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# ECO-PHYSIOLOGICAL VARIATION AND ADAPTATION MECHANISMS OF *Azadirachta indica* A. JUSS IN NIGERIA

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## ABSTRACT

This study assessed diverse foliar morphological and anatomical characteristics exhibited by *Azadirachta indica* towards adapting to various climatic conditions in Nigeria. The study covered three ecological zones of Nigeria, namely: Rainforest (Akure), Derived Savanna (Ekiti) and Guinea Savanna (Ilorin). In each ecological zone, ten trees at not less than 50 meters apart were randomly selected and ten leaf samples were collected from each of the selected trees. The number of leaflets on each leaf was counted. Out of the ten leaf samples, two leaves were randomly selected and all their leaflets were measured using Leaf Area Meter. Variables measured include: Leaflet Surface Area (LSA), Leaflet Length (LL), Leaflet Width (LW), Apex Length (AL) and Degree of Obliqueness (DO). Leaflets were also subjected to further analyses, including: estimation of stomata density, epidermal cell density on both adaxial and abaxial surfaces and cuticle thickness. It was revealed that there were significant reductions in all the foliar morphological variables from rainforest to derived savanna and guinea savanna ecological zones, respectively. The mean densities recorded for stomata and epidermal cells varied across the ecological zones, likewise, the cuticle thickness. Highest stomata density and cuticle thickness was recorded in this species occurring in lowland rainforest (33.33) and guinea savanna (28.16μm) respectively. Whereas, very Low stomata density was observed in this species occurring in derived savanna (25.00). High stomata density in the leaflets of this species found in the rainforest may be a mechanism developed to adjust to the low light intensity in this ecological zone to aid photosynthesis. High cuticle thickness observed in Guinea savanna may be associated with control of water loss as a result of high temperature. More so, low stomata density in the derived savanna may also be associated with less water availability and high need to conserve water by controlling evapo-transpiration from the surface of the leaflets. This study has added more knowledge to ecophysiological variation and adaptation mechanism of *Azadirachta indica*.

**Keywords:** Folia Morphology, Anatomic Variation, Adaptation Mechanism and Ecological Zones.

## INTRODUCTION

Climate change is altering the availability of resources and the conditions that are crucial to plant performance (Nicotra *et al.*, 2010). One way in which plants will respond to these changes is through environmentally induced shifts in phenotype (phenotypic plasticity). According to Kelly *et al.*, (2012) phenotypic plasticity can be broadly defined as the ability of a genotype to produce different phenotypes in response to different environmental conditions. According to Auld *et al.*, *(*2009), in nature where environmental conditions continually vary, individual is confronted with the challenge of taking full advantage of fitness under diverse conditions. Species inhabit a large geographical array or a variety of different habitats within a limited area, or both, phenotypic plasticity has been broadly recognized as a significant feature for plants to cope with varying environmental heterogeneity (Lamarque *et al.*, 2013, Valladares *et al.*, 2007). Coleman *et. al.*, (1994) also buttress that environment-induced phenotypic variation (phenotypic plasticity) in plants is often considered to be a functional response that maximizes fitness in variable environments. These plastic responses include changes in behavior, physiology, morphology, growth, life history and demography, and can be expressed either within the lifespan of a single individual or across generations (Miner *et al*, 2005). When populations experience suboptimal conditions, the mechanisms involved in the regulation of phenotypic variation can be challenged, resulting in increased phenotypic variance. This kind of disturbance can be diagnosed by using morphometric tools to study morphological patterns at different hierarchical levels (Lazic *et al.*, 2015).

Generally, the anatomical structure of leaves is regulated by many environmental factors such as temperature, water availability and light intensity (Fernandes *et al.*, 2014; Weih and Karlsson, 1999). In responding to a wide range of environmental stimuli such as light, temperature, and water status, there are alterations in the anatomical characteristics of leaves, which consequently affect the behavior and function of certain structural components. These responses are known as adaptation strategies; it is the way in which plant species relates with environmental factors for survival. Technically, strategies a particular plant species develop in interacting with its site environment may vary from that of a different species.

Leaves play key roles in plant function and long-term adaptation to the environment. Although, comprising basically of epidermis, stomata, and mesophyll, leaves exhibit apparent differences in area, thickness, and shape among different species, as a result of phylogenetic relationships and adaptation to specific environments (Farquhar *et al.*, 2002). Few researchers have investigated how morphological traits of the leaf economic spectrum, such as leaf area, and specific leaf area, vary across large geographical scales and ecosystems and adapt to environmental factor (Zheng *et al*., 2013). However, it remains unclear whether variations in leaflets morphological and anatomical traits in *Azadirachta indica* are associated with adaptation to different environments across a large geographical scale in Nigeria. Hence, the need for this study.

## MATERIALS AND METHODS

### Study Area

The research was carried out in Akure, Ondo State (Rainforest), Otun Ekiti, Ekiti State (Derived Savanna) and Ilorin, Kwara State (Guinea Savanna) as presented in Fig. 1 - 3.

### Sample Collection

Samples were collected in three ecological zones which were purposefully selected owing to more population of this species. In each ecological zone, ten trees at not less than 50 meters apart were randomly selected and ten leaves were collected from each of the selected trees. Each tree assessed in different ecological zones were more than 50 meters apart this is because closely related species show more similar patterns of plasticity than distantly related species, regardless of non-native or invasive status (Cook-Patton and Agrawal, 2011). The number of leaflets on each leaf were counted and recorded. Subsequently, two leaves were selected randomly from the ten leaves and all the leaflets in the leaves were measured using Leaf Area Meter. Variables measured include: leaflet surface area (LSA), leaflet length (LL), leaflet width (LW), apex length (AL) and degree of obliqueness (DO).

