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EXPLORATION OF SOME ANATOMICAL CHARACTERS IN GROUPING THE THREE NIGERIAN *CLEOME* L. SPECIES INTO C_3 AND C_4 PHOTOSYNTHETIC PATHWAYS

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ABSTRACT

The leaf anatomical and epidermal characteristics of three species of Cleome were studied in a bid to group them into the C_3 and C_4 photosynthetic pathways and contribute data to better understand possible range of quantitative anatomical characters of C_4 plants that may be peculiar to dicotyledons. The characters investigated are intercellular air spaces, inter-stomatal distance, inter-veinal distance, Kranz tissue, leaf thickness, maximum lateral cell count criterion, mesophyll thickness, one cell distant count criterion, stomatal density, stomata index, stomata size and vein density. The presence of Kranz tissue, inter-veinal distance of less than 150 µm, maximum lateral count of two to five and mesophyll thickness were major features of the C_4 Cleome species. This showed that C. gynandra is a C_4 species while C. rutidosperma and C. viscosa are C_3 species. Among the epidermal characters studied, only the stomatal density was useful in the delimitation of the species.

KEY WORDS: *C_3 plants, C_4 plants, Cleome, leaf anatomy, photosynthesis*

INTRODUCTION

The family Cleomaceae, comprising about 10 genera and 300 species, is a relatively small family of flowering plants in the order Brassicales (Jansen, 2004). The family was formerly part of the Capparaceae before DNA analysis suggested the genera are closer to the Brassicaceae (Kasem, 2016). The genus *Cleome* L. is the largest in the family, comprising about 150 to 200 species of herbaceous annual or perennial plants with wide cosmopolitan distribution in tropical and subtropical regions of the world (Huxley, 1992; Jansen, 2004). One hundred and fifty *Cleome* species have so far been described in the tropical regions of the world (Sharma and Balakrishnan, 1993). Three species, *C. gynandra*, *C. rutidosperma* and *C. viscosa* have been reported from Nigeria (Akobundu and Agyakwa, 1987). These species occur mainly near human settlements and as pantropical weeds of cultivated fields. Edeoga *et al*. (2009) carried out comprehensive studies on the morphological characters of the vegetative, floral and palynology of the three species. They reported, in part, the nature of their leaf anatomy without linking them to their respective photosynthetic pathways.

Photosynthesis is an important biological process in the life cycle of plants. Over the years, plants have devised means of optimizing this process in the presence of harsh environmental conditions and limited resources (Wang, 2004; Taylor *et al*., 2010). There are generally three pathways that plants take to carry out photosynthesis which are the C_3, C_4 and the Crassulacean Acid Metabolism (CAM) (Ehleringer *et al*., 1997; Wang, 2004). Notwithstanding, there are reports of groups of plant that do not exactly fit in any of these categories which have been designated as C_3-C_4 intermediate group (Voznesenskaya *et al*., 2010). In terrestrial plants, the C_3 pathway is the commonest as only about 3% of them use the C_4 pathway (Voznesenskaya *et al*., 2017). In plants such as *Cleome*, there are evidences of C_3, C_4 and intermediate C_3-C_4 metabolism. (Marshall *et al*. 2007). It is note-worthy that differences occur in the biochemical processes involved in the photosynthetic pathways of these plants. These differences are essentially linked to the anatomical structures of the leaves – the main site of photosynthesis (Bruhl and Wilson, 2007; Ueno *et al*., 2006). One of such differences is the Kranz tissue composed of the mesophyll and bundle sheath, two morphologically and functionally distinct cells.

The Kranz tissue was initially reported to be peculiar to C_4 species (Hattersley *et al.*, 1982). It was reported to have developed as a response to inadequate supply of CO_2 and have been used in several studies to differentiate C_4 from C_3 species (Renvoize, 1987; Faniyan et al., 2013; Ayeni *et al*., 2015). However, other studies have reported several C_4 species lacking the Kranz anatomy (Sage, 2002; Voznesenskaya et al., 2002; Edwards *et al*., 2004). Therefore, several anatomical characters must be considered in order to have a reliable classification of plant species into different photosynthetic pathways (Sage, 2002; Ajao *et al.* 2017). Other anatomical characters that have been used in classifying species into the C_3 and C_4 pathways include intercellular air spaces, inter-stomatal distance, leaf thickness, maximum lateral cell count, mesophyll thickness, one cell distant criterion, stomatal density, stomatal index and stomatal size (Bruhl and Wilson, 2007; Diana *et al*., 2007; Kim, 2012; Faniyan *et al*., 2013; Ayeni *et al.,* 2015; Ajao *et al*. 2017). Others are leaf vein density and interveinal distance (Nelson and Dengler, 1997; Nelson *et al*., 2005). For monocots, several studies have been carried out to reach a particular range of anatomical characters that indicate C_3 or C_4 metabolism. However, studies on dicots are relatively few. Therefore, the present study aims to employ the three Nigerian species of *Cleome* as dicotyledons and group them into either the C_3 or C_4 photosynthetic pathways using several anatomical characters suggested by previous authors already cited. It also aims at contributing to available data on quantitative anatomical characters that may indicate C_4 pathway in the dicotyledons.

