



Studies on anatomical characters indicating C₃ and C₄ photosynthetic metabolism in the genus *Boerhavia* L. (Nyctaginaceae)

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ABSTRACT: The C₃ and C₄ photosynthetic pathways in dicotyledons were investigated with the four species of *Boerhavia* occurring in Nigeria using light microscopy. The study is not yet well reported on dicotyledons as done for monocotyledons. The features cross-examined were stomata index, stomata size, inter-stomatal distance, stomatal density, interveinal distance, intercellular air spaces, leaf thickness, mesophyll thickness, Kranz tissue, one cell distant count criterion, maximum lateral cell count criterion, vein density and vein distance. Based on these features, these species (*B. erecta*, *B. coccinea* and *B. repens*) were grouped into C₄ while *B. diffusa* was grouped as a C₃ plant. In particular, interveinal distance less than 166µm and maximum lateral count ranging 2 to 6 will help in grouping C₄ dicotyledons species while those that were greater than these values are useful in grouping C₃ and plants.

KEY WORDS: *Boerhavia*, C₃ Plant, C₄ Plant, Kranz tissue, Leaf anatomy, Photosynthesis.

INTRODUCTION

Boerhavia L. is a genus in the family Nyctaginaceae comprising of 40 species worldwide (Fosberg 1978; Fosberg, 1999; Spellenberg, 2003) and four species in Nigeria (Hutchinson and Dalziel, 1972). Fosberg (1978) divided the species into two groups, *B. diffusa* group and *B. repens* group, on the basis of the mode of inflorescence. In *B. diffusa* group the inflorescences are strictly terminal and paniculate, while in *B. repens* group the inflorescences are axillary and pedunculate cymes or umbels. *Boerhavia erecta* and *B. coccinea* were reported as been newly evolved (Chou *et al.*, 2004; Chen and Wu, 2005).

The genus *Boerhavia* contains species that show C₃ and C₄ photosynthetic pathways (Gentry, 1993; Jorgensen and Ulloa, 1994; Ehleringer *et al.*, 1997; Muhaidat *et al.*, 2007). Plant species generally follow one of the three photosynthetic pathways such as Crassulacean Acid Metabolism (CAM), C₃ and C₄ (Ehleringer *et al.*, 1997; Wang, 2004). The most common are the C₃ and C₄, while CAM species are found in succulent plants (Ranson and Thomas, 1961). Several authors have affirmed variations occurring in the photosynthetic pathways are strictly associated with differences in ultrasound characteristics, physiological, ecological and most importantly anatomical features of such plants (Takeda *et al.*, 1980; Hibberd and Quick, 2002; Bruhl and Wilson, 2007). This is because the leaves of C₄ plants have anatomical features different from those of C₃ plants, and the difference in biochemical function is associated with the structural features of leaves (Ueno *et al.*, 2006).

There are number of anatomical features which have been used previously to distinguish the plant species possessing C₃ and C₄ photosynthetic pathways, these include stomatal density, interstomata distance, stomatal index and stomatal size, Kranz tissue, interveinal distance (Oguro *et al.*, 1985; Soros and Dengler, 1998; Nelson *et al.*, 2005; Bruhl and Wilson, 2007; Martins and Alves, 2009; Taylor *et al.*, 2010; Kim, 2012; Faniyan *et al.*, 2013; Ayeni *et al.*, 2015). Other features include leaf thickness, mesophyll thickness, intercellular air spaces, one cell distant criterion and maximum lateral cell count (Dengler *et al.*, 1994; Soros and Dengler, 1998; Nelson *et al.*, 2005; Bruhl and Wilson, 2007; Diana *et al.*, 2007; Faniyan *et al.*, 2013; Ayeni *et al.*, 2015). Leaf vascular characters such as leaf vein density and vascular spacing which are highly diverse across C₃ and C₄ flowering plant species (Nelson and Dengler, 1997) were also considered because of their importance in leaf physiological functions such as photosynthesis and water use efficiency (Sack and Holbrook, 2006; Brodribb *et al.*, 2007; Mckown *et al.*, 2010).