Also, three leaflets were selected randomly from 3 randomly selected trees in each ecological zone for anatomical study. In the leaflet epidermal study, standard median portions (midway between the tip and the base) were taken from each sample. The portions were put into Nitric Acid, in glass Petri-dishes, and kept in an oven at 60ºC for 20 minutes. Each sample was washed thoroughly in 4-5 changes of water. The abaxial and the adaxial epidermis were separated by means of fine forceps and dissecting needle. The epidermis were then stained in Safranin-O, and counter stained in Toluedene blue for five minutes, washed with 4-5 changes of water to remove excess stain and then mounted in 25% glycerol. The scrape method of Metcalfe (1968) was utilized in getting the epidermal peels of some of the accessions. The entire leaves were placed face down on a slide and flooded with a commercial bleaching agent (containing Sodium Hypochlorate). The material was then carefully scraped with a new razor blade until the epidermis was reached. The bleaching agent acted as lubricant and at the same time helped to soften the cell layers as they were scraped off. After scraping, the scraped portion was carefully cut and the peels were stained in Safranin-O and then mounted in 25% glycerol for examination under the light microscope. Photomicrographs of the epidermis were made for both the adaxial and abaxial surfaces. Trichome type(s), crystal type(s), shapes of the epidermal cells, numbers of epidermal cells, stomata types and stomata frequency were all noted for both leaf surfaces. Also, stomata frequency per square millimetre and stomata index (I) were estimated for the two leaf surfaces using the formula below as proposed by Wilkinson (1979):

$$SI = \frac{S}{(S+E)} \times 100$$

Where S = number of stomata per unit area

E = number of ordinary epidermal cell in the same area

SI = stomata index

The length and breadth of the stomata was measured using ocular micrometer and the measurements were converted to microns using the stage micrometer.

The anatomy of sampled leaflets was studied by cutting transverse sections of the leaflet. All sections were made with the aid of Reichert Sliding Microtome at a thickness of 8 - 10 microns.

The sections were stained in Alcian blue for 3-5 minutes, rinsed thoroughly in water to remove excess stain and counterstained in Safranin O solution for 3-5 minutes. The sections were again washed with water and treated in series of ethanol dilution: 50%, 70%, 80%, 90% and 100% to enhance the dehydration process. The dehydrated sections were transferred into absolute xylene to remove any remaining trace of water and ethanol. These made sections clear and prevented cloudiness of the slide, as well as the drying of the slide. Sections were therefore mounted in 25% glycerol. Photomicrographs of all anatomical features were made with the aid of Accu-scope Trinocular Microscope (Accu-scope 33001 LED Trinocular Microscope with 3.2 MP CMOS digital camera). All measurements were made with the aid of ocular micrometer and final figures derived with ocular constant. For the determination of the cuticle thickness of the leaflets, transverse sections of the leaves were cut at thickness 20 ųm using Reichert sliding microtome. Specimens were processed using standard anatomical procedures as described previously (Illoh, 1995; Adedeji and Jewoola, 2008; Saheed and Illoh, 2010). All microscopic measurements were made with the aid of an ocular micrometer inserted in the eyepiece of the microscope. These measurements were later multiplied by the ocular constant with respect to the objectives under which they were taken.

## DATA ANALYSIS

The Data obtained was subjected to one way analysis of variance in complete randomized design (CRD) using Statistical Package for Social Sciences (SPSS) version 21 to test for significant differences among the variables measured across the ecological zones. Where significant differences exist, Duncan's new multiple range test (DMRT) follow-up procedure was carried out.

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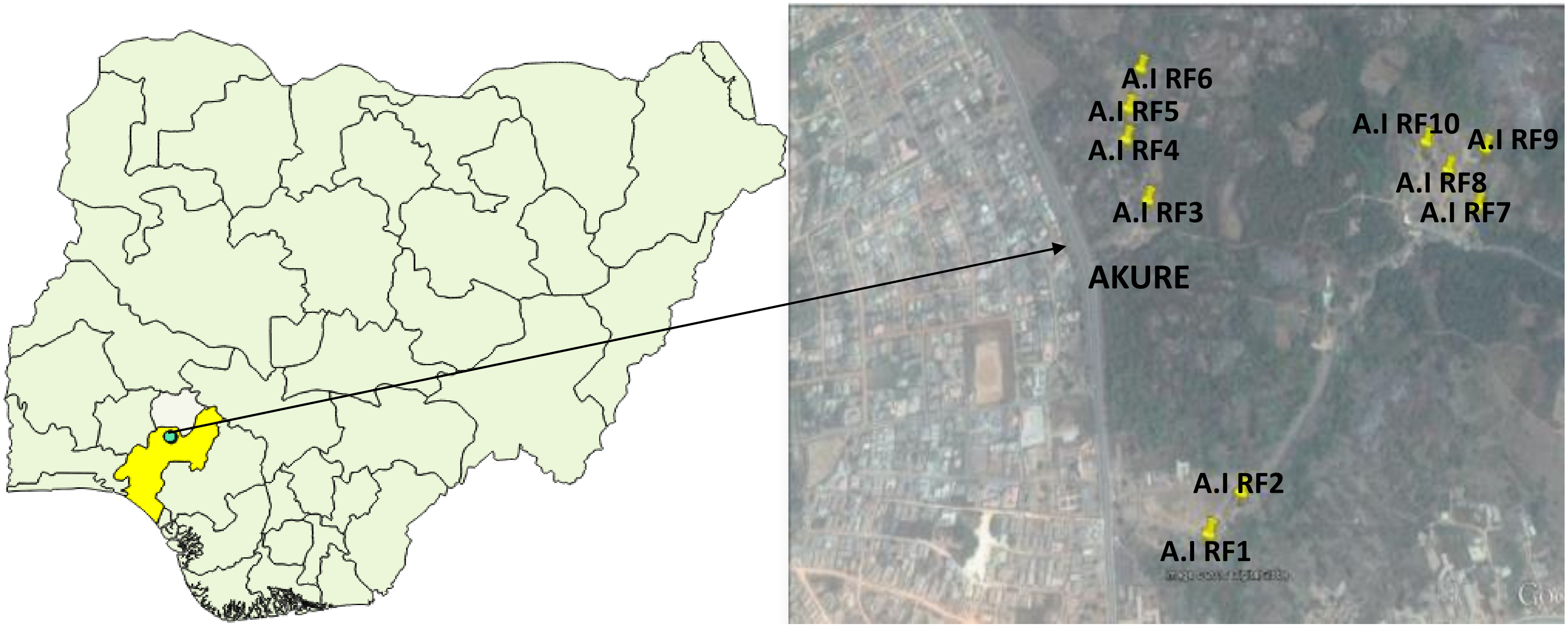


