied revealed the highest divergence in the *A. squamosa* while the least angle of divergence is found in *A. reticulata*. A closer look also suggested a nearly same angle of divergence in between *A. reticulata* and *A. senegalensis*. Another feature of the lamina architecture found to be of significant importance is areola shape. A more like polygonal areola shape is recorded in all species with other shapes like square, rectangle and triangle. This character shows high potential for defining affinity in the genus. Though, the polygonal areola shape agreed with *A. muricata* and *A. reticulata*, which was also the position of Folorunso and Modupe (2007). Vein of a particular size, class also display some degrees of uniformity course and pattern of distribution in relation to other classes (Hickey, 1973). The vein course in Folorunso and Modupe (2007) report showed straight branched vein course, there were levels of agreement as reported in this study. Linear nerves ending among the *Annona* species has been consistent though with additional types. *A. squamosa* maintain only linear nerves ending all through the species.

In conclusion, characters like stomata type, position of stomata in the leaf surface, vein course, angle of divergence, areola shape, nerves ending among others have been of greater help to delimit the genus even to the species level. A combination of this anatomical data set has been used to prepare an indented dichotomous key for separating the species. This report will assist in resolving the problem of adulteration when the medicinal species are cluttered or fragmentary in the Nigerian herbal markets.

An Indented Dichotomous Key for Separating the Four Species of *Annona* occurring in Nigeria

1a. Epidermal cell shape polygonal on adaxial surface……...….2

2a. Stomata brachyparacytic present…….....*A. reticulata*

2b. Stomata brachyparacytic

absent……..……..……3

3a. Gland duct present, anticlinal wall thick pitted on both surfaces.....……..*A. muricata*

3b. Gland duct absent, anticlinal wall thin non-pitted on both surfaces..… *A. senegalensis*

