To the Graduate Council:

I am submitting herewith a thesis written by Emily Robertson Whittier entitled "Histological Distribution of Orange G-Specific Cytoplasmic Globules and Safranin-Aniline Blue-Specific Cytoplasts in the Shoot of Kalanchoe blossfeldiana." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ornamental Horticulture and Landscape Design.

Effin T. Graham

Effin T. Graham, Major Professor

We have read this thesis and recommend its acceptance:

Gary L. McDaniel

D B Williams

Accepted for the Council:

L Evans Rich

Vice Chancellor
Graduate Studies and Research

Ag-Vet
Thesis
77
.W475
cop.2

HISTOLOGICAL DISTRIBUTION OF ORANGE G-SPECIFIC CYTOPLASMIC GLOBULES AND SAFRANIN-ANILINE BLUE-SPECIFIC CYTOPLASTS IN THE SHOOT OF KALANCHOE BLOSSFELDIANA

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Emily Robertson Whittier

December 1977

1346419

ACKNOWLEDGMENT

I would like to express my appreciation to the faculty of the Department of Ornamental Horticulture and Landscape Design. Also, appreciation goes to my major professor, Dr. E. T. Graham, and to the other members of my committee, Dr. G. L. McDaniel and Dr. D. B. Williams.

ABSTRACT

Large spherical cellular inclusions in Kalanchoe blossfeldiana were selectively stained using aqueous safranin followed by citrate buffered aniline blue-orange G pH 3.5. The globules were stained bright orange. Other cellular inclusions that filled entire cells were brightly stained shades of pink, red, or purple. Tissue fixed in 5% glutaraldehyde and 2% calcium acetate revealed the globules and inclusions throughout the shoot. Globules were present in the pith parenchyma cells and in the layer of cortex just outside the vascular tissue from just below the apical meristem to the crown. Globules were also found in parenchyma cells in petioles and in mesophyll cells surrounding vascular tissue in leaves. Cellular inclusions in the form of whole dense protoplasts were more frequently found in a continuous layer just under the epidermis in the stem, and in the leaves were concentrated immediately under the epidermis. Both the globules and whole cell inclusions were also randomly scattered through stem and leaf tissue. Various histochemical tests did not identify the composition of either the globules or the whole cell inclusions, but eliminated polysaccharides, protein, and unsaturated lipids as the major structural components of the globules. Some whole-cell inclusions in fresh sections gave positive reactions for tannin, protein, and polysaccharides.

TABLE OF CONTENTS

SECTION		PAGE
I.	INTRODUCTION	1
II.	LITERATURE REVIEW	3
III.	MATERIAL AND METHODS	6
IV.	RESULTS AND DISCUSSION	9
BIBLIOGRAPHY		21
VITA		24

LIST OF FIGURES

FIGURE		PAGE
1.	Two Cross Sections of Kalanchoe Stem Showing Orange Globules and Other Cellular Inclusions in the Pith Parenchyma Cells .	12
2.	Cross Sections of Stem Showing Orange Globules in Clusters and the Honeycomb Appearance of Cellular Inclusions	13
3.	Sections of Whole Leaf Showing Orange Globules and Inclusions Stained with Safranin-Aniline Blue-Orange G, Embedded in Paraffin	14
4.	Longitudinal Sections of Stem Embedded in Paraffin and Stained with Safranin-Aniline Blue-Orange G Showing Whole Cell Inclusions	15
5.	Four Views of Cellular Inclusions Located Near the Epidermis of Leaf Sections, Showing the Variety of Colors, Embedded in Paraffin and Stained with Safranin-Aniline Blue-Orange G . .	17

I. INTRODUCTION

The plant Kalanchoe blossfeldiana contains many cellular inclusions throughout its vegetative tissues. Histological observation reveals numerous globules of various sizes which can be selectively stained with orange G in many cells of the plant. Other cells are completely filled with brightly stained material.