**Plate 1: Map of Nigeria Showing Where Samples were Collected in Ondo State**
**NOTE: A.I -** *Azadirachta indica,* **RF** – Rainforest.

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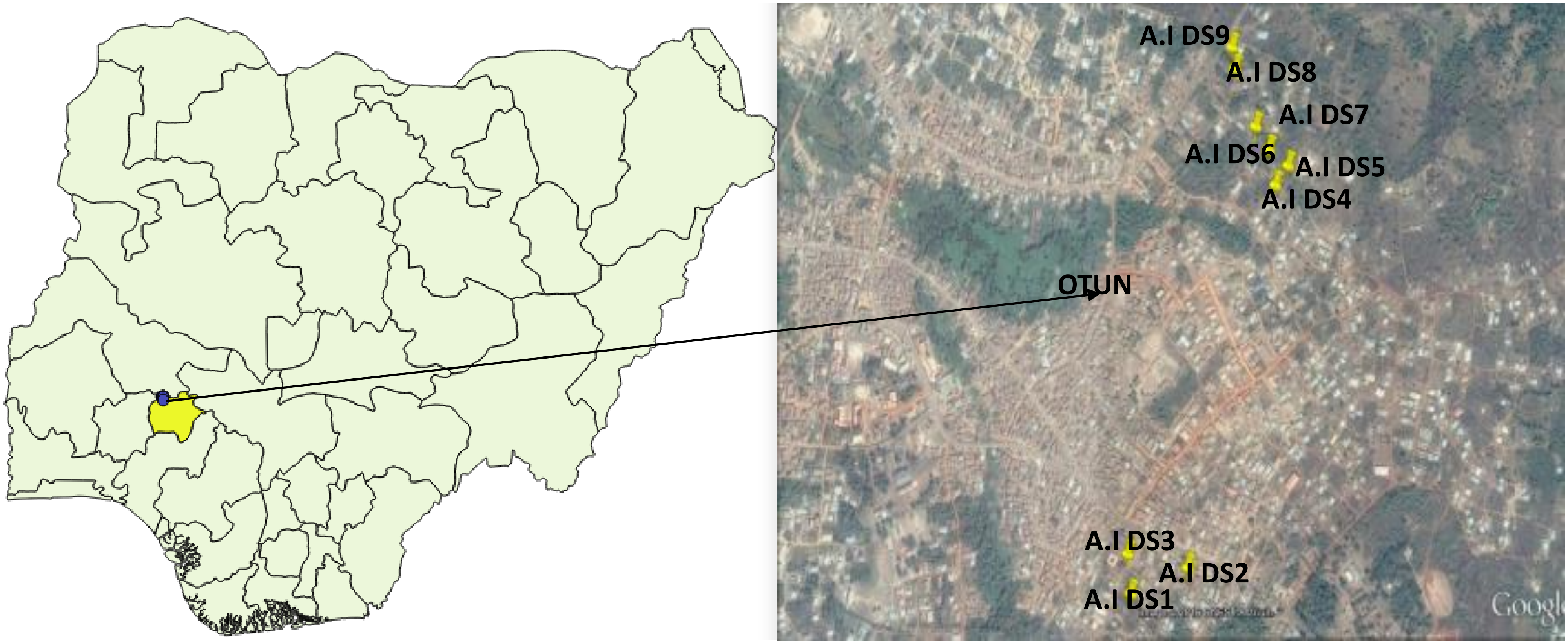


**Plate 2: Map of Nigeria Showing Where Samples were Collected in Ekiti State**
**NOTE: A.I -** *Azadirachta indica*, **DS** – Derived Savanna

