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OCCURRENCE, TYPES AND LOCATION OF CALCIUM OXALATE CRYSTALS IN *VERNONIA AMYDALINA* DEL (*ASTERACEAE*)

[1]Nwosu, Sunday Iroham; [2]Stanley, H. O and [3]Okerentugba, P.O.
[1]Department of Plant Science and Biotechnology, Faculty of Science, University of Port Harcourt, Nigeria.
[2 & 3] Department of Microbiology, Faculty of Science, University of Port Harcourt, P.M.B 5323
Choba, Port Harcourt, Nigeria.

ABSTRACT
In this study, calcium oxalate (CaOx) crystals were investigated and their morphology and distribution determined by light microscopy in the stem, leaf and petiole tissues of *Vernonia amygdalina* Del. of the family *Asteraceae*. Calcium Oxalate crystals were identified in cleared tissues by histochemical technique suing silver nitrate and hydrogen peroxide. Druses and styloids were observed in stem epidermal and pith cells, and leaf mesophyll cells of *V. amygdalina*. The distribution of calcium oxalate crystals on the epidermal and pith cells is suggestive of their mechanical support in *V. amygdalina*. This study provides additional chemo-taxonomic evidence in the identification of *Vernonia amygdalina*.

KEY WORDS: Calcium oxalate crystals, Asteracace, *Vernonia amygdalina*, Histochemistry and chemo-taxonomy.

INTRODUCTION

Vernonia amygdalina Del. of the family Asteraceae is a perennial, small tree between 1m and 6m in height. It is commonly found in tropical African countries, including Nigeria. It is commonly called "bitter leaf" because of its bitter taste, which however, can be reduced by boiling or macerating the leaves in several changes of water. In Nigeria, it is known variously as "Onugbu" in Igbo Language, "Ewuro" in Yoruba" "Oriwo: in Bini, Etidot" in Cross River State, "Ityuna" in Tiv and "Chusar doki" or fatefata in Hausa. In Nigeria, the leaves are used as vegetable and as spices in the popularly known bitter-leaf soup. The leaves can be taken as an appetizer and the water extract taken as digestive tonic. It is consumed by the female Hausas in their belief that it makes them more sexually attractive. The leaves have also been used in Ethiopia instead of hops to make Tela beer and also to make beer in Nigeria. It is also found in homes in villages as fence-post and pot-herb.

Some wild chimpanzees in Tanzania had been observed to use *V. amygdalina* for the treatment of parasite- related diseases (Huffman *et al;* 1989). Okoli *et al.* (2007) had reported the efficacy of garlic, pawpaw, bitter leaf and mistletoes for the treatment of hypertension in Nigeria. *Vernonia amygdalina* has been reported to possess antibiotic, antimicrobial and antimalarial properties. The sap of the leaves of *V. amygdalina* was found to show inhibitory capacities against *Staphylococcus epidernidis, S. aureus, Escherichia coli* and *Pseudomonas aeroginosa* (Ijeh *et al.*, 1996). A 60% methanoic extract of the leaves gave antimicrobial activity against *Bacillus subtilis, Klebsiela pneumonia, Pseudomonas aeroginosa, Proteus vulgaris, Shigella dysenterial* and *S. aureus* (Akinpelu, 1999). Both vernolide and vernodalol isolated from *Vernonia amygdalina* showed activities against the *Gram-positive Bacillus cereus, Staphylococcus epidermidis, Streptococcus pyrogens and Micrococcus kristinae;* and Gram-negative *Salmonella pooni* (Erasto *et al*., 2006). *Vernonia amygdalina* had been reported to show oxytocic property. In Malawi, the leaves have been used to induce uterine mobility and control post-parturn hemorrhage by traditional birth- attendants (Bullougb and Leary, 1982). There are no literature reports on histochemical localization of calcium oxalate crystals in *Vernonia amygdalina* Calcium oxalate crystals are found in over 215 plant families (Mc Nair 1932, Franceschi and Horner, 1980, Ward *et al.*, 1997) and distributed in organs such as stems, roots, leaves, floral structures and seeds (Franceschi and Horner, 1980; Tilton and Horner, 1980; Horner and Wagner 1980;1992; Prychild and Rudall, 1999; Horner, *et al*., 2000., Leristen and Horner, 2006). The shapes of calcium oxalate crystals may vary and are commonly described as raphides, druses, styloids, prisms and crystal sand (Franceschi and Horner, 1980; Franceschi and Nakata, 2005). The distribution and shapes (raphides druses) of these crystals have been used as taxonomic characters for a number of plant families. For example, the presence of rephides has been used to separate sub-families of Rubiaceae (Lersten, 1974) and subgenera of *Prunus* (Lersten and Horner, 2000) in the dicots, and in monocots crystals have been used as a synapomorphy for some families (Prychid and Rudall, 1999). Crystals often from in the vacuole of cells called crystal idioblasts, which are specialized for crystal formation (Foster, 1956). In some plants, crystals accumulate in the vocuoles of other cell types, such as mesophyll, storage parenchyma and epidermal cells (Farnceschi and Horner, 1980). In other cases, they are deposited in cell walls as seen in gymnosperms (Franceschi and Horner, 1980, Hudgins *et al.*, 2003).