1b. Epidermal cell shape irregular on adaxial surface……..*A. squamosa*

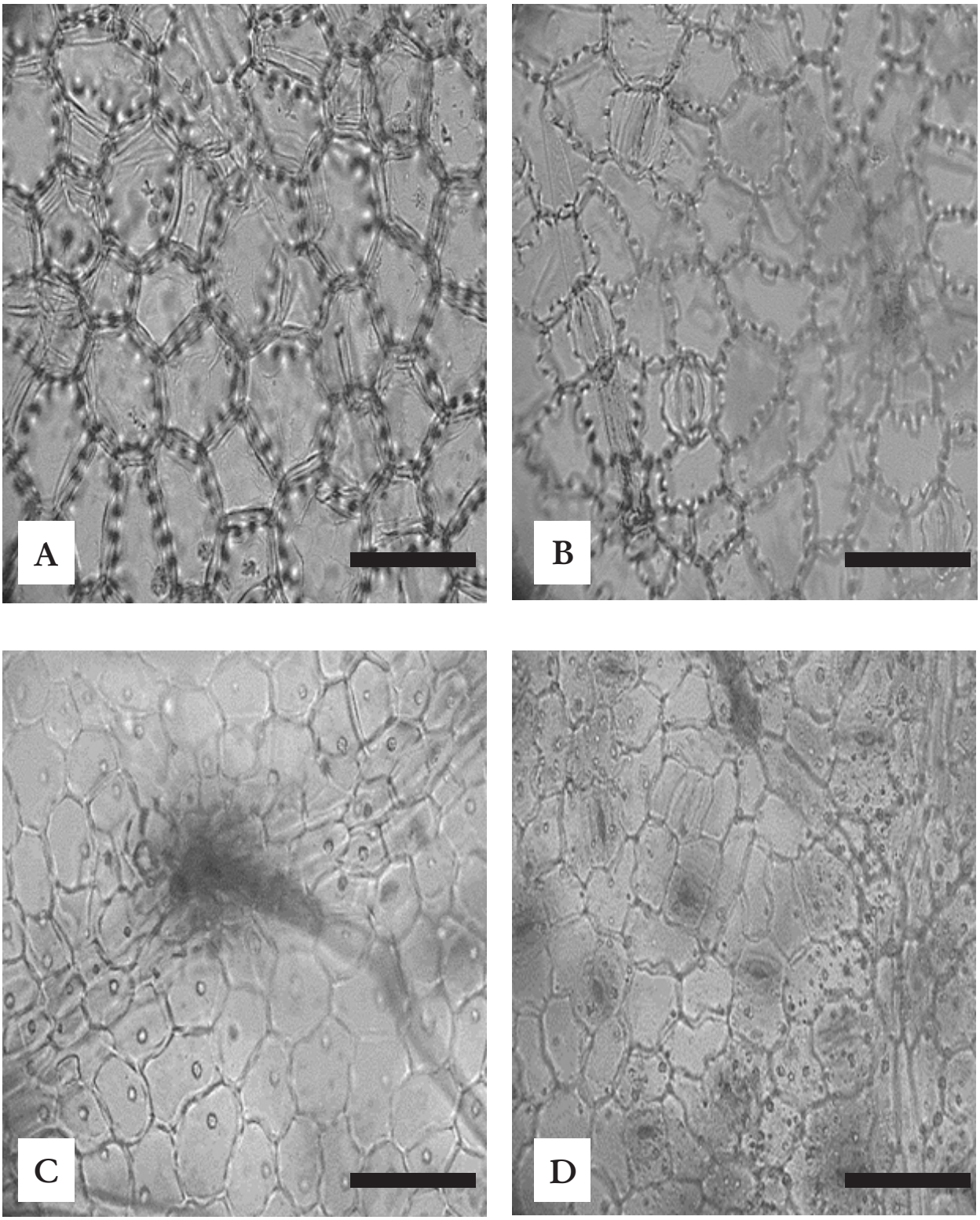

Figure 1: Leaf Epidermal Characterstics from Nigerian Species of *Annona*

A, B: *A. muricata,* C, D: *A. reticulata*. Adaxial on the left, abaxial on the right. Scale bar = 50 μm.