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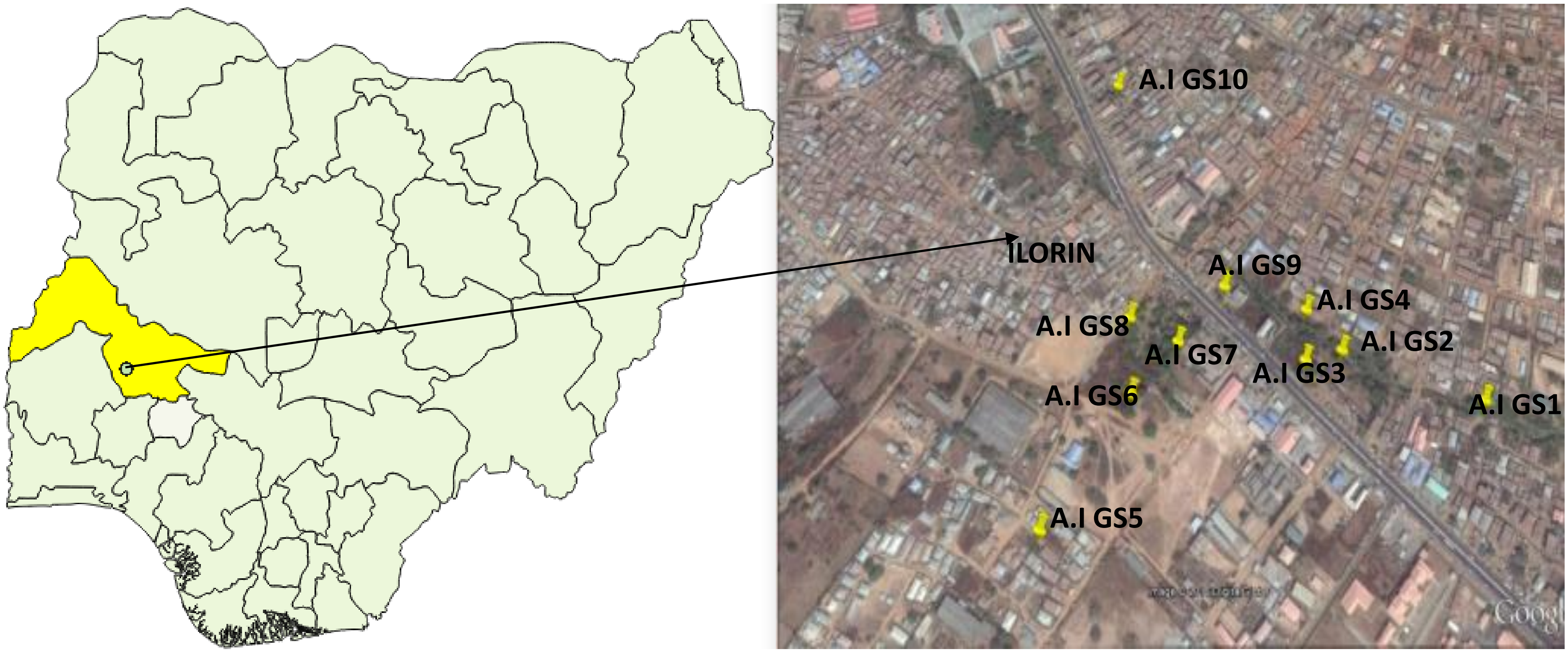


**Plate 3: Map of Nigeria Showing Where Samples were Collected in Kwara State**

**NOTE: A.I -** *Azadirachta indica,* **GS –** Guinea Savann

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## Results and Discussion

### Foliar Morphological Characteristics

The Analysis of Variance (ANOVA) for comparing leaflets morphological characteristics revealed significant differences among the three ecological zones (Table 2). The leaflet surface area was found to be significantly higher in Rainforest trees (10.67 $m^2$), followed by Derived Savannah (9.56 $m^2$) and Guinea Savanna (7.60 $m^2$) respectively. Also, leaflet length was significantly higher in Rainforest trees (7.56 cm), followed by Derived Savannah (6.72 cm) and Guinea Savanna (6.27 cm). Leaflet width was significantly higher in Rainforest trees (2.94 cm), than in Derived Savannah (2.77 cm) and Guinea Savanna (2.48 cm) trees respectively. Additionally, leaflet apex was significantly higher in Rainforest trees (1.17 cm), followed by Derived Savannah (1.07 cm) and Guinea Savanna (0.97 cm) trees respectively. Moreover, the degree of obliqueness recorded significantly higher value in Rainforest trees (0.56 cm), followed by Derived Savannah (0.52 cm) and Guinea Savanna (0.52 cm) trees respectively.

**Table 1: Summary Result for Foliar Morphological Characteristics**

| Ecological Zones | LSA± SE($cm^2$) | LL ± SE(cm) | LW± SE(cm) | AL± SE(cm) | DO± SE |
|---|---|---|---|---|---|
| Guinea Savanna | $7.60\pm0.15^c$ | $6.27\pm0.09^c$ | $2.48\pm0.04^c$ | $0.97\pm0.013^c$ | $0.52\pm0.011^c$ |
| Derived Savanna | $9.56\pm0.17^b$ | $6.72\pm0.10^b$ | $2.77\pm0.04^b$ | $1.07\pm0.01^b$ | $0.52\pm0.01^b$ |
| Rainforest | $10.67\pm0.25^a$ | $7.56\pm0.11^a$ | $2.94\pm0.05^a$ | $1.17\pm0.01^a$ | $0.56\pm0.01^a$ |

*Means with the same superscript are not significantly different from each other.*
*Note: LSA - Leaflet Surface Area, LL - Leaflet Length, LW - Leaflet Width, AL - Apex Length, DO - Degree of Obliqueness and SE – Standard Error*

### Anatomical Features of Leaflets of *A. indica* from the Derived Savannah Ecosystem

The Photomicrographs revealing anatomical features in the leaflet of *A. indica* found in Derived savannah are presented in Plate 1, 2, 3 and 4. On the adaxial surface, epidermal cells are polygonal with straight anticlinal walls, mean epidermal cells was 31 per square millimetre. Stomata are absent, simple unicellular trichomes were present, though they were sparse in distribution. Mean epidermal cells on the abaxial surface was 33 per square millimetre, while, mean stomata density was 25 per square millimetre, mean stomata index 21.3%. Also, simple

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and long unicellular trichome was present. The mean thickness of the upper cuticle was 10.67 µm, palisade mesophyll cells one layered and composed of closely packed cylindrical cells of irregular lengths, Spongy mesophyll cells largely irregular in shape with intercellular air spaces, and the mean thickness of the lower cuticle was 10.17 µm.