Although their functional significance in plant development remains unclear, various functions have been attributed to them, including calcium regulation in plant

cells (Franceschi 1989; Kostman and Franceschi, 2000. Volk, *et al.*, 2002), protection against herbivory (Molano-Flores, 2001), detoxification of heavy metals or oxalic acid (Franceschi and Nakata 2005), tissue strength, light-gathering and reflection (Franceschi and Honer, 1980; Kuo-Huang, *et al.*, 2007). The aim of this study is to provide additional information on the presence of calcium oxalate crystals as chemo-taxonomic character of *Vernonia amygdalina* and its distribution in relation to functions.

MATERIALS & METHODS

Plants were collected from cultivated garden in Rumuigbo, Obio/ Akpor Local Government Area of Rivers State (Nigeria). For light microscopy, fresh specimens were fixed in formal acetic alcolol (1:1:18v/v mixture of formaldehyde glacial acetic acid and 70% ethanol) at room temperature overnight. The fixed specimens were then rinsed twice in distilled water. Hand-sections were made from fixed stems, petioles and leaves. The samples were treated with 2.5% JIK commercial bleach (2.5% sodium hypochlorite) for 4 hours. After washing in distilled water, the sections were stained with equal mixture of 5% silver intrate ($AgNO_3$ and 30% hydrogen peroxide (H_2O_2) under intense light, supplied with a 60-watt electric bulb held at a distance of about 20cm from the slides. The staining lasted for about 30 minutes (Okoli, 1992). Crystals were examined in cleared tissues with a bright-field microscopy (Olympus, Tokyo, Japan). Selected images were captured with a Yasika digital camera. The terminologies for calcium oxalate crystals used in this study were as described by Franceschi and Horner (1980); Franceschi and Nakata (2005).

RESULTS

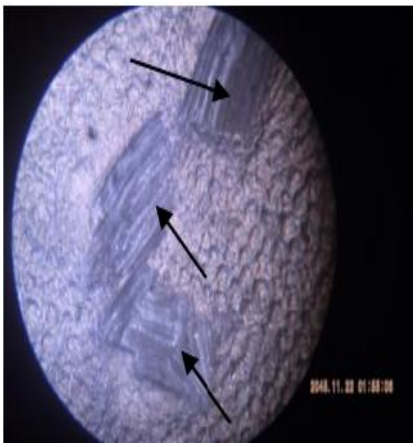
The calcium oxalate crystals in all cleared tissues were easily observed since the clearing technique removed all the cytoplasmic inclusions except for cell walls and crystals. They were examined by LM (brightfield). Their morphogy and distribution in tissues are shown in Table 1.

TABLE 1: Morphology (type) and locations of calcium oxalate crystals in the tissues of *Vernonia amygdalina*

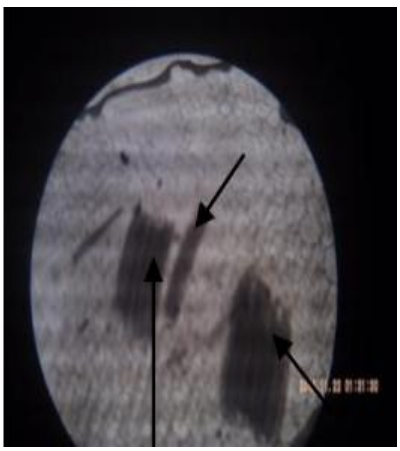
Tissue	*V. amygdalina* (Bitter type)					*V.amygdalina (Sweet type)*				
	Vascular bundles	Pith	Epider-mis	Cortex	Mesophyll	Vascular bundles	Pith	Epide-rmis	Cortex	Meso-phyll
Stem	Druse	Druse		Druse	-	Styloids	Styloi-ds	-	-	-
Leaf	Druse	-	-	-	Druse	-	-	-	-	Styloids
Petiole	Druse	Druse	-	Druse	-	-	Styloids	-	-	-

The morphology of calcium oxalate crystals in the tissues examined were druse and styloids and varied in their distributions. While calcium oxalate crystals are located on the pith of both stem and petiole, they are located on the leaf mesophyll. No crystals were observed in leaf epidermal cells. The druse calcium oxalate crystals were observed in the tissues of *Vernonia Amygdalina* (bitter type), while the styloid crystals were observed in the *Vernonia amygdalina* (sweet type).