MATERIAL AND METHODS

Sample collection and identification

Three Nigerian species of *Cleome* used in this study were C. *gynandra* L., *C. viscosa* L. and *C. rutidosperma* DC. Ten fresh samples of each species were collected from different locations in Ile-Ife, Osun State, southwestern Nigeria. The collected samples were identified at the Department of Botany Obafemi Awolowo University Herbarium (IFE) and also using information from the Flora of West Tropical Africa by Hutchinson and Dalziel (1972). Table 1 shows the geographical coordinates of the locations where samples were collected.

TABLE 1: SPECIES OF *CLEOME* USED IN THIS STUDY AND THEIR LOCATIONS OF COLLECTION

Species	Collection sites/GPS coordinate	Location
Cleome gynandra	7º28N, 4º33E) Ile Ife (	Osun State, Nigeria
Cleome viscosa	OAU (7º34N, 4º68E)	Osun State, Nigeria
Cleome rutidosperma	7º28N, 4º33E) Ile Ife (	Osun State, Nigeria

Foliar anatomical studies

Foliar epidermal study: Sizeable portions of mature fresh leaves for each species studied were cut from the median part following the methods of Ogundare and Saheed (2012) and Ajao et al. (2017). Epidermal peels were made from both abaxial and adaxial surfaces of the leaves and parameters such as stomatal density, stomatal index, inter-stomatal distance and stomatal size were measured. The stomatal density (SD) was determined as the number of stomata per square millimeter (Stace, 1965). For each character, 3-4 leaves of each species were examined. To study vein density and spacing, the whole leaf lamina of each species was cleared and viewed under the compound microscope (BIOBASE LCD Biological Microscope Model – DM-125).

In the foliar transverse section studies, sizeable leaf portions were incised from mature fresh leaves for each species and fixed in 50% ethanol for 2 hours. The leaf portions were embedded in paraffin wax and transverse section were cut using sledge microtome at thicknesses of 20 µm. The specimens were then processed using previously established procedures (Saheed and Illoh, 2010; Ajao *et al.* 2017). Sections were viewed for the presence or absence of Kranz tissue while maximum lateral and one cell distance counts were made. Measurements were taken for leaf thickness, mesophyll thickness, inter-veinal distance and proportion of intercellular air spaces. Measurements were also taken for the leaf thickness, mesophyll thickness and inter-veinal distance.

Appropriate scopes were used for each of the studies and photomicrographs were taken under the microscope. Quantitative characters were measured with the aid of a calibrated ocular micrometer inserted in the eye-piece of the microscope. Data were collected from 20 measurements (n = 20) and appropriate statistical methods were applied for the interpretation.

RESULTS AND DISCUSSION

Table 2 presents a summary of all anatomical characteristics examined in all the species studied. In the transverse section, Kranz tissue was present only in *C. gynandra* (Fig 1), while it was absent in the rest (Figs 2 and 3). Leaf anatomy showed that *C. gynandra* has the lowest inter-veinal distance of 139.0±10.2 µm when compared to *C. rutidosperma* (207.4±9.4 µm) and *C. viscosa* (211.4±11.7 µm). The maximum lateral cell count (MLCC), which refers to the number of chlorenchymatous mesophyll cells in the intervening bundle sheaths of adjacent vascular bundle, was also investigated. *C. viscosa* and *C. rutidosperma* have a MLCC of six to ten while *C. gynandra* have two to five. One cell distance criterion was observed only in *C. gynandra*, where there was only one cell separating the chlorenchymatous mesophyll cell from the nearest parenchymatous bundle sheath cell. The results for leaf thickness and mesophyll thickness were similar to that of inter-veinal distance as *C. gynandra* has the lowest mean values among all the species studied (Table 2). In contrast, the proportions of intercellular airspace in all the three species were in the same range with overlapping mean values.