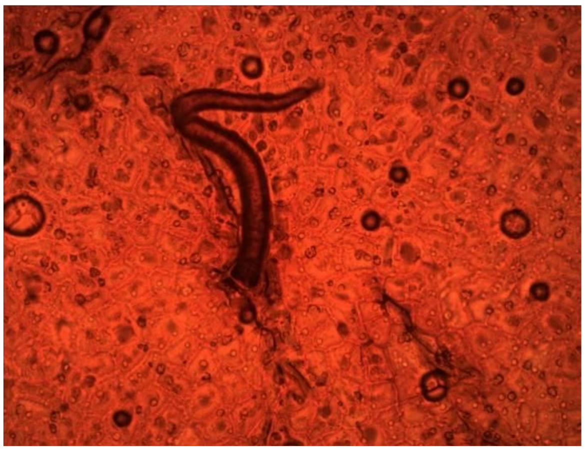

**Plate 1: Adaxial surface view (lamina) of leaflet's stomata**

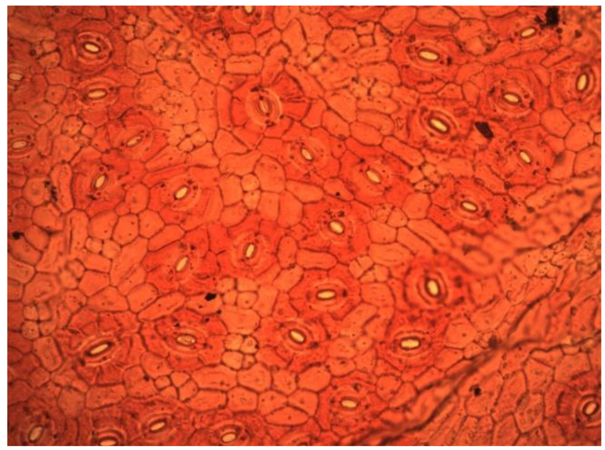

**Plate 2: Abaxial surface view (lamina) of leaflet's stomata**

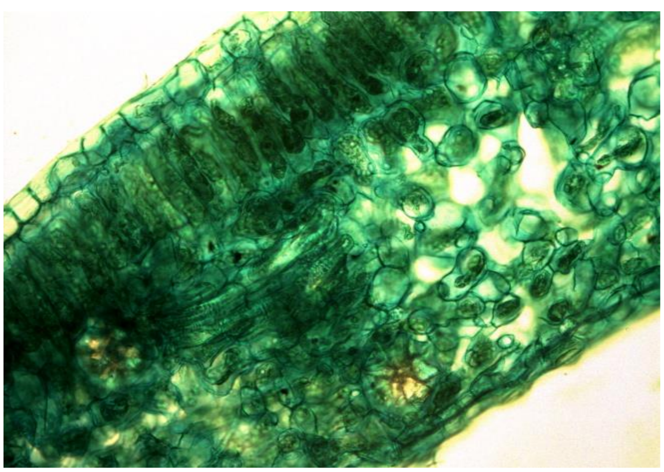

**Plate 3: Transverse Section of leaflet's palisade and spongy mesophyll cells**

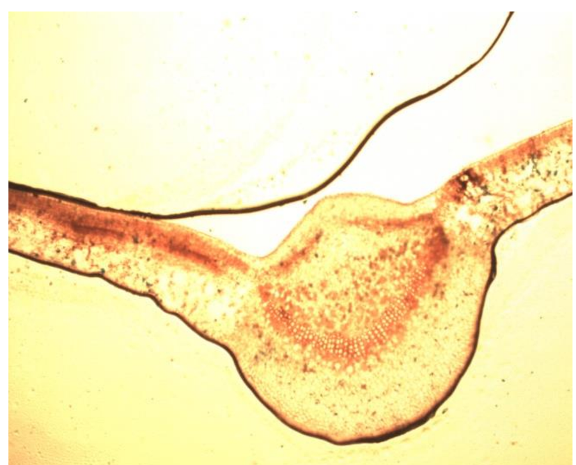

**Plate 4: Transverse Section of Leaflet's (Midrib) Vascular Bundle and vascular ring**

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**Anatomical Features of the Leaflet of *A. indica* in the Rainforest Ecosystem**

The Photomicrographs showing the anatomical features in the leaflet of *A. indica* found in Rainforest ecosystem is presented in Plate 5, 6, 7 and 8. On the adaxial surface, epidermal cells were polygonal with variable sizes, ranging between triangular and heptagonal, mean epidermal cells was 41.83 per square millimetre, stomata were absent, simple unicellular trichomes were present. Mean epidermal cell on the abaxial surface was 33.43 per square millimetre, mean stomata density was 32.33 per square millimetre, and mean stomata index 28.42%. The mean thickness of the upper cuticle was 12 µm, and mean thickness of the lower cuticle was 11.56 µm.