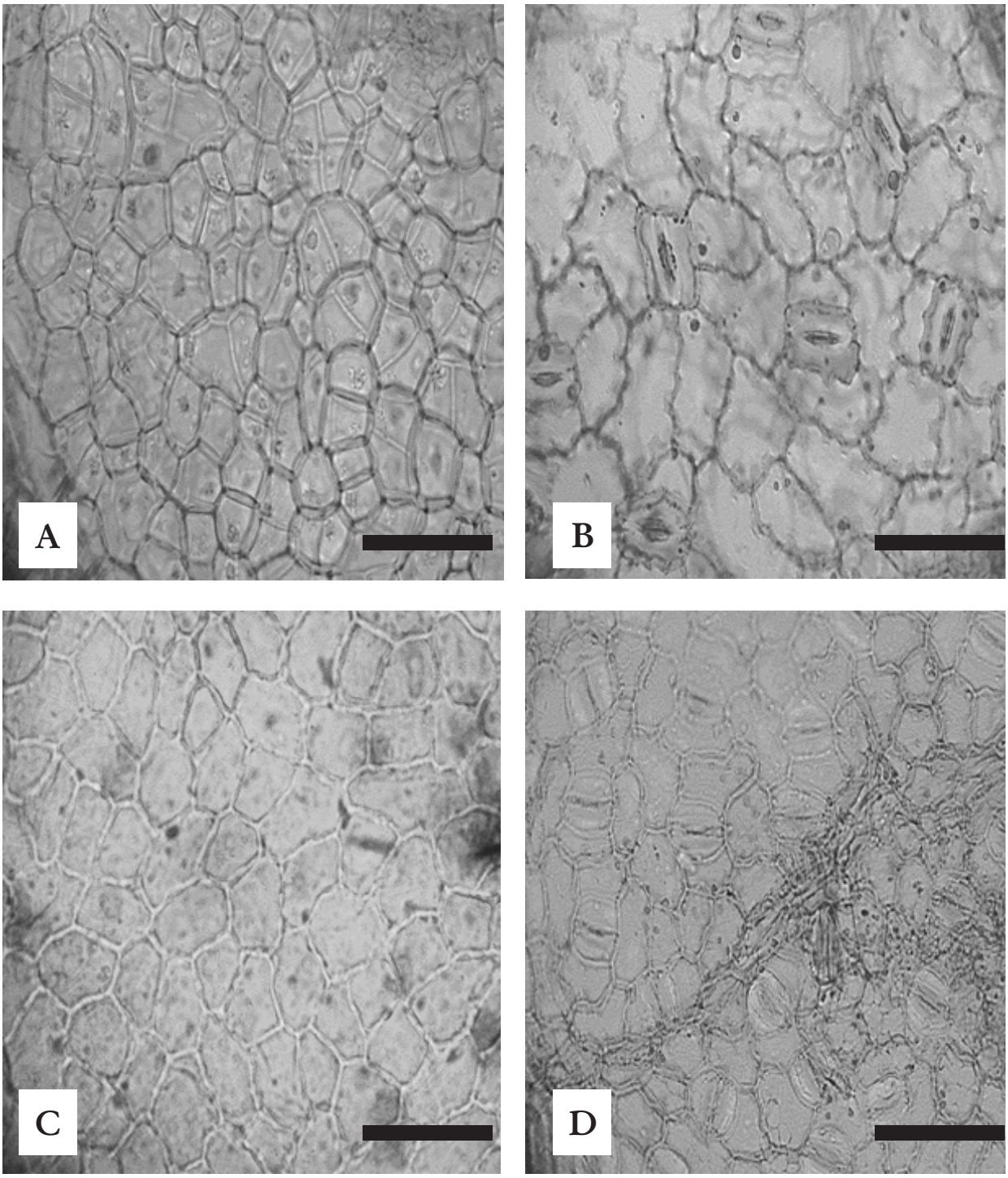

Figure 2: Leaf Epidermal Characterstics from Nigerian Species of *Annona*
A, B: *A. senegalensis,* C, D: *A.squamosa.* Adaxial on the left, abaxial on the right. Scale bar = 50 μm.

Table 2: Qualitative Epidermal Characters of the Species of *Annona*

Species	Surface	E.C.S	A.W.P	Stomatal Type	Trichome	Crystal type
A. senegalensis	Adaxial	Polygonal	Straight	-	Absent	Druses, abundant
	Abaxial	Polygonal	Straight-curved	Paracytic	Absent	Druses, sparse
A. reticulata	Adaxial	Polygonal	Curved	-	Present	Druses, abundant
	Abaxial	Polygonal	Curved	Paracytic,Brachyparacytic	Absent	Druses, abundant
A. muricata	Adaxial	Polygonal	Straight	-	Present	Druses, sparse
	Abaxial	Polygonal	Straight	Paracytic	Present	Druses absent
A. squamosa	Adaxial	Polygonal	Straight	-	Absent	Druses, sparse
	Abaxial	Irregular	Curved	Paracytic	Absent	Druses absent

E.C.S- Epidermal Cell Shape, A.W.P- Anticlinal Wall Pattern.

Table 3: Quantitative Epidermal Characters of the Species of *Annona*

Species	Surface	Cell number per field	Cell Length (μm)	Cell Width (μm)	AWT	Stomata Length (μm)	Stomata Width (μm)	Stomatal Number	Stomatal Index
A. senegalensis	**Adaxial**	102(188±5)200	16.0(17.9±0.3)20.0	15.0(16.1±0.2)18.0	1.0(1.0±0.0)1.0	-	-	-	
	Abaxial	151(174±4)204	17.0(18.6±0.4)23.0	14.0(15.6±0.2)17.0	1.0(1.0±0.0)1.0	8.0(8.9)10.0	1(1±0)1	18(26)35	13%
A. reticulata	**Adaxial**	127(135±1)143	16.0(18.1±0.4)22.0	13.0(14.8±0.3)17.0	1.5(1.5±0.0)1.5	-	-	-	
	Abaxial	81(90±1)93	11.0(13.7±0.4)16.0	14.0(15.8±0.3)17.0	1.5(0.5±0.0)1.5	9.0(5.1)9.0	1(1±0)1	19(20)22	18.2%
A. muricata	**Adaxial**	100(122±2)144	17.0(19.4±0.3)21.0	15.0(16.0±0.2)18.0	1.0(1.0±0.0)1.0	-	-	-	
	Abaxial	112(130±2)145	18.0(19.9±0.4)23.0	14.0(15.5±0.3)17.0	1.5(1.5±0.0)1.5	9.0(9.7)9.0	1(1±0)1	31(35)39	21.2%
A. squamosa	**Adaxial**	56(73±2)82	19.0(23.1±0.6)26.0	16.0(9.2±0.5)22.0	1.5(1.5±0.0)1.5	-	-	-	
	Abaxial	62(71±2)96	22.0(15.6±0.8)31.0	15.0(7.0±0.3)18.0	1.0(1.0±0.0)1.0	9.0(9.6)9.0	1(1±0)1	15(20)22	22%