TABLE 2: SUMMARY OF LEAF ANATOMICAL CHARACTERS OF THE STUDIED *CLEOME* SPECIES

Species	**Kranz tissue**	**Interveinal Distance (µm)**	**MLCC**	**OCDC**	**Leaf Thickness (µm)**	**Mesophyll Thickness (µm)**	**Intercellular Airspace (%)**
C. rutidosperma	Absent	207.4±9.4	6-10	Absent (2)	193.1±5.8	156.4±5.4	14.5±2.1
C. gynandra	Present	139.0±10.2	2-5	Present (1)	157.6±3.7	119.6±3.3	13.8±0.8
C. viscosa	Absent	211.4±11.7	6-10	Absent (2)	229.0±4.9	202.8±5.5	13.8±1.1

MLCC = Maximum Lateral Cell Count OCDC = One Cell Distance Count Criterion

A summary of the epidermal characteristics studied are presented in Table 3. On the adaxial surface, *C. viscosa* (43.47±2.9 %) has the highest stomatal indices. However, there was no clear difference in the stomatal indices of all the species on the abaxial surface. On the other hand, *C. viscosa* has the lowest stomatal density on both the abaxial (9.64±0.8 mm^{-2}) and adaxial (6.96±0.5 mm^{-2}) surfaces, followed by *C. gynandra* and *C. rutidosperma.* For the stomatal size, *C. gynandra* have a stomatal size of 32.16±2.0 $µm^2$ on the adaxial and 24.0±3.7 $µm^2$ on the abaxial, which is the lowest among the three species studied because the stomatal sizes of the other two species were at least 40 $µm^2$ on both surfaces (Table 3). Interstomatal distances revealed varying results for the abaxial and adaxial surfaces. While *C. gynandra* has the lowest interstomatal distance on the adaxial surface, *C. viscosa* has the lowest on the abaxial. Examination of vein densities showed that *C. gynandra* has the highest with 4.9 mm^{-2} while *C. rutidosperma* and *C. viscosa* have vein densities of 1.8±0.2 mm^{-2} and 1.6±0.2 mm^{-2} respectively.

TABLE 3: SUMMARY OF LEAF EPIDERMAL CHARACTERS OF THE STUDIED *CLEOME* SPECIES

Species	**Stomatal Index (%)**		**Stomatal Density (mm^{-2})**		**Stomatal Size ($µm^2$)**		**Interstomatal Distance (µm)**		**Vein Density (mm^{-2})**
	AD	AB	AD	AB	AD	AB	AD	AB	
C. rutidosperma	39.39±1.4	58.97±1.9	14.92±0.9	20.36±1.3	66.52±3.8	50.92±5.5	22.44±1.7	25.76±1.8	1.8±0.2
C. viscosa	43.47±2.9	58.82±0.7	6.96±0.5	9.64±0.8	58.0±2.7	40.05±3.7	22.16±2.2	17.98±1.6	1.6±0.2
C. gynandra	37.50±0.8	55.56±2.3	12.76±1.1	14.36±0.9	32.16±2.0	24.0±3.7	20.24±1.9	29.56±1.1	4.9±0.4

AD = Adaxial Surface AB = Abaxial Surface

The wreath-like arrangement of the mesophyll and bundle sheath (Kranz tissue) in C_4 species helps to minimize the loss of CO_2 and reduce the possibility of photorespiration (Gutierrez *et al*., 1974; Nelson *et al*., 2005). Thus, this is considered a strong indication of the C_4 photosynthetic pathway. It has been used in numerous anatomical surveys of leaves to identify C_4 photosynthetic pathway for many plant species (see Renvoize, 1987; Hattersley *et al*., 1982; Faniyan *et al*., 2013; Ayeni *et al*., 2015; Ajao *et al*., 2017). Of all the species in this study, Kranz tissue was present only in *C. gynandra* and absent in the remaining two species. This result suggests that *C. gynandra* is C_4 while *C. rutidosperma* and *C. viscosa* are C_3.