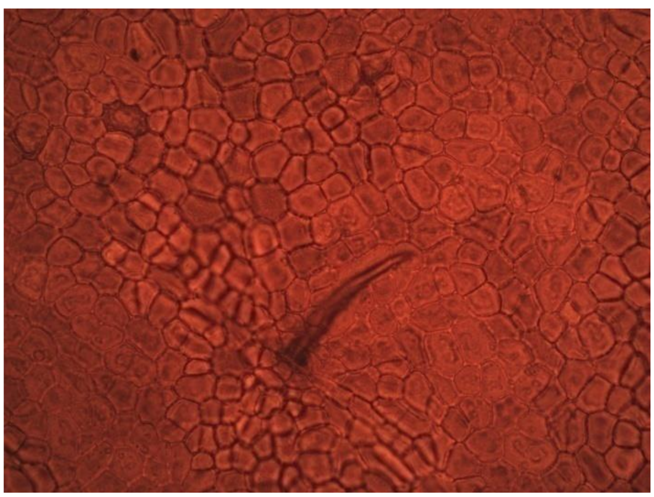

**Plate 5: Adaxial surface view (lamina) of leaflet's stomata**

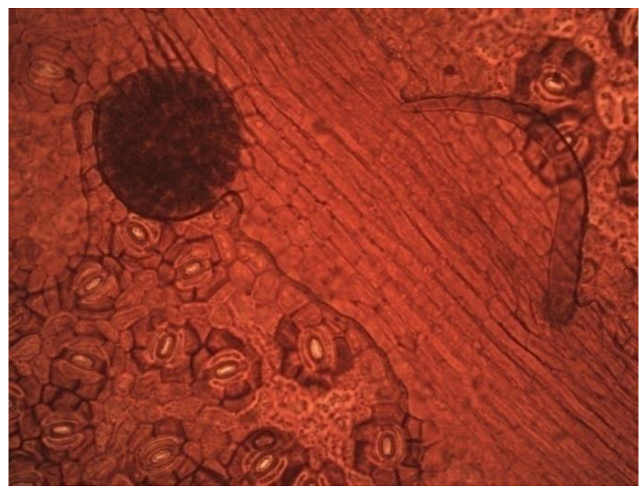

**Plate 6: Abaxial surface view (lamina) of leaflet's stomata**

**Plate 7: Transverse Section of Leaflet's (Lamina) palisade and spongy mesophyll cells**

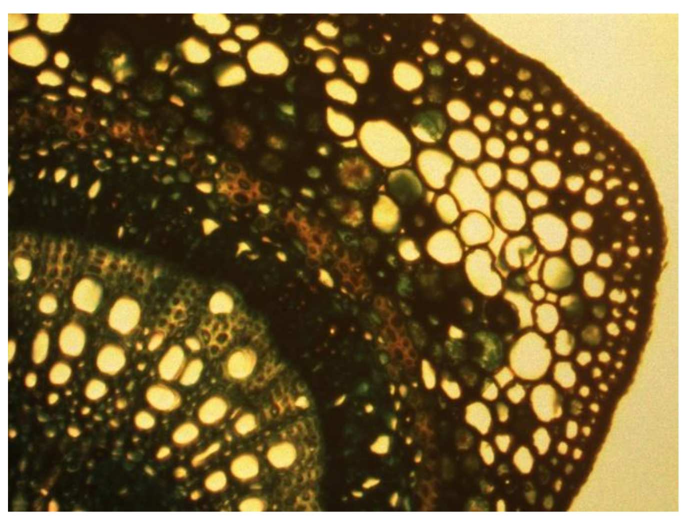

**Plate 8: Transverse Section of Leaflet's (Midrib) Vascular Bundle and vascular ring**

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The Photomicrographs revealing anatomical features in the leaflet of *A. indica* found in Derived savannah is presented in Plate 9, 10, 11 and 12. Epidermal cells on the adaxial surface were mostly pentagonal, mean epidermal cell was 38.17 per square millimetre, stomata was absent, simple unicellular trichomes were present. The mean epidermal cell on the abaxial surface was 31.33 per square millimeter, mean stomata density was 28.17 per square millimeter, and mean stomata index 18.92%, simple unicellular

**Anatomical Features of the Leaflet of *A. indica* in the Guinea Savannah**

Epidermal cells on the adaxial surface are mostly pentagonal; mean epidermal cell is 38.17 per square milimeter, stomata absent, simple unicellular trichomes present. Mean epidermal cell on the abaxial surface is 31.33 per square millimeter, mean stomata density is 28.17 per square millimeter, and mean stomata index 18.92%, simple unicellular trichome present. Mean thickness of upper cuticle is 12.67µm, and mean thickness of lower cuticle is 12.5µm.