Most important anatomical feature is Kranz tissue which is a structure of two morphologically and functionally distinct types of photosynthetic cells: mesophyll and bundle sheath. The Kranz tissue has been commonly used in numerous anatomical surveys of leaves to identify C₄ photosynthetic pathway for many plant species (Renvoize, 1987; Hattersley *et al.*, 1982; Faniyan *et al.*, 2013; Ayeni *et al.*, 2015). The unique leaf anatomy of C₄ plants provides several advantages for efficient CO₂ fixation which is required in C₄ photosynthetic pathway because C₃ bundle sheath cells

**Table 1:** *Boerhavia* species collected and their locations in Southwestern Nigeria.

Species	Sample sites/ GPS coordinate	Location
<i>Boerhavia coccinea</i> Mill	OAU (7°34' N, 4°68' E), Ede (7°44' N, 4°26' E)	Osun
<i>Boerhavia diffusa</i> L.	OAU (7°34' N, 4°68' E), Ede (7°44' N, 4°26' E)	Osun
<i>Boerhavia erecta</i> L.	OAU (7° 34' N, 4°68' E), Ede (7° 44' N, 4°26' E)	Osun
<i>Boerhavia repens</i> L.	Ajibode Ahoo (7°27' N, 3°53' E)	Oyo

enveloping C₄ mesophyll cells minimizes the loss of CO₂ from C₃ photorespiration (Nelson *et al.*, 2005). There appears to be few exceptions, perhaps they are the intermediate species, because some C₃ plants have been shown to possess Kranz tissue (Kanai and Kashiwagi, 1975), while some C₄ plants have been reported not to possess the Kranz tissues (Sage, 2002; Voznesenskaya *et al.*, 2002; Edwards *et al.*, 2004). Intermediate species however present caution in directly linking the occurrence of Kranz tissue to C₄ metabolism, hence it is insufficient to base classification of taxa as a C₄ species on this character alone.

We suggest that any reliable classification that will be based on anatomical evidence should put into consideration many characters. A similar position has been stressed previously by Bruhl *et al.* (1987) and Sage (2002) that there is need for some caution when using isolated characters as predictors of photosynthetic pathway in plants due to the presence of intermediate species. The current study focuses on the application combined anatomical characters for the grouping the Nigerian species of the genus *Boerhavia* into either C₃ or C₄ photosynthetic pathways. This is with the hope of unravelling those characters that may be peculiar to dicotyledons.

MATERIALS AND METHODS

Collection of Plant material

All the four described Nigerian species of *Boerhavia* which are; *B. coccinea* Mill, *B. repens* L., *B. diffusa* L., and *B. erecta* L. were used in this study. Fresh samples of all the four species were collected from different locations in Southwestern Nigeria. (The geographical coordinates of all the locations are shown in Table 1), a total of 45 plants were sampled with at least 10 individuals representing each species. Identification was carried out at the Department of Botany Obafemi Awolowo University Herbarium (IFE) and Forestry Herbarium, Ibadan (FHI) in addition to the information from the Flora of West Tropical Africa by Hutchinson and Dalziel (1972). Herbarium abbreviations are after Holmgren *et al.* (1981).

Leaf Anatomical Studies

Fresh mature leaves of all the species were used. In every plant sampled, data was taken from 2-3 leaves. For foliar epidermal study, sizeable portions of mature fresh leaves for each species studied were cut from the median part (i.e. mid-way between base and petiole)

following the previously established protocols (Adedeji and Jewoola, 2008; Saheed and Illoh, 2010; Ogundare and Saheed, 2012). Epidermal peels were made for both abaxial and adaxial surfaces of the leaf and stomatal density, stomatal index, inter-stomatal distance and stomatal size were measured. Clearing of whole leaf lamina was carried out for the purpose of studying vein density and vein spacing.

