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Comparative foliar epidermal studies in *Capsicum annuum* L. and *Capsicum frutescens* L.

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Abstract

The study evaluated the degree of relatedness and variability in foliar epidermal features of four varieties of two closely related cultivated *Capsicum* species,*C. annuum* L. and *C. frutescens* L., in West Africa using light microscopy, scanning electron microscope (SEM) and energy dispersive X-ray (EDX) spectroscopy. The varieties used in the study were *C. annuum* var. *abbreviatum*,*C. annuum* var. *acuminatum*, *C. annuum* var. *grossum* and *C. frutescens* var. *baccatum*. In general, the results of foliar epidermal traits revealed similarities and overlaps in the four varieties, and some diagnostic traits that could be employed in taxonomic decision. Across the varieties, stomatal type was anisocytic and epidermal cells were irregularly shaped with wavy anticlinal walls. Non-glandular trichomes were only found on the abaxial and adaxial surfaces in *C. annuum* var. *abbreviatum* and *C. frutescens* var. *baccatum* although varied in type from unicellular to multicellular. The four varieties also exhibited similarity in major elemental compositions as revealed by EDX. *Capsicum frutescens* var. *baccatum* could be distinguished from the three varieties of *C. annuum* by the presence of bromine, chlorine, and nitrogen in its elemental composition spectroscopy. The study shed more light on the degree of relatedness and variability among the varieties of the cultivated *Capsicum* species which could be exploited for selection and breeding purposes.

Keywords: Anisocytic stomata, *Capsicum*, Foliar Epidermis, Relatedness, Trichome

Introduction

The genus *Capsicum* which belongs to the family Solanaceae, originated from the Central and South America (Bosland and Votava, 2000). The members have several universal English names which include hot pepper, chile pepper, chili, bell pepper, chilliand sweet pepper. *Capsicum* species are important and popular vegetables worldwide. They consist of a range of bioactive compounds and essential nutrients (Farhan et al., 2014; Olatunji and Afolayan, 2018). Pepper fruits contain various compounds like capsaicinoids, carotenoids, fatty oils, steam volatile oil, protein, mineral elements and vitamins among others (Bosland and Votava, 2000; Olatunji and Afolayan, 2018). The mature fruits of pepper are particularly high in vitamin C and other constituents that have positive nutritional values in human (Marin et al., 2004).

Capsicum consists of about 35 species out of which five,*C.annuum* L., *C. chinenses* Jacqs., *C. frutescens* L., *C. pubescens* R. and*C. baccatum* L.,are widely cultivated (Carolina et al., 2016).In West Africa, the genus is represented by two cultivated species, *viz.*, *C. annuum* and *C. frutescens* with four varieties. As the two species are morphologically and closely related, there has been considerable debate on their status as two separate species or just varieties of one species. Linnaeus (1753) originally reported *C. annuum* and *C. frutescens* as the only two species in the genus

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based on their form as annual (*C. annuum*) or perennial (*C. frutescens*). In the study of South American pepper, Bukasow (1930) rejected Linneaus' classification and considered the *C. annuum* form to be just a variety of *C. frutescens*. Till date, the distinction of these two plants as separate species or varieties of one species is unresolved.

Micromorphological and anatomical foliar epidermal traits displayed important roles in the identification and delineation of plants especially at the generic and species levels (Saheed and Illoh, 2010; Jimoh and Olowokudejo, 2018). Several authors had stressed the importance of anatomical characters in differentiating plant species (Stuessy, 2009; Ajayan et al., 2015). Taxonomically, foliar epidermal structures are important characters that are used in delineating plants. According to Bano et al. (2015), trichomes are epidermal attachments that vary in plants; they provide additional taxonomic tools because of their morphological distribution and diversity.