AWT-Anticlinal wall thicknes

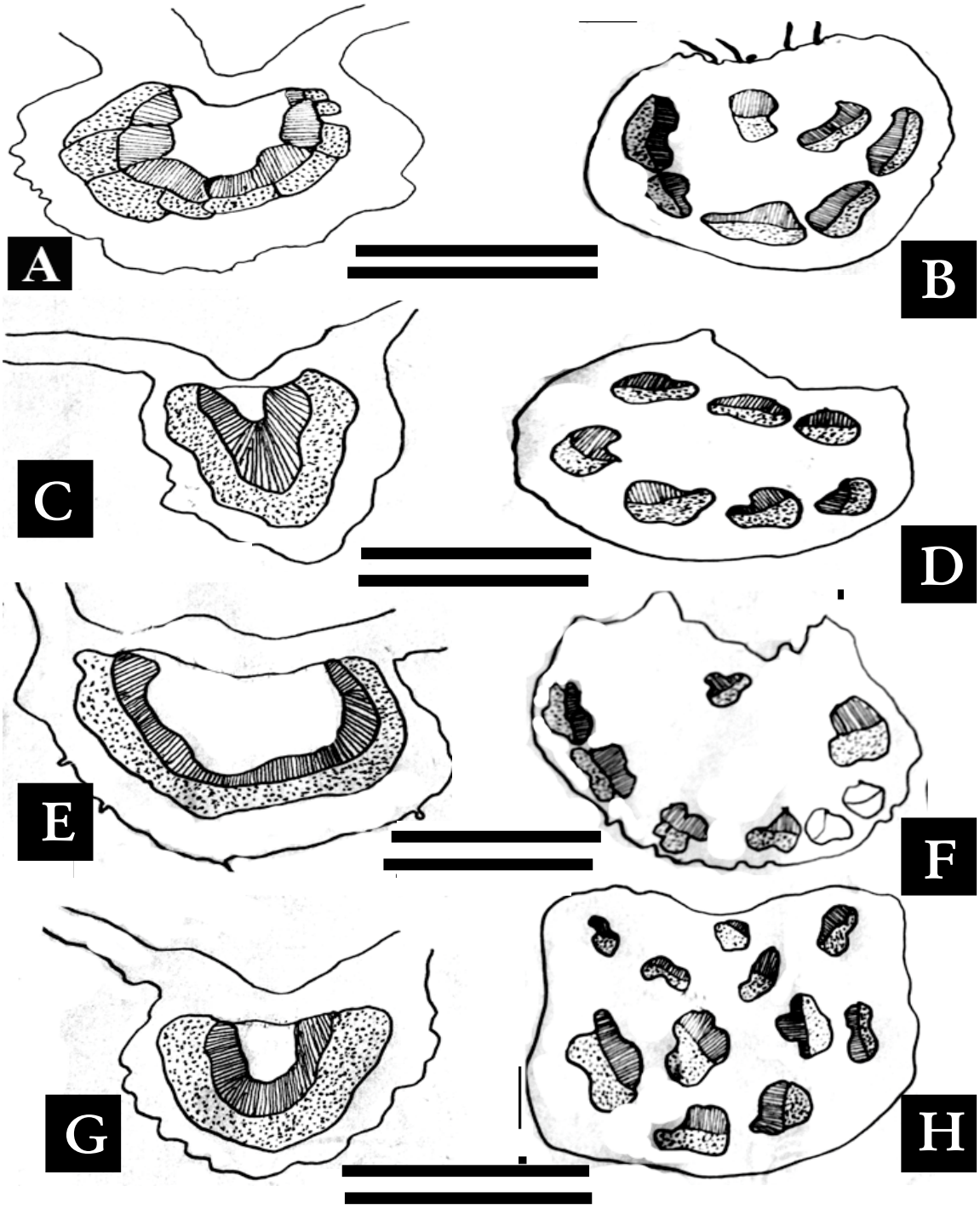

Figure 3: Midrib and Petiole Anatomical Characters of Four Species of *Annona* occurring from Nigeria A and B- *A. senegalensis,* C and D- *A. reticulata*, E and F- *A. muricata*, G and H- *A. squamosa.* Scale bar= 50 μm.

Key: = Xylem = Phloem

Table 4: Qualitative Characters of Midrib and Petiole Anatomy of *Annona* species

	Midrib				Petiole				
Species	Shape on Adaxial surface	Shape on Abaxial surface	Outline	Trichome	Shape on Adaxial surface	Shape on Abaxial surface	Outline	Vascular bundle location	Gland duct
A. senegalensis	Narrow U-shaped	Crescentiform	Uneven	Present	Crescentiform	Crescent	Not even	Central	Absent
A. reticulata	Furrowed	Hollow	Uneven	Absent	Crescentiform	U-shaped	Even	Central	Absent
A. muricata	Wide U-shaped	Crescentiform	Uneven	Absent	Crescentiform	Concave	Not even	Central	Present
A. squamosa	Wide V-shaped	Crescentiform	Uneven	Absent	Crescentiform	Concave	Not even	Central	Absent