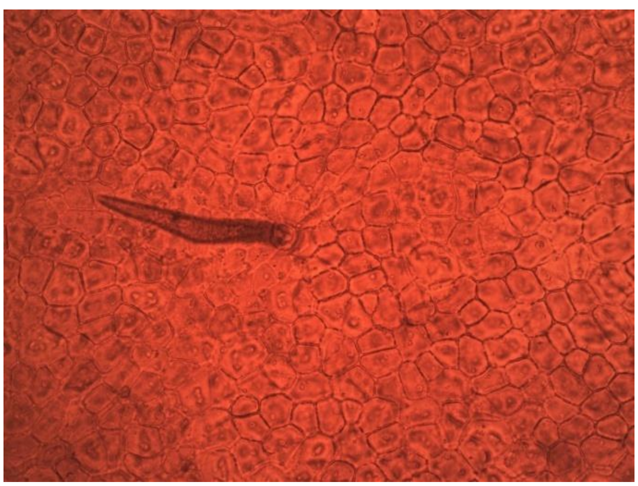

**Plate 9: Adaxial surface view (lamina) of leaflet's stomata**

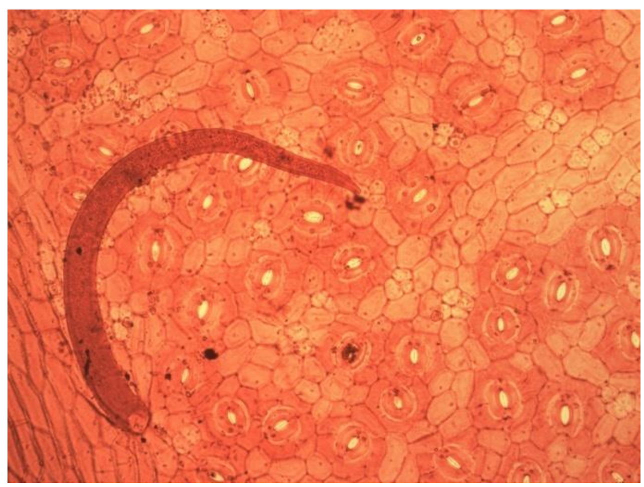

**Plate 10: Abaxial surface view (lamina) of leaflet's stomata**

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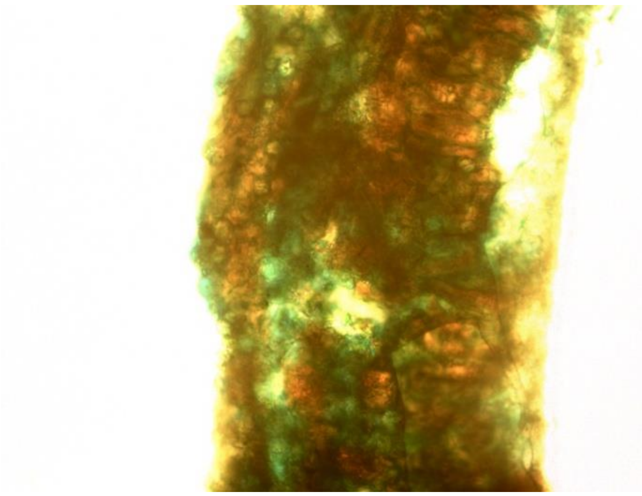

**Plate 11: Transverse Section of Leaf's (Lamina) palisade and spongy mesophyll cells**

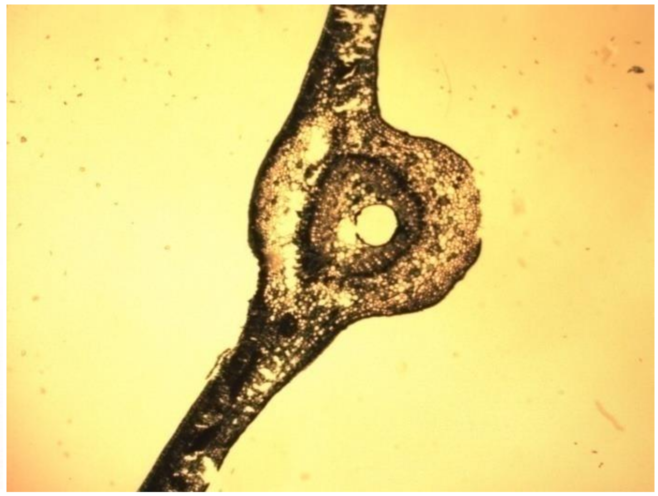

**Plate 12: Transverse Section of Leaflet's (Midrib) Vascular Bundle and vascular ring**

Savanna respectively (Table 2). However, there was no significant difference in the density of epidermal cells on the abaxial surface. Significant differences in the density of stomata in the leaves across the ecological zones were also recorded. It was discovered that the mean stomata density was significantly higher in rainforest trees, than in Guinea Savanna and Derived Savanna trees respectively. Although high cuticle thickness was found in both upper and lower cuticle of *A. indica* leaflets in Guinea Savanna, no significant difference was recorded across the ecological zones.

**Table 2: Summary of Anatomical Characteristics of *A. indica* Leaflets in Nigeria**

| Ecological Zones | ECAd | ECAb | SD | UCT(µm) | LCT(µm) |
|---|---|---|---|---|---|
| Rainforest | 41.8333$^{a}$ | 33.4333$^{a}$ | 32.3333$^{a}$ | 12.0000$^{a}$ | 11.5633$^{a}$ |
| Guinea Savanna | 38.1667$^{ab}$ | 31.3333$^{a}$ | 28.1667$^{ab}$ | 12.6667$^{a}$ | 12.5000$^{a}$ |
| Derived Savanna | 31.0000$^{b}$ | 33.0000$^{a}$ | 25.0000$^{b}$ | 10.6667$^{a}$ | 10.1667$^{a}$ |

*Means with the same superscript are not significantly different from each other.*
*Note: ECAd - Epidermal Cells (Adaxial), ECAb - Epidermal Cells (Abaxial), SD – Stomata Density,*
*UCT - Upper Cuticle thickness, LCT - Lower Cuticle thickness*

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## DISCUSSION

### Variations in Leaflets Morphological Traits

Leaflet surface area was found to vary among the three ecological zones. This is expected as the variations in leaf size along climatic gradients may result from the greater evaporative demand of larger leaves due to enhanced thickness of the boundary layer for energy and gaseous exchange (Royer *et al.,* 2005 cited in Xu *et al.,* 2009). The implication of this result was discussed by Xu *et al.,* (2009) that leaf surface area is an important parameter which dictates growth rate, the larger the specific leaf area, the larger the area for capturing light per unit of previously captured mass. It implies that neem tree in rainforest will be doing well in their overall wellbeing because they can capture more light to generate food (sugar) while neem tree in the guinea savanna will capture less light which can lead to producing less food; their overall wellbeing will definitely be less when compared to neem trees in the rainforest. The result supported Gordon statement that leaves growing in sunny environments are smaller than leaves growing in shaded environments (Gordon, 2015). Xu *et al.,* (2009) buttressed that their study highlighted that leaflet size was obviously restricted with a short supply of water.

Leaflet length and leaflet width showed that neem tree from Rainforest ecological zone has the highest mean length and width followed by leaflets from the Derived Savanna. Leaflet length and width in Derived Savanna was closely followed by Leaflet length and Leaflet width in the Guinea Savanna respectively. This result showed that leaflet length and leaflet width increased along with the expansion of leaflet surface area (Xu *et al.,* 2009). This is against the statement of Niinemets *et al.,* (2007) that leaf elongation characterizes the overall slenderness of the leaves. It reflects the integrated changes of leaf major and minor axes. Leaflets from the Rainforest ecological zone has the highest mean of leaflet length, but maintains the highest mean leaflet surface area while that of Guinea Savanna are smaller in length and has the lowest mean leaflet surface area.