Based on the size of fruit and morphological differences between and within the cultivated pepper, four varieties are recognized *viz.,C. annuum* var. *abbreviatum*, *C. annuum* var. *acuminatum*, *C. annuum* var. *grossum* and *C. frutescens* var. *baccatum*, which are locally known as rodo, sombo, tatase and wewe respectively. This study was therefore aimed at examining and comparing the ultrastructural morphology of the leaves of the varieties of the cultivated *Capsicum* species using light microscopy, scanning electron microscope (SEM) and energy dispersive X-ray (EDX) spectroscopy. This is with the view to assessing possible relatedness and dissimilarity among the varieties and species for proper identification and classification.

Material and Methods

Nigeria is a major producer and consumer of the cultivated varieties of the *Capsicum* species in West Africa, therefore, mature fruits of the four cultivated varieties of *C. annuum* and *C. frutescens* were obtained from the markets in major geographical zones in Nigeria. Seeds in the fruits were first removed, sun-dried and stored at room temperature of about 15-25 ^{o}C in paper bags and later used for planting. Sowing was done in plastic pots in the greenhouse of the University of Fort Hare. At early flowering stage, fresh, mature and fully expanded leaves were harvested for micromorphological study. The voucher specimen of each variety was collected and deposited at the University of Ilorin's herbarium.

Light Microscopy

Leaf epidermal peels were obtained from the adaxial and abaxial surfaces of fresh matured leaves of the *Capsicum* varieties. The peels were mounted on glass slide, covered and observed under a Zeiss light microscope. Photomicrographs were taken with a digital camera (DCM 510, 5 M pixels, CMOS chip) that was fitted to the microscope.

SEM and Energy Dispersive X-ray Spectroscopy (SEM-EDX)

Fresh leaves of each variety of *Capsicum* species were cut into segments of 4–6 mm length andwere fixed for 24 hours in 6% w/v glutaraldehyde (pH 7.3) and rinsed with 0.05 M sodium cacodylate buffer (pH 7.5). Each sample was later rinsed in distilled water and dehydrated in a graded series of ethanol 10–100% for 20 min per rinse. The samples were dried in a Hitachi HCP-2 critical point dryer and mounted on aluminum stubs with double-sided carbon coated sputter coating with gold palladium (Elko IB-3 Ion Coater). The samples were examined at varying magnifications using JEOL (JSM-6390LV) scanning electron microscope (SEM) that was operated at 10–15 kV accelerated voltage. The energy-dispersive X-ray spectroscopy (EDX) involved both fixing and dehydration procedure as in SEM. The elemental analysis was done using energy dispersive X-ray analyzer (Thermo Electron Corporation, 6733B-IUUSN, USA) which was coupled to the SEM.

Electron images were captured using Noran system six imaging software.

Results and Discussion

Leaves are the most varied organs of flowering plants that give valuable criteria for classification of plants species. The microscopic and anatomical features of foliar epidermal studies have become very helpful for identification especially in angiosperm classification at species level in many genera (Adedeji et al., 2007; Bello et al., 2017; Ohikhnena et al., 2017). Light microscopic studies of the four varieties of *Capsicum* species revealed same shape of epidermal cells on the adaxial and abaxial sides of their leaves. They all had irregularly shaped epidermal cells with wavy anticlinal walls (Figures 1 & 2).

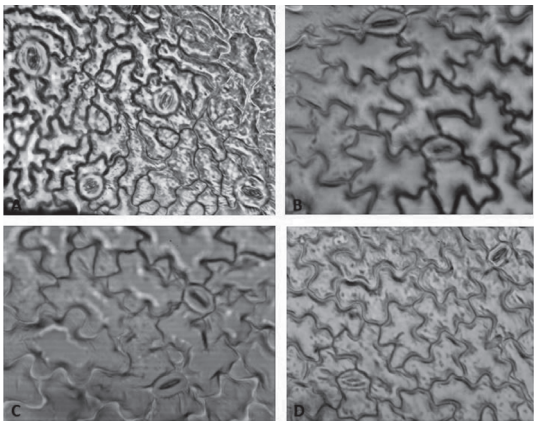

Figure 1. Light micrograph of adaxial leaf surface of four varieties of *Capsicum* species at X40 objective. (A) *C. annuum* var. *abbreviatum* (B) *C. annuum* var. *acuminatum* (C) *C. annuum* var. *grossum* (D)*C. frutescens* var. *baccatum*