For the purpose of studying leaf internal tissues, transverse sections of the paraffin wax-embedded leaf samples were cut using a sledge microtome set at 20µm thickness. Specimens were processed using standard procedures (Illoh, 1995; Adedeji and Jewoola, 2008; Saheed and Illoh, 2010) and they were viewed for the presence or absence of Kranz tissue while counts were made for maximum lateral cell count (the number of chlorenchymatous mesophyll cells intervening between bundle sheaths of laterally adjacent vascular bundles) and one cell distant count (no chlorenchymatous mesophyll cell is separated from the nearest parenchymatous bundle sheath cell by more than one other chlorenchymatous mesophyll cell). Measurements were taken for the leaf thickness, mesophyll thickness, interveinal distance (The interveinal distance, representing the average distance between vein centers) and proportion of intercellular air spaces. Sections were viewed with light microscope Leica DM 500 and captured with Amscope camera attached to ACCUScope microscope.

All 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 basic statistical methods were applied for the interpretation.

RESULTS

Transverse section of the leaf showed the presence of Kranz tissue in all the species studied with the exception of *B. diffusa* (Fig. 1). The leaf anatomical structures of *B. coccinea*, *B. erecta* and *B. repens* have the chlorenchymatous mesophyll cells intervene between bundle sheaths of adjacent vascular bundles (maximum lateral count) ranging from two to six while that of *B. diffusa* ranges from five to ten. One cell distant criterion was absent only in *B. diffusa* (Fig. 1C & D) while it occurs in others. *Boerhavia coccinea*, *B. erecta* and *B. repens* (Fig. 1A, B, E, F, G & H) have interveinal distances of 153±7.8µm, 152 ± 13.7µm and 166 ± 31.5µm respectively while the highest interveinal