1. Styloid Crystal in the leaf mesophyll cells of *V. amygdalina (arrows)*

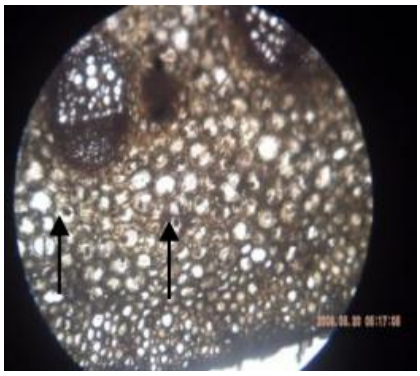


2. Styloid Crystals in the petiole pith cells of *V. amygdalina*

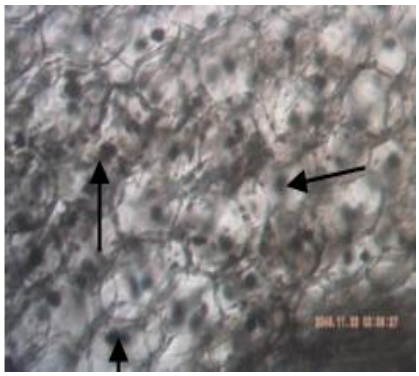


3. Styloid Crystals in the stem pith cells of *V. amygdalina (arrows)*

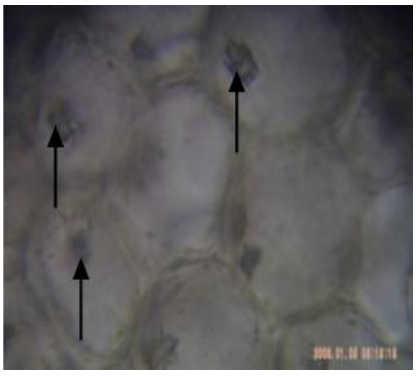
FIGURES 1-3. Types and locations of Calcium Oxalate Crystals in the leaf, petiole and stem tissues of *Vernonia amygdalina* (Sweet type)



4. Druse Crystals in the leaf pith cells of *V. amygdalina (arrows)*



5. Druse Crystals in the petiole pith cells of *V. amygdalina (arrows)*



6. Druse Crystals in the stem pith cells of *V. amygdalina (arrows)*

FIGURES 4-6. Types and locations of Calcium Oxalate Crystals in the leaf, petiole and stem tissues of *Vernonia amygdalina* (bitter type)

DISCUSSION

Calcium oxalate crystals are found at all taxonomic levels in photosynthetic organisms, from small algae to higher plants (Franceschi and Nakata, 2005). The crystals were formed from endogenously synthesized oxalic acid and Ca^{2+} (ions) taken from the environment, and they were produced and accumulated in species-specific morphologies. Several studies have described calcium oxalate crystals in Asteraceae (Dormer, 1961, Horner, 1977; Meric and Dane, 2004; Meric, 2008). The present study examined the morphology and location of calcium oxalate crystals in *Vernonia amygdalina* (of the Vernoneae tribe). Druses, (Spherical aggregates of calcium oxalate crystals) and styloids were observed in the stem pith cells of *Vernonia amygdalina*. It can be suggested that the large druses in the stem pith of *V. amygdalina* provide a storage site for calcium within the cells. Druses were also present in the mesophyll cells of the leaves which make the primary photosynthetic tissue in *V. amygdalina*. Kuo-Huang, *et al.* (2007) indicated that calcium oxalate crystals are involved in dispersing light to the chloroplasts in the photosynthetic parenchyma cells of leaves. Druses of calcium oxalate crystals were observed in the stem epidermal cells. The function of crystals in the stem epidermal cells may be to provide additional strength to the tissues. Calcium oxalate crystals may have an ecological role in plants as a defense mechanism, and a physiological role accumulating excess calcium (Molawo-Flores, 2001). Raphide crystals are generally thought to play an ecological role as herbivore defence mechanism (Sakai, *at al*., 1972, Sunell and Healey, 1985; Perera *et al.*, 1990, Ward *et al*., 1997). These crystals are found in large cells that are filled with mucilage and in most cases, are dead at maturity. Accumulation of mucilage increases pressure against the cell wall. This facilitates release of crystals when the cell wall is damaged by grazing (Esau, 1965). Caterpillar larvae of the beet armyworm *Spodoptera exigua* Hubner, show a clear feeding preference for tissue from calcium oxalate- deficient (Cod) mutant lines Cod5 and Cod6 in choice test comparisons with wild-type *Medicago truncatula* Gaertn. Compared to their performance on mutant lines, larvae feeding on wild-type plants with abundant calcium oxalate crystals suffer significantly reduced growth and increased mortality. Food utilization measurements show that, after consumption, calcium oxalate also interferes with the conversion of plant material into insect biomass during digestion. Calcium oxalate crystals have been thought to have a physiological role sequestering excess calcium within plant cells (Borchert, 1984, 1985, 1986; Franceschi, 1987, 1989; Fink, 1991; Webb, 1999). High concentrations of calcium can interfere with many cell processes (*eg.* Calcium - dependent signaling microsketal dynamics) in plants (Webb, 1999).The morphology and distribution of crystals is constant within a species. This indicates that their presence, morphology and distribution in a species are under genetic control (Ilarslan *et al*., 2001; Franceschi and Nakata, 2005). Thus, the constancy of crystals, type and distribution may be considered a taxonomic character for classification of species (Franceschi and Nakata, 2005). This study was aimed at determining the types and locations of calcium oxalate crystals in *Vernonia amygdalina* as a taxonomic evidence in its classification. All the investigated tissues of the plant (*V. amygdalina*) contained druse crystals, except stem pith which contains druse and styloids crystals. Further research will shed more light on the taxonomic significance of calcium oxalate crystals as an anatomical feature in the tribe Vernoneae.

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