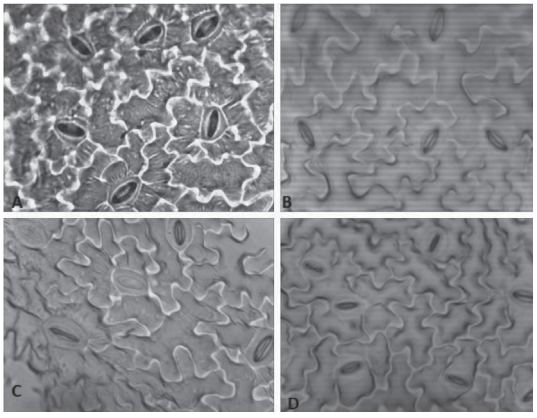

Figure 2. Light micrograph of the abaxial leaf surface of four varieties of *Capsicum* species at X 40 objective. (A) *C. annuum* var. *abbreviatum* (B) *C. annuum* var. *acuminatum* (C) *C. annuum* var. *grossum* (D)*C. frutescens* var. *baccatum*

The leaves of all varieties were amphistomatic and were characterized by anisocytic stomata that were more abundant on the abaxial surfaces (Figures 3 & 4). The stomata were randomly arranged on both epidermal surfaces. The stomatal distribution agreed with earlier works of several researchers of the *Solanaceae* family (Adedeji et al., 2007; Mbagwu et al., 2007; Zhigila et al., 2015; Bello et

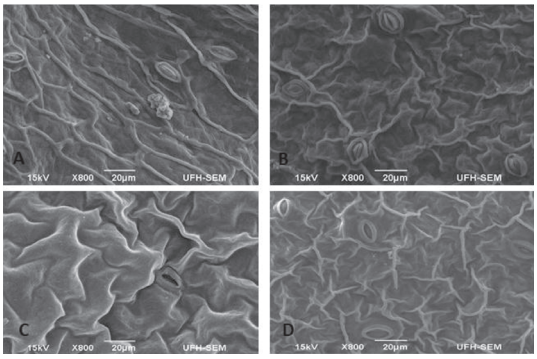

Figure 3. SEM micrograph of the adaxial leaf structure of the four *Capsicum* varieties showing stomatal distribution (A) *C. annuum* var. *abbreviatum* (B) *C. annuum* var. *acuminatum* (C) *C. annuum* var. *grossum* (D) *C. frutescens* var. *baccatum*

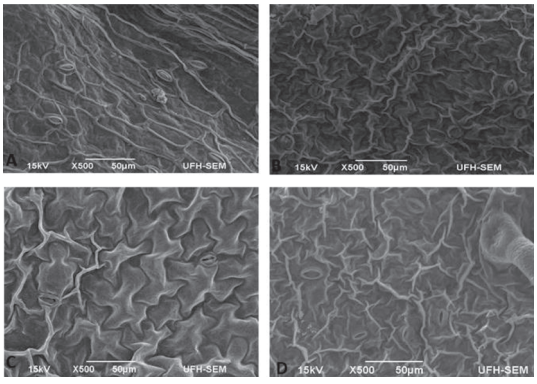

Figure 4. SEM micrograph of the abaxial leaf surface of the four *Capsicum* varieties showing stomatal distribution (A) *C. annuum* var. *abbreviatum* (B) *C. annuum* var.

al., 2017). The abundant distribution of stomata on the abaxial epidermal surface is a common phenomenon in most angiosperms. This type of stomata distribution helps in reducing the rate of transpiration and in addition, improves exchange of gases by providing larger surface area for diffusion of carbondioxide into the leaf and removal of excess water vapor from the leaf in a moisture laden environment(Ashafa et al., 2008). Stomata complex type was anisocytic in all the varieties studied which reflected their phylogenetic relationship.