The Rainforest ecological zone has longer apex and it is more oblique. Trees from Guinea Savanna has shorter apex length and less oblique. These findings were supported by Lovett and

Haq (2000); Wright and Westoby (1999); Yates *et al.,* (2010) that a narrow leaf with smaller petiole size is viewed as an adaptation to sunny and dry environments for controlling water balance. This result corresponds with the findings of Niinemets *et al.,* (2006). They opined that changes of petiole length were consistent with changes of leaflet area and dry mass, which indicated that there was a positive relationship between leaf petiole length and leaf size. Due to the economics of light interception and biomechanical requirements, increases in leaf size are often bound to enhanced biomass investment in the petiole. The implication is that the elongation of petioles will achieve optimal leaf display to deal with the denseness of the canopy, but it may not be the major way to reduce self-shading, as the plants face a trade-off between the need for increasing interception areas and support structures. Increasing the investment in petioles needs to synthesize more xylogens, and longer petioles will lead the leaf to bend (Pickup *et al.,* 2005).

**Variations in Anatomical Traits**

Though, a study conducted by Mensah (2012), showed relatively thick cuticle, with a double layer of palisade mesophyll in *Azadirachta indica* leaves from the coastal savanna zone of Ghana, which may probably be the feature for its adaptation to withstand the drought stress. All the leaf samples across the various ecological zones in Nigeria possessed trichomes, however, Hairiness has been reported to be more likely concerned with protection from excessive insulation than from high transpiration (Yanney-Wilson, 1963; Schulze *et al*., 1987). Also, in a study conducted by Pyakurel and Wang (2014), revealed that paper birch leaves had a higher adaxial hair density with decreasing annual precipitation. As though, it can be said that cuticle layer grew thicker with reduced water availability, especially, the upper cuticle layer. This can be an explanation for the thick cuticle layer observed in leaflets in the Guinea Savanna, this characteristic is associated with the need to prevent too much water loss to avoid desiccation and maintain the water use efficiency.

Leaf stomata are primarily known to be the principal means of gas exchange in plants. The more stomata per unit area, the more $CO_2$ can be taken up, and the more water can be released, which was observed in the leaflets from the Rainforest. Thus the higher stomata density can greatly amplify the potential for behavioral control over water loss and $CO_2$ uptake. Generally, photosynthetic apparatus are only designed to function well over a rather narrow range of temperatures. When heated, cytochromes, pigments, and membranes critical to phosphorylation and carbon fixation rapidly denature. To avoid this, plants may open the stomata and evaporate water, which will reduce the leaf temperature. Thus, one may hypothesize that leaves in the sun should have a higher stomata density than leaves in the shade. But, on the other hand, if water is not available, such as under drought conditions, excessive evaporation might lead to desiccation and an equally severe disruption of photosynthetic function. Thus, one may expect plant leaves exposed to drought conditions to have fewer stomata in sunlit environments.

The most significant variations between the anatomical characteristics of the *Azadirachta indica* leaflets from the various ecological zones in Nigeria were in the stomata density, stomata index, epidermal cell density, and cuticle thickness. Leaflets from Rainforest showed a relatively thin cuticle layer with high stomata density and index, and more epidermal cells. Considering the climatic conditions of the vegetation zone; high sunlight and water availability, the high stomata density recorded in the Rainforest may be a mechanism developed to adjust to the light intensity in this ecological zone so as to aid photosynthesis, as higher stomata conductance would enhance photosynthesis; increased intake of $CO_2$. Higher stomata density in the Rainforest may be associated with high relative humidity therefore, there is less need for transpiration control, and meanwhile, more $CO_2$ can be absorbed by the leaves to increase photosynthesis. The derived savanna is a belt that is characterized by more sunlight and lower humidity; stomata density in the leaflets is very low as they may be responsible for enhancing water use efficiency and adapting to a drought condition. Less stomata would lead to a reduction in water loss from the leaves incorporated with reduction in gaseous exchange due to the low amount of stomata openings in the leaves. Although, a relatively thinner cuticle layer was discovered in this region, this might not help in controlling water loss from the leaves. However, leaves in the Guinea Savanna had numerous stomata on the lower epidermis plus thick cuticle. High stomata density with a thick cuticle can be said to be the best mechanism in preventing water loss, as well as, gaining more $CO_2$ for photosynthesis. By this, photosynthesis is not affected by the process of transpiration control. The characteristics exhibited in this region are very efficient for surviving in drought areas. In addition, single layer of palisade mesophyll was the structure observed in the leaflets across the ecological zones in Nigeria, while, Mensah (2012) reported a double layer of the palisade mesophyll in the leaves of *A. indica* in the savanna zones of Ghana.

## CONCLUSION

This research work affirmed that leaf sizes are restricted by environmental conditions. The leaf can be considered as a microcopy of the plant, and the variations of leaf morphology can reflect the plant capacity to acquire, use and conserve resources. In the different ecological zones, varying stomata density and epidermal cell density was observed. This explains that the trees in each ecological zone adapts to the environment in its own way, the best possible for it to balance water loss through transpiration and water use efficiency, and also increases $CO_2$ absorption. Trees in the Guinea Savanna were characterized with density of epidermal cells on the adaxial surface and stomata density lower than that of the Rainforest but, higher than the trees in Derived Savanna. It also exhibited the thickest cuticle layer among the three eco-zones. Presence of thicker cuticle may be associated with need to control water loss due to hot climate which may cause dryness of the leaves. This study has contributed to the understanding of optimum habitat conditions and the ecophysiological adaptations of *A. indica* in Nigeria.

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