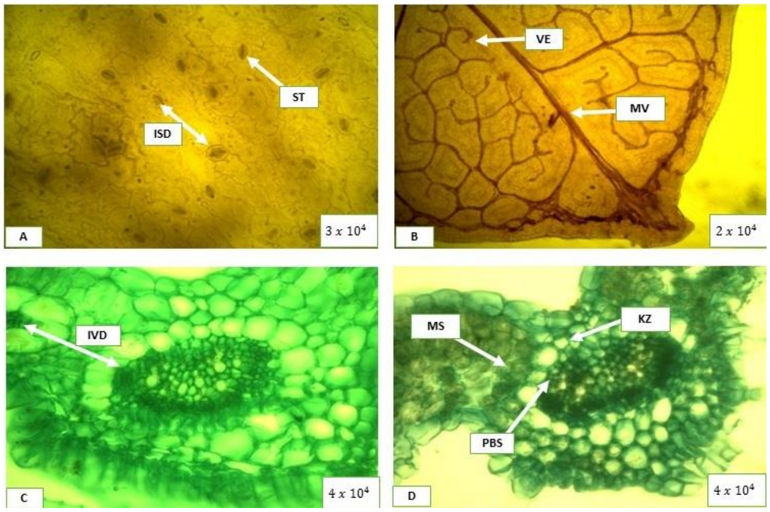

FIG 1: Leaf transverse section of the *C. gynandra*.
ISD: interstomatal distance, ST: stomata, MV: main vein, VE: vein ending, IVD: interveinal distance, MS : mestome sheath, PBS: parenchymatous bundle sheath, KS : Kranz sheath.

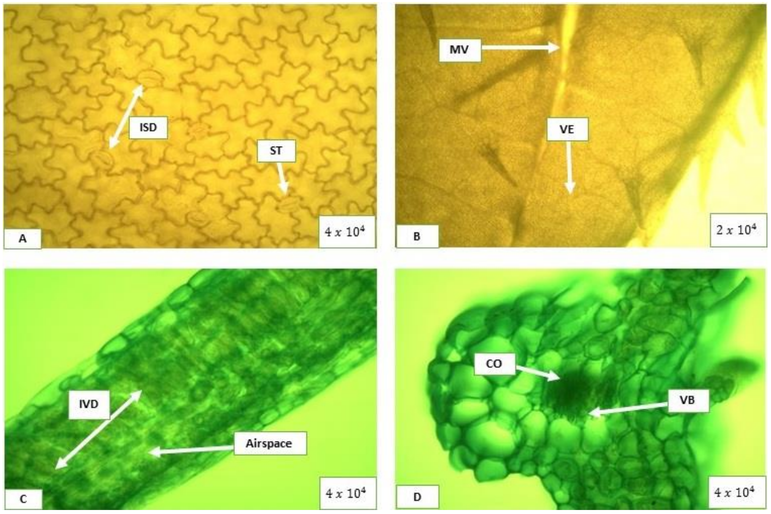

FIG 2: Leaf transverse section of the *C. rutidosperma*.
ISD: interstomatal distance, ST: stomata, MV: main vein, VE: vein ending, IVD: interveinal distance, CO: collenchyma, VB: vascular bundle.

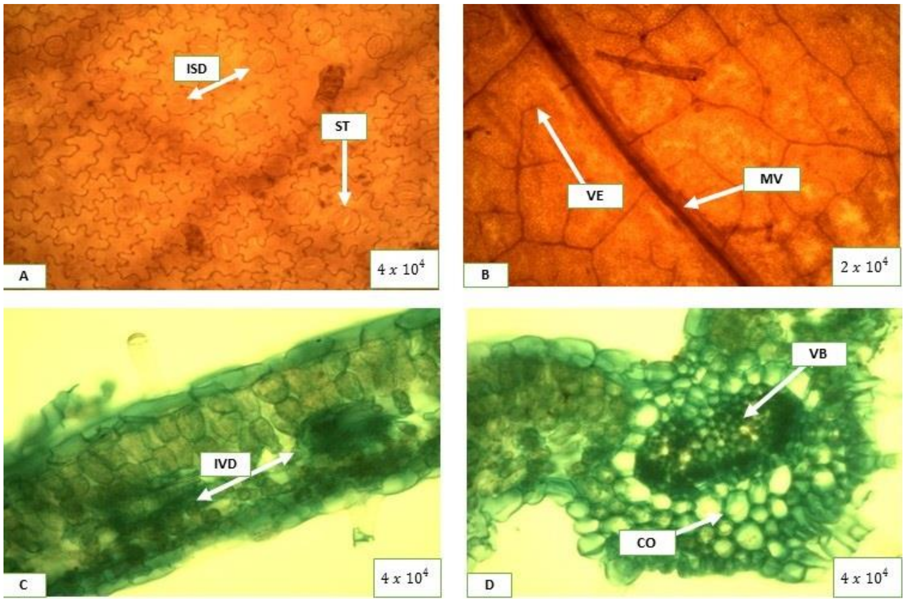

FIG 3: Leaf transverse section of the *C. viscosa*.
ISD: interstomatal distance, ST: stomata, MV: main vein, VE: vein ending, IVD: interveinal distance, CO: collenchyma, VB: vascular bundle.