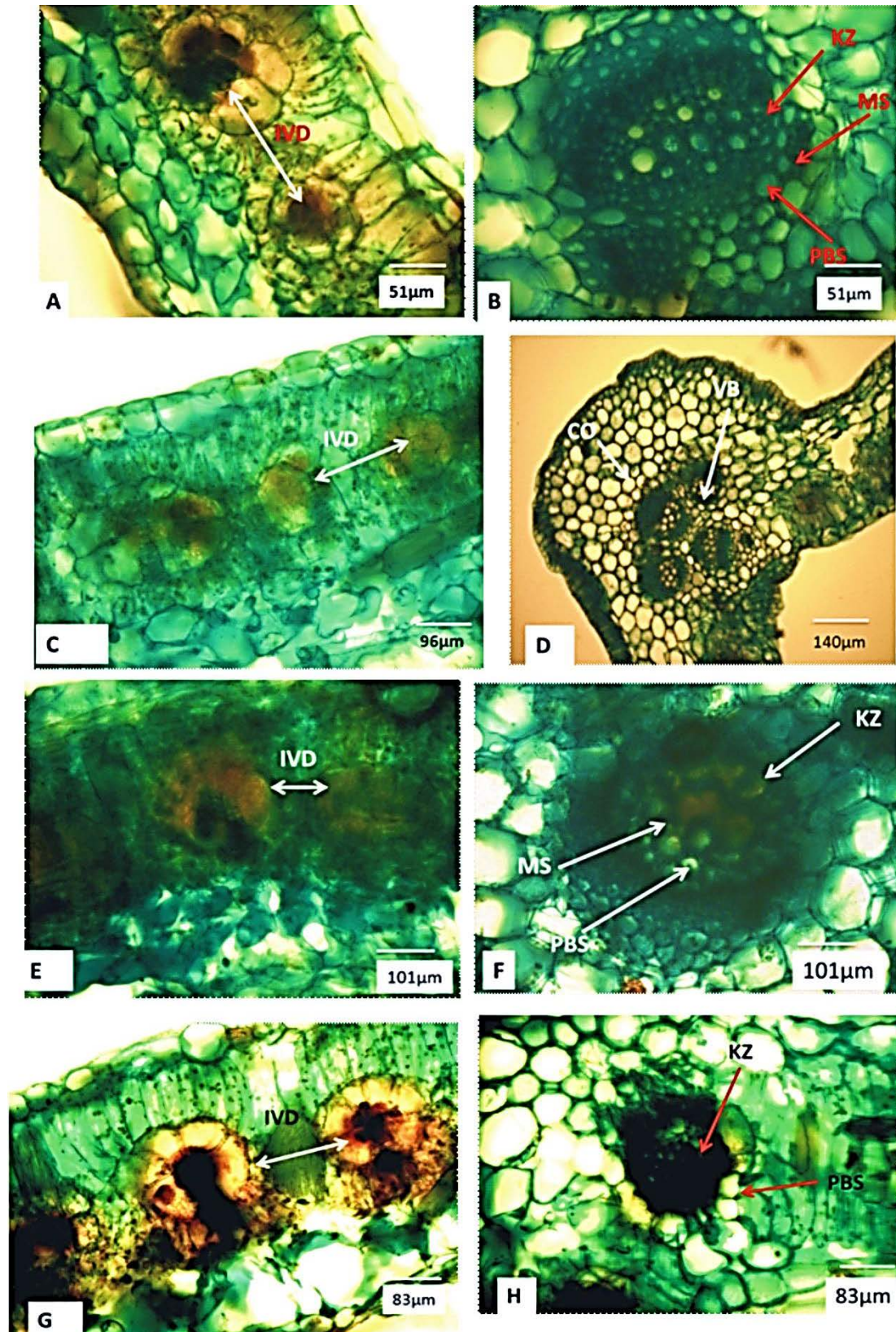


Fig. 1: Leaf transverse section of the *Boerhavia* species; (A)-(B) *B. coccinea* (C_4 species), (C)-(D) *B. diffusa* (C_3 species), (E)-(F) *B. erecta* (C_4 species), (G)-(H) *B. repens* (C_4 species); IVD: interveinal distance, VB : vascular bundle, MS : mesophyll sheath, PBS: parenchymatous bundle sheath, KS : Kranz sheath.

**Table 2:** Summary of Leaf Anatomical Characters of *Boerhavia* Species Studied.

Species	KT	IVD(μm)	MLCC	OCDC	LT(μm)	MT(μm)	IAS%
<i>B. coccinea</i>	Present	153.3 \pm 7.8	3-6	1	340.0 \pm 10.4	281.0 \pm 10.7	11.2 \pm 0.3
<i>B. diffusa</i>	Absent	192.0 \pm 23.0	5-10	2	422.0 \pm 11.3	347.0 \pm 8.6	20.6 \pm 8.6
<i>B. erecta</i>	Present	152.0 \pm 13.7	2-6	1	390.0 \pm 6.1	319.0 \pm 6.4	12.5 \pm 0.3
<i>B. repens</i>	Present	166.0 \pm 31.5	3-6	1	317.0 \pm 7.1	272.0 \pm 6.6	11.7 \pm 0.4

KT= Kranz Tissue; IVD= Interveinal Distance; MLCC= Maximum Lateral Cell Count; OCDC= One Cell Distant Count; LT= Leaf Thickness; MT= Mesophyll Thickness; IAS= Intercellular Airspaces. Values are means + Standard Error