The type of fixation and the staining method used are important in differentiating these inclusions within the cell. The globules, especially, seem to be preserved only in a noncoagulent type fixative such as pH neutral glutaraldehyde. A new staining method has been developed at the Department of Ornamental Horticulture and Landscape Design, University of Tennessee, to distinguish the various globules and cytoplasmic staining affinities in paraffin sections (Personal communication with Dr. E. T. Graham). The sections are first stained in safranin O and then in a combination stain of aniline blue-orange G. This technique stains the globules a bright orange while whole cells having cytoplasm of either different density or of different composition are stained various shades of pink, red, blue, and purple.

The K. blossfeldiana, as a species of Crassulaceae, is characterized by Crassulacean acid metabolism or dark carbon dioxide fixation. It is also a photoperiodic plant, induced to bloom under short day conditions. Any relationship between carbon dioxide fixation or photoperiodism and cytoplasmic structure would have great physiological significance. Since the cytoplasmic globules and inclusions in Kalanchoe have only been

recently found, however, a survey of the anatomical distribution of these cellular structures is an essential first step toward further investigation of their physiological significance.

Preliminary observations have indicated that the cellular entities in question are to be found in leaf and stem tissue. The purpose of this investigation, therefore, was to determine the histological distribution of orange G-specific globules and cells with dense cytoplasm in the vegetative shoot of Kalanchoe blossfeldiana.

II. LITERATURE REVIEW

There is very little information on cellular globules resembling those under consideration in this thesis. Shumway and associates (1976, 1972, 1970) using electron microscopy have observed protein globules in tomato and tobacco plants. These globules were quite small about 1 micron in diameter. They occurred in vacuoles and were believed to be proteinaceous. Attempts to demonstrate them in light microscopy were unsuccessful. On the contrary, the exploratory work leading to this thesis clearly demonstrated the Kalanchoe globules in the light microscope.

Gifford and Stewart (1968) reported the presence of spherical membrane-bound inclusions in the proplastids and vacuoles of cells in Byrophyllum and Kalanchoe. These globules were studied by electron microscopy and were very small, 0.7 to 1.2 microns in diameter. Fixation with glutaraldehyde not followed by post fixation in permanganate created "ghost" images of the globules in the cells.

Akers, Anderson, and Blum (1977a, b) observed vacuolar bodies within all organs of Spartina alterniflora except the apical meristem. These vacuolar bodies which were visible in the light microscope were located in the mesophyll and outer bundle sheath cells of the leaves. In the root, rhizome, peduncles, and glumes, the vacuolar bodies were located in parenchyma cells. Various histochemical tests were performed on the vacuolar inclusions. Tests for lipids, proteins, tannins and glycols gave negative results. The only positive results were obtained

with 2, 4, dinitrophenylhydrazine which stained the bodies yellow, an indication of aldehydes.

Esau (1977) stated that the Crassulaceae family contains large amounts of tannin and idioblasts. Rost (1969, 1965) reported that Crassula argenta (Jade plant) contained large amounts of tannin in the mesophyll and just below the epidermis of the leaves. These were located in fresh sections stained with ferric sulfate as described by Jensen (1962). Rost also reported that these tannin cells were bright red in paraffin sections stained with safranin. Tannin is a broad term for polyhydroxyphenolic substances found in plant cells and there are many methods reported for their detection and differentiation. Most methods require making fresh sections of the tissue and then treating them with various chemicals. Reeve (1959) reported successful use of the nitroso test (Jensen, 1962) when measured photometrically to distinguish different types of tannin compounds. More recently Parham and Kaustinen (1976) found that tannins can be differentiated in material fixed in glutaraldehyde, post-fixed in osmium tetroxide and embedded in epoxy. They found that tannin deposits stained by Sudan black B were brownish-orange and were easily distinguished from lipid bodies of similar size which stained dark blue to black, and from starch grains which were unstained. Chafe (1963) stated that tannin deposits were stained by osmium tetroxide in tissue fixed in glutaraldehyde for electron microscopy and showed that cells containing tannin could be located by using glutaraldehyde and