The stomatal apparatus was ellipsoid and found openon both the adaxial and abaxial surfaces in all the varieties. The subsidiary cells were 2- 4 in number (Tables 1&2). The mean stomatal density differed among the varieties ranging from 69.01 mm^{-2} (*C.annuum* var. *abbreviatum*) to 103.53 mm^{-2}(*C. annuum* var. *acuminatum*) on the adaxial surface (Table 1) and from 106.01 mm^{-2} (*C. annuum* var. *acuminatum*) to 155.47 mm^{-2} (*C. frutescens* var. *baccatum*) on the abaxial surface (Table 2) (Figures 3 & 4). The longest mean stomatal pore length on the adaxial surface was recorded in *C.annuum* var. *abbreviatum* (13 µm) while the shortest was recorded in *C. annuum* var. *acuminatum* (12 µm).

The pattern of epidermal cell wall is another feature that is taxonomically important and used to delineate species. The four varieties studied exhibited similarity in the pattern of epidermal cell wall (wavy) on both the adaxial and abaxial surfaces and were irregular in shape. This pattern was characteristic and most dominant in the genus *Capsicum* (Dias et al., 2013; Zhigila et al., 2015). The mean number of epidermal cell count on the adaxial surface ranged from 10±1 in both *C.annuum* var. *abbreviatum* and *C. frutescens* var. *baccatum* to 12±1 in both *C. annuum* var. *acuminatum* and *C. annuum* var. *grossum*. On the abaxial surface, the highest mean number of epidermal cells was recorded in *C. frutescens* var. *baccatum* while the lowest was recorded in *C. annuum* var. *grossum* (Tables 1&2). The mean number of epidermal cells which was in the same range on both leaf surfaces in the four varieties indicatedclose relationship among the varieties

Many researchers have stressed the importance of trichomes as a morphological feature for

*Table 1***.** Characters of the adaxial (upper) leaf epidermis of the four varieties of *Capsicum* species viewed under light microscope

	C. annuum var. *abbreviatum*	*C. annuum* var. *acuminatum*	*C. annuum* var. *grossum*	*C. frutescens* var. *baccatum*
Cell shape	Irregular	Irregular	Irregular	Irregular
Anticlinal walls	Wavy	Wavy	Wavy	Wavy
Stomata type	Anisocytic	Anisocytic	Anisocytic	Anisocytic
Number of Epidermal Cell (per mm^2)	10 ± 1^a	12 ± 1^a	12 ± 1^a	10 ± 1^a
Stomata pore length (µm)	13 ± 2.90^a	12 ± 1.00^a	12.67 ± 0.58^a	12.33 ± 3.21^a
Stomata density (no./mm^2)	69.01 ± 0.50^d	103.53 ± 1.20^a	79.01 ± 2.90^c	86.27 ± 0.69^b
number of subsidiary cell	4 ± 0^a	4 ± 0^a	4 ± 0^a	4 ± 0^a

Table 2: Characters of the abaxial (lower) leaf epidermis of the four varieties of *Capsicum* species

	C. annuum var. *abbreviatum*	*C. annuum* var. *acuminatum*	*C. annuum* var. *grossum*	*C. frutescens* var. *baccatum*
Cell shape	Irregular	Irregular	Irregular	Irregular
Anticlinal walls	Wavy	Wavy	Wavy	Wavy
Stomata type	Anisocytic	Anisocytic	Anisocytic	Anisocytic
Number of Epidermal Cell (per mm^2)	11.67 ± 1.53^a	11.33 ± 0.56^a	9 ± 2^b	12.67 ± 0.58^a
Stomata pore length (µm)	14.58 ± 0.59^a	15.1 ± 1.81^a	15.08 ± 2.36^a	14.06 ± 1.57^a
Stomata density (no./mm^2)	134.27 ± 0.6^b	106.01 ± 1.4^d	120.14 ± 0.5^c	155.47 ± 1.2^a
number of subsidiary cells	3 ± 1^a	3.67 ± 0.58^a	3 ± 1^a	3 ± 1^a