Table 3: Summary of Leaf Epidermal Characters of *Boerhavia* Species Studied

Species	S.I (%) n=20		SD(mm ⁻²) n=20		ISD (μm) n=20		S.S (μm^2) n=20		V.D(mm ⁻²)	V.S(μm)
	AD	AB	AD	AB	AD	AB	AD	AB		
<i>B. coccinea</i>	87.1 \pm 1.7	86.7 \pm 0.9	6.4 \pm 0.3	5.6 \pm 0.3	72.5 \pm 6.6	82.2 \pm 7.1	322.1 \pm 10.1	447.0 \pm 8.9	11.5 \pm 0.4	186.0 \pm 11.7
<i>B. diffusa</i>	81.4 \pm 0.9	84.7 \pm 1.0	4.8 \pm 0.3	3.3 \pm 0.2	109.0 \pm 8.1	93.7 \pm 8.8	381.4 \pm 18.9	523.0 \pm 20.4	6.9 \pm 0.3	303.0 \pm 19.0
<i>B. erecta</i>	82.0 \pm 0.8	79.1 \pm 2.7	11.2 \pm 0.4	11.8 \pm 0.3	66.7 \pm 9.9	75.5 \pm 10.7	253.3 \pm 5.8	167.0 \pm 9.4	11.1 \pm 1.5	225.0 \pm 9.6
<i>B. repens</i>	82.5 \pm 1.0	84.3 \pm 1.1	11.8 \pm 0.4	7.4 \pm 0.4	60.5 \pm 5.4	57.5 \pm 5.9	180.0 \pm 4.9	292.0 \pm 14.0	10.5 \pm 0.4	189.0 \pm 5.5

S.I= Stomatal Index; SD=Stomatal Density; ISD=Interstomatal Distance; S.S= Stomatal Size; V.D=Vein Density;V.S= Vein Spacing; AD=Adaxial; AB=Abaxial. Values are means +| Standard Error

distance of $192 \pm 23.0\mu\text{m}$ was seen in *B. diffusa* (Table 2). The same trend was observed for leaf thickness and mesophyll thickness (Table 2) where *B. diffusa* has the highest mean values compared to other species. For the proportion of intercellular air spaces, *B. diffusa* has the highest mean proportion of $20.6 \pm 8.6\%$ while the remaining species such as *B. coccinea*, *B. erecta* and *B. repens* have mean proportion of $11.2 \pm 0.3\%$, $12.5 \pm 0.3\%$, and 11.7 ± 0.4 respectively (Table 2).

The result of the vasculature of the leaves reveals that *B. diffusa* has the lowest mean vein density and highest mean vein spacing compare to other species studied (Table 3). Epidermal characters also varied greatly among the species studied. *Boerhavia diffusa* has stomata density that is less than 5mm^{-2} on both abaxial and adaxial surfaces; it also has lower stomatal index on the adaxial surface (Table 3), while other species have stomata density greater than 5mm^{-2} . Highest interstomatal distance on both abaxial and adaxial surfaces was observed in *B. diffusa* (Table 3). The last epidermal character considered in this study is the stomatal size, and the result showed that *B. diffusa* has the highest stomatal size of $523 \pm 20.4\mu\text{m}^2$ and $381 \pm 18.9\mu\text{m}^2$ at both abaxial and adaxial surfaces respectively in comparison to other species studied (Table 3).

Discussion

This study has investigated the usefulness of combination of leaf anatomical characters in the grouping of *Boerhavia* species into different photosynthetic pathways. Of all the species studied, Kranz tissue is present in *B. coccinea*, *B. erecta* and *B. repens* but absent in *B. diffusa*. This suggests that *B. coccinea*, *B. erecta* and *B. repens* have C_4 photosynthetic structure while *B. diffusa* is a C_3 species. Kranz tissue has been used in numerous anatomical

surveys of leaves to distinguish plant having C_4 photosynthetic pathway (Renvoize, 1987; Hattersley *et al.*, 1982; Faniyan *et al.*, 2013; Ayeni *et al.*, 2015). The occurrence of Kranz tissue presents a unique anatomical feature in C_4 plants and it is suggested to confer several advantages for efficient CO_2 fixation in such species (Nelson *et al.*, 2005). Kranz tissue surrounds the C_3 bundle sheath cells with C_4 mesophyll cells which help in minimizing the loss of CO_2 thus conferring C_3 photorespiration advantage (Nelson *et al.*, 2005).