Interveinal distance is the average distance between the vein centres (Li *et al.*, 1999). It has been reported that veins in C_4 species are always closer together than vein in C_3 species (Crookston and Moss, 1974; Takeda *et al*. 1980; Dengler *et al.*, 1994). This is because closer spacing of veins ensures a more efficient transport of photosynythates between cells (Monson *et al*., 1984). Shorter interveinal distances indicate a well–developed veinal system. This suggests that it assists C_4 plants in the regulation of the balance between photosynthesis and water usage (Oguro *et. al.*, 1985). In this study, *C. gynandra* has interveinal distance of 139.0±10.2 µm while *C. rutidosperma* and *C. viscosa* have interveinal distances of 207.4±9.4 µm and 211.4±11.7 µm respectively. This result suggested that *C. gynandra* is C_4 because of its relatively smaller interveinal distance while the other two species are C_3. The results of this study conform with reports from the work of Ajao *et al*. (2017) and Faniyan *et al*. (2013) that interveinal distance of C_3 plants are greater than those of their C_4 counterparts although Takeda *et al*. (1980), Dengler *et al*. (1994), Li and Jones (1994) and Ayeni *et al*. (2015) proposed that a species is C_4 if its leaf interveinal distance is less than 130µm and C_3 if greater, among monocots. This study therefore stregntened the proposition of Ajao *et al*., (2017) that interveinal distance greater than 130 µm but less than 166 µm may be a characteristic of many C_4 dicots.

The maximum lateral count, which is expressed as the number of chlorenchymatous mesophyll cells intervening between bundle sheaths of laterally adjacent vascular bundle (Hattersley and Watson, 1975), was also investigated. Previous studies on monocots (Hattersley and Watson 1975) proposed that two to four chlorenchymatous mesophyll cells intervene between bundle sheath of laterally adjacent vascular bundles of C_4 species, whereas this number is more than four in C_3 species. This criterion has been successfully used in Cyperaceae (Bruhl and Wilson, 2007; Ayeni *et a*l., 2015) and Poaceae (Soros and Dengler, 1998) to distinguish between C_3 and C_4 plants. In this study, the maximum lateral count of *C. gynandra* ranges from two to five while those of *C. rutidosperma* and *C. viscosa* range from six to ten. This result agrees with Ajao *et al*. (2017) on dicotyledonous species of *Boerhavia*, but it disagrees with reports for monocots (Hattersley and Watson, 1975; Bruhl and Wilson, 2007; Ayeni *et a*l., 2015). The result however suggested *C. gynandra* to be C_4 and the other two to be C_3 because lower maximum lateral count in species of the same genus is considered an indication of C_4 photosynthesis. As suggested by Ajao *et al.* (2017), this study reveals that C_4 dicots may have a higher maximum lateral counts than monocots.

One-cell-distance count theory states that in C_4 species, no chlorenchymatous mesophyll cell is separated from the nearest parenchymatous bundle sheath by more than one other chlorenchymatous mesophyll cell (Hattersley and Watson, 1975). In *C. rutidosperma* and *C. viscosa*, two cells separated their chlorenchymatous mesophyll cells from the nearest parenchymatous bundle shealth. This suggests them to be C_3 species, using this criterion while *C. gynandra*, with only one cell separating the chlorenchymatous mesophyll from the nearest parenchymatous mesophyll cell is C_4. This corresponds with many previous work (Bruhl and Wilson, 2007; Soros and Dengler, 1998; Ayeni *et a*l., 2015; Ajao *et al*, 2017 and Faniyan *et al*., 2013).