delineation and systemic comparisons. The biological function of trichomes has been of scientific interest for a long time. This includes, among others, protection against insect herbivores, lowering of leaf temperature and storage of phytochemicals (Ashafa et al., 2008; Albert and Shama, 2013). According to Adedeji et al. (2007), the presence, absence and type of trichomes had been successfully employed in the delineation of plants in some species. Trichome density could also be reasonably used for the identification of plant species (Esseit et al., 2012). In this study, trichomes were found on both surfaces only in *C.annuum* var. *abbreviatum* and *C. frutescens* var. *baccatum.* The occurrence of trichomes in these varieties only suggested that they were more closely related than other varieties. No trichome was seen in *C. annuum* var. *grossum* and in *C. annuum* var. *acuminatum.* In *C.annuum* var. *abbreviatum,* simple, non-glandular multicellular trichomes were observed on the adaxial surface with long, thin apical end while bicellular non-glandular trichomes were seen on the abaxial surface (Figure 5). In *C. frutescens* var. *baccatum*, non- glandular trichomes were present on both the adaxial and abaxial surfaces of the leaf which were unicellular or bicellular (Figure 5). The differences in the type of trichomes in the two varieties was in agreement with the report of Aworinde et al. (2014) that there was pronounced hair diverseness in the genus which had been of substantial significance in comparative investigations in flowering plants. *C. annuum* var. *abbreviatum* and C. *frutescens* var. *baccatum* could, therefore, be identified based on the presence and type of trichomes.

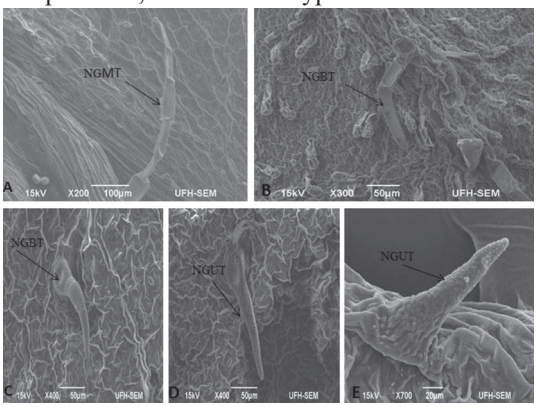

*Figure 5.*SEM Micrograph of the different trichomes found in the *Capsicum* species (A) Non-glandular multicellular trichome on the adaxial surface of *Capsicum annuum* var. *abbreviatum* (B) Non-glandular bicellular trichome on the abaxial surface of *Capsicum annuum* var. *abbreviatum* (C) Non-glandular bicellular trichome on the adaxial surface of *Capsicum frutescens* var. *baccatum* (D) Non-glandular unicellular trichome on the adaxial surface of *Capsicum frutescens* var. *baccatum* (E) Non-glandular unicellular trichome on the abaxial surface of *Capsicum frutescens* var. *baccatum*

The spectra of the energy dispersive X-ray spectroscopy and the percentage atomic

Table 3. Percentage atomic compositions of the elements in the foliar epidermis of four varieties of *Capsicum* species revealed by EDX spectroscopy

Elements	*C. annuum* var. *abbreviatum* (Rodo)	*C. annuum* var. *acuminatum(*Sombo)	*C. annuum* var. *grossum* (Tatase)	*C. frutescens* var. *baccatum* (Wewe)
Carbon (C)	37.32±1.08	7.93±0.84	36.23±0.98	35.32±0.71
Oxygen (O)	23.31±0.40	3.19±0.33	16.45±0.35	19.25±0.46
Sodium (Na)	1.14±0.12	0.54±0.08	0.50±0.05	0.51±0.05
Magnesium (Mg)	0.44±0.08	0.49±0.06	0.24±0.03	0.40±0.04
Silicon (Si)	0.10±0.04	5.37±0.22	0.07±0.04	0.02±0.02
Sulfur (S)	0.50±0.11	2.13±0.36	0.58±0.11	0.62±0.11
Phosphorus (P)	0.27±0.11	0.78±0.30	0.64±0.10	0.32±0.10
Potassium (K)	1.59±0.15	1.77±0.18	0.36±0.05	4.26±0.18
Calcium(Ca)	1.18±0.08	0.14±0.14	0.01±0.01	1.19±0.09
Gold (Au)	19.60±4.56	77.68±10.90	32.97±4.05	17.15±3.60
Bromine (Br)	-	-	-	7.60±2.22
Beryllium (Be)	15.55±0.82	-	11.96±0.60	8.63±0.36
Chlorine (Cl)	-	-	-	0.21±0.05
Nitrogen (N)	-	-	-	4.92±1.51