Table 5: Character of Leaf Lamina Architecture of *Annona* species

Species	Angle of divergence	Nerves ending	Areole shape	Vein course	Venation
A.senegalensis	83-100	0-1, curved, linear	Polygonal –triangular	Straight	Eucamptodromous
A.reticulata	76-138	1-2, linear	Polygonal -rectangular	Straight to curved	Eucamptodromous
A.muricata	73-106	1-2, curved, linear	Polygonal –square	Straight to loop	Craspedodromous
A.squamosa	113-125	linear	Polygonal	Straight	Bronchidodromous

Leaf Architecture

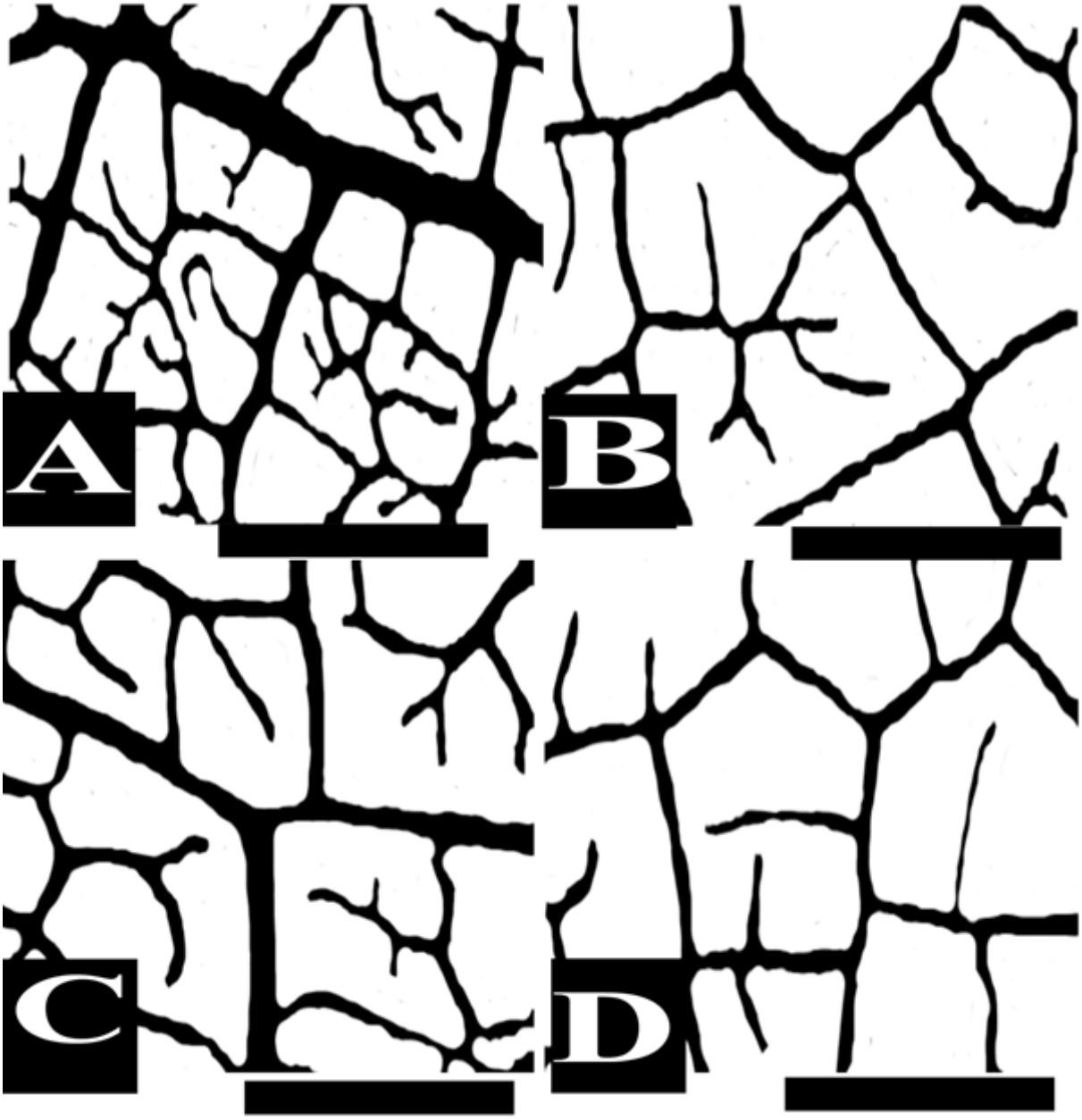

Figure 4: Leaf Lamina Architectural Patterns under Light Microscope
A- *A. senegalensis*, B- *A. reticulata*, C- *A. muricata*, D- *A. squamosa.* Scale 50 μm.

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