Another anatomical character investigated is the leaf thickness, which is the distance between the upper and lower cuticle of the leaf. In C_3 plants, higher leaf thickness has been reported in comparison to their C_4 counterparts (Nelson *et al.*, 2005; Nelson and Sage, 2008; Faniyan *et al*., (2013). This is because the thinner leaves in C_4 species help in quick diffusion of metabolites across cells of its tissues, an important requirement in C_4 metabolism (Dengler *et al*., 1994). The result obtain in this study shows that *C. rutidosperma* and *C. viscosa* has leaf thickness of 193.13±5.75 μm and

229±4.87 μm respectively while *C. gynandra* has a leaf thickness of 157.6±3.72 μm supporting that *C. gynandra* is C_4 while the other two are C_3. This is in conformity with the results of Ajao *et al.* (2017), Li *et al* (1999) and Bruhl and Wilson (2007) working on species in different genera. This same trend was observed for mesophyll thickness where *C. gynandra* has the lowest mesophyll thickness of 119.6±3.6 μm in comparison to 156.38±5.4 μm in *C. rutidosperma* and 202.75±5.5 μm in *C. viscosa*.

This study also investigated the proportion of intercellular airspace. Observations revealed that the intercellular air spaces found in the three species exhibit no clear pattern that could be employed for their grouping. *C. rutidosperma, C. gynandra* and *C. viscosa* have intercellular airspaces of 14.5±2.1 %, 13.8±0.8 % and 13.8±1.1 % respectively. This results disagrees with some of the previous studies where intercellular spaces was said to be significantly higher in C_3 plants than in C_4 (Diana *et al* 2007; Nelson *et al* 2005, Faniyan *et al* 2013). It is known that Diana *et al.* (2007) had previously shown that among C_4 plants, the intercellular concentration of CO_2, which is an important process in C_4 metabolism, is largely controlled by intercellular air spaces. Therefore, the C_4 species of *Cleome* may have other mechanisms of controlling CO_2 concentration which should be further investigated.

Some useful patterns were also observed in the leaf epidermal studies. Stomatal size has been shown to be higher in C_3 than C_4 species (Taylor *et al.*, 2010). Data from this study shows that *C. gynandra* has the lowest stomatal size among the three species (Table 3), on both the adaxial and abaxial surfaces. This suggests that *C. gynandra* is a C_4 species. The result is in line with the report of Ajao *et al.* (2017) where stomata size was succesfully used in grouping species of *Boerhavia* into C_3 and C_4 photosynthetic pathways. Leaf vein density data also revealed that *C. gynandra* has a higher vein density than the other two species (Table 3). High vein density in leaves is considered to be critical in the metabolism of C_4 species (Sinha and Kellogg, 1996). Results from the genus *Flaveria* (McKown and Dengler, 2007) and *Boerhavia* (Ajao *et al.*, 2017) corroborated this idea as the C_4 species in their studies have higher vein densities. Consequently this result confirmed that *C. gynandra* is C_4 while the other two are C_3.

Stomatal density, expressed as the stomata per unit leaf area (Oguro *et al*., 1985), was studied. Lin *et al*. (1986) opined that the ratio of stomata density is always small in C_3 plants compared to those of C_4 species. This was corroborated by Ajao *et al.* (2017), even though some previous reports in some genera such as *Panicum* (Oguro *et al.*, 1985), *Euphorbia* (Faniyan *et al.*, 2013) and *Cyperus* separated their photosynthetic pathways. However, this confirms the naturalness of the species grouping into a genus. This is because stomatal characteristics provide reliable evidence upon which taxonomic decisions can be based. This same conclusion can be made about the stomatal indices and inter-stomatal distances because there were no clear differences in all values obtained.

CONCLUSIONS

This study revealed that anatomical characters of the leaf proved to be an effective means of grouping the three plant species into the renowned two photosynthetic pathways. However, among the epidermal characters studied, only the stomatal size was found to be useful in the delimitation of these species because there is no clear pattern of differences in the stomatal densities, stomatal indices and the inter-stomatal distances of the species. Until now, anatomical characters studied were found to be useful in delimiting plants species, based on their photosynthetic pathways. However, this study revealed that intercellular airspace is not useful in the genus *Cleome* and probably in many other genera. The rest of the anatomical characters investigated revealed that *C. rutidosperma* and *C. viscosa* are C_3 species while *C. gynandra* is a C_4 species. Considering the results of this study and those of previous studies, it is safe to conclude that interveinal distance of less than 166µm is characteristic of dicots as opposed to 130 µm that was previously reported for monocots. It is noteworthy that though this is the first study on delimitation of Nigerian species of *Cleome* into photosynthetic pathways. Further studies are required to complement and address issues raised herein.

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