The 'maximum cell distant count theory' i.e. the one-cell-distant benchmark is one of the standard criteria investigated in this study. It states that in C_4 species, not more than one other cell separates a chlorenchymatous mesophyll cell from the nearest parenchymatous bundle sheath cell (Hattersley and Watson, 1975). *Boerhavia diffusa* has two cells separating the chlorenchymatous mesophyll cells from the nearest parenchymatous bundle sheath thereby suggesting it to be C_3 species but only one cell separates the chlorenchymatous mesophyll from the nearest parenchymatous mesophyll cell in other species suggesting they are C_4 species. This corresponds with the works of Bruhl and Wilson (2007), Soros and Dengler (1998), Ayeni *et al* (2015) and Faniyan *et al* (2013) who have all reported that 'One cell criterion accurately predicts C_4 pathway in species of *Cyperus* and *Euphorbia*.

The 'maximum lateral cell count' is another criterion investigated in this study and it is expressed as the number of chlorenchymatous mesophyll cells intervening between bundle sheaths of laterally adjacent vascular bundles (Hattersley and Watson, 1975). The criterion as expressed in grasses showed that between two to four chlorenchymatous mesophyll cells intervene between bundle sheaths of laterally adjacent vascular bundles in C_4 species but in C_3



species, more than four chlorenchymatous mesophyll cells intervene between bundle sheaths of laterally adjacent vascular bundles. *Boerhavia coccinea*, *B. erecta* and *B. repens* may be regarded as belonging to the former group, C_4 plants, with a range of two to six chlorenchymatous mesophyll cells while *B. diffusa* having a range of five to ten chlorenchymatous mesophyll cells belongs to the later C_3 plant group. This still shows that a lower number of chlorenchymatous mesophyll cells intervening between bundle sheaths of adjacent vascular bundles occur in C_4 species while a higher number is found in C_3 species.

Hattersley and Watson, 1975; Soros and Dengler, 1998; Bruhl and Wilson, 2007; Ayeni *et al.*, 2015 also reported in monocotyledons that lower number of chlorenchymatous mesophyll cells intervene between bundle sheaths of adjacent vascular bundles in C_4 species but the number is usually higher in the C_3 species. This same pattern occurs in the four species of *Boerhavia* studied. However, there is difference in the number of intervening chlorenchymatous mesophyll cells and it is assumed that the difference is due to the plants are dicotyledons. This assertion should be probed further in other dicotyledonous species so as to understand the mechanism better in the group. Future research is needed in this area to establish whether or not the pattern is generic, specific or group constant in angiosperms.

Interveinal distance is expressed as the average distance that exists between vein centres (Li *et al.*, 1999). Closer spacing of veins is known to confer an efficient transport of photosynthetic products between cells of species where they occurs (Monsoon *et al.*, 1984). Earlier studies on monocots have shown that leaf interveinal distance of a plant species is less than $130\mu\text{m}$ if such a species is C_4 and greater if it is C_3 (Takeda *et al.*, 1980; Dengler *et al.*, 1994; Li and Jones, 1994; Ayeni *et al.*, 2015). This clearly shows that a relatively lower interveinal distance is a characteristic of a C_4 species while a higher interveinal distance is for C_3 species. The values found in this study were quite above the limit of $130\mu\text{m}$ reported for monocots which suggests that dicots tend to have higher values. This finding however, conforms to the report of Faniyan *et al* (2013) on *Euphorbia*, another dicotyledonous plant.

The leaf and mesophyll thickness shows that though these characters vary among the species studied, however, *B. diffusa* still has the highest mean values when compared to other species studied. The results of this study and those of earlier ones (Oguro *et al.*, 1985; Nelson *et al.*, 2005; Nelson and Sage, 2008; Faniyan *et al.*, 2013) depict that a general increase in leaf and mesophyll thickness is characteristic of C_3 species when compared to C_4 species. The thinner is thought to help diffusion of metabolites more quickly across tissues, this is a very important requirement in C_4

metabolism (Dengler *et al.*, 1994). A similar pattern was observed for intercellular air spaces where *B. diffusa* displayed a higher value when compared to the other three species. The report from this study is also in agreement with those of Nelson *et al.* (2005) on monocotyledons and Faniyan *et al.* (2013) on dicotyledons where C_3 plants are reported to exhibit higher proportion of intercellular air spaces than their C_4 counterparts. Diana *et al.* (2007) had shown that in C_4 plants, the intercellular concentration of CO_2 , which is largely controlled by intercellular air space at which the photosynthetic rate equals the rate of respiration, is lower than that of C_3 plants.