composition found in the foliar epidermis of the four varieties of *Capsicum* are presented in Table 3 and figures 6, 7, 8 and 9. EDX gives accurate information on the chemical composition of sub-cellular structures in plants (Wyroba et al., 2015).

The EDX analysis showed the presence of carbon, oxygen, sodium, magnesium, silicon, sulphur, phosphorus, potassium, calcium and gold in all the varieties. Beryllium (Be) was found in *C.annuum* var. *abbreviatum*, *C. annuum* var. *grossum* and *C.*

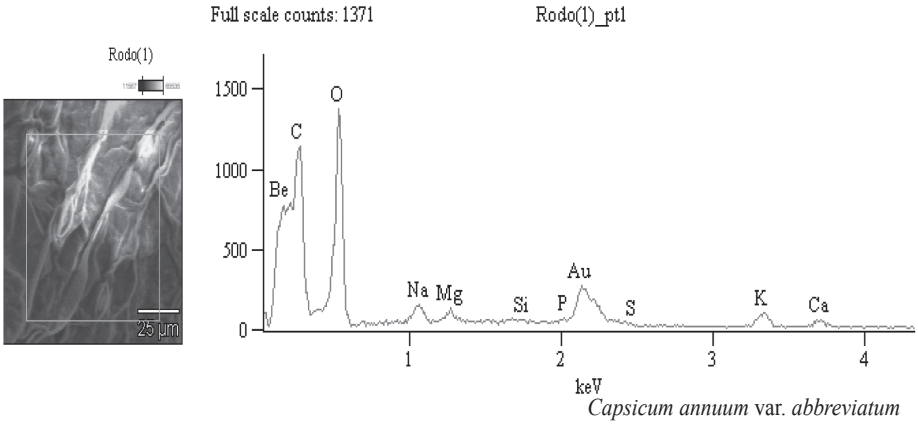

Figure 6. EDX photomicrograph and spectra of the foliar epidermis of *Capsicum annuum* var. *abbreviatum*

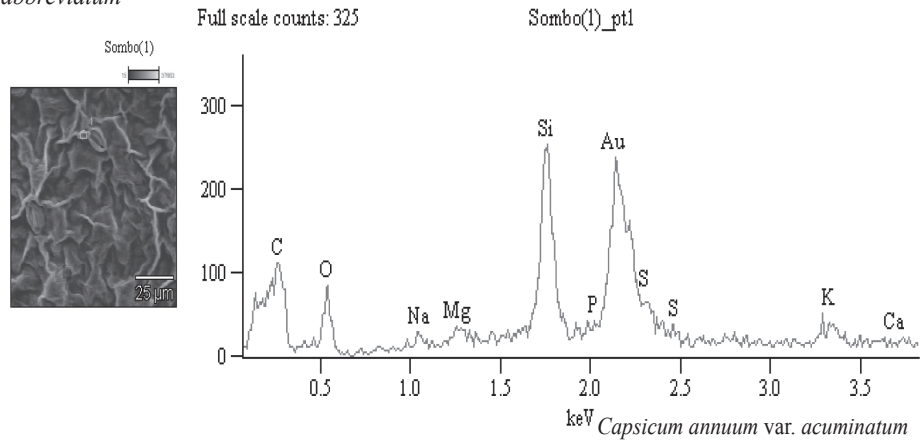

Figure 7. EDX photomicrograph and spectra of the foliar epidermis of *Capsicum annuum* var. *acuminatum*