Leaf vascular traits are important in the direct determination of the leaf physiological functions such as photosynthesis and water use efficiency (Sack and Holbrook, 2006; Brodribb *et al.*, 2007; McKown *et al.*, 2010). In *B. diffusa*, lowest mean vein density and highest mean vein spacing compare to other species suggests is a C_3 species. In line with Takeda and Fukuyama (1971) and Crookston and Moss (1974), vein spacing in C_3 and C_4 species revealed that veins are consistently more closely packed with denser vascular system in C_4 species than C_3 leaves. Close veins in leaves is speculated to be a critical step in the evolution of the C_4 pathway in angiosperms (Sinha and Kellogg, 1996). However, this has only been demonstrated in the eudicot genus *Flaveria* (McKown and Dengler, 2007). The greater vein density observed in C_4 compared to C_3 species probably involves modifications to minor vein patterning rather than major veins (Ueno *et al.*, 2006; McKown and Dengler, 2007; Muhaidat *et al.*, 2007).

Apart from the leaf anatomical features described above, the leaf epidermal features studied were equally taxonomically informative. Epidermal characters such as stomatal density, stomatal size and interstomatal distance in both abaxial and adaxial surfaces were particularly useful in this study. Only *B. diffusa* has stomata density that is less than 5mm^{-2} on both abaxial and adaxial surfaces, other species have stomata density that is greater than 5mm^{-2} . This further suggests that *B. diffusa* is a C_3 species while others are C_4 . Our findings on this corroborated the report of Lin *et al* (1986) which stated that the ratio of stomata density is always small in C_3 plants compared to those of C_4 species. In addition, Kim (2012) reported that in the last 50 years, reduction in stomatal density was greater in C_3 plants than C_4 plants while Taylor *et al.* (2010) reported that stomatal conductance in C_4 is lower than that of C_3 as a result of stomatal size which is shown to be higher in C_3 than C_4 . Our data showed that *B. diffusa* has the highest stomatal size on both surfaces in comparison to other species studied; thus confirming the grouping of the species as a C_3 plant. Another interesting physiological process which the study will assist its



additional understanding is the stomatal behaviour. The rates of stomatal opening and closure, or the stomatal conductance of the plant has been shown to differ among C_3 and C_4 plants. According to Huxman and Monson (2003) stomata of C_3 plants open and close more directly to light while the stomata of C_4 plants respond more directly to internal CO_2 conditions which may now resulted in higher stomatal size in C_3 species than C_4 counterpart.

The anatomical features of the leaves of the studied species are good indices for the photosynthetic grouping. Although the features described are those of a few representatives of dicotyledons which hitherto have not been described as yet for any plants in this category. There are several articles covering some aspects of the features studied in many monocotyledons. However, the specificity of the characters in dicotyledons requires further studies in future research. In the study, the features studied appeared useful for grouping three species (*B. erecta*, *B. coccinea* and *B. repens*) into C_4 plants and one *B. diffusa* into C_3 plant. The features are additional identification criteria for the studied species. This study therefore proposes characters, possibly for the first time as we are not aware of any existing ones, which may be peculiar only to dicotyledons. Finally, the findings reported have strengthened the opinion of Fosberg (1978) that the genus *Boerhavia* should be divided into two groups which are *B. repens* (C_4) group and *B. diffusa* (C_3) group. However, carbon isotope discrimination, oxygen suppression, photosynthetic products, carbon dioxide compensation and photorespiration should be investigated to take the decision reached here a bit further.

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