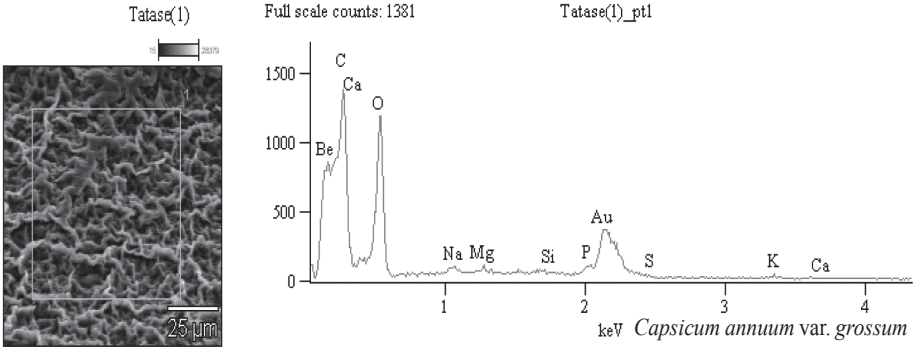

Figure 8. EDX photomicrograph and spectra of the foliar epidermis of *Capsicum annuum* var. *grossum*

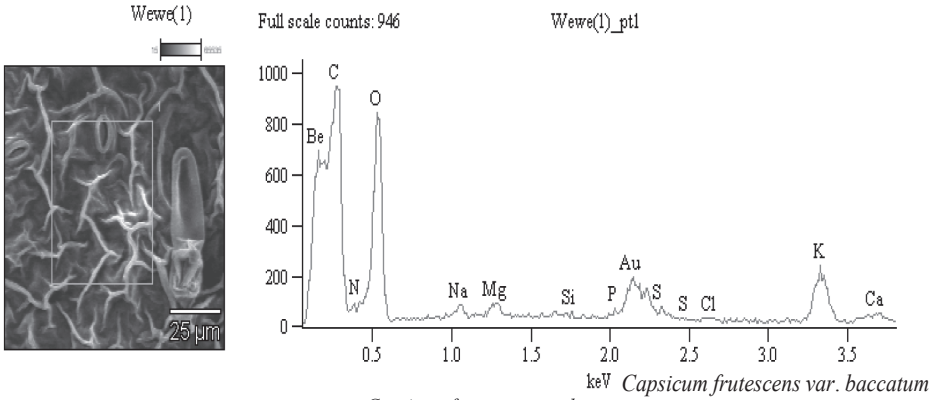

Figure 9. EDX photomicrograph and spectra of the foliar epidermis of *Capsicum frutescens var. baccatum*

frutescens var. *baccatum* only while bromine, chlorine and nitrogen were found only in *C. frutescens* var. *baccatum*. The presence of the different elements on the foliar epidermis might be the result of crystal deposits on the leaf. Crystals have been reported to occur in almost all plants and they are found on the surface of leaves, stem, fruits, seed, and flowers; with various shapes and sizes (Lersten and Horner, 2008; Nakata, 2012). They play important roles such as mechanical support to plants, providing tolerance to heavy metals and protection against herbivores. Hartley et al. (2015) reported that closely related species might have similar foliar epidermal elemental compositions which might be employed in delineation of the plants. The similarity in the elemental composition in all the varieties studied was an indication of their relatedness. However, *C. frutescens* var. *baccatum* could be distinguished from the other three varieties by the presence of bromine, chlorine and nitrogen in its elemental composition. The presence of gold in high amounts in all the varieties could be attributed to the spur plating during coating of the samples.

In general, the results from this study revealed similarities in the foliar epidermal features of the four varieties which justified the evolutionary and phylogenetic relatedness among them. However, the differences observed mainly in trichome occurrence and morphology did not provide enough evidence to conceive that the four varieties were separate species. Foliar epidermal traits needed to be considered along with information from cytological studies, and ultimately molecular profiling, in the ongoing effort to fully understand the phylogenetic relationship among the cultivated *Capsicum* species in this part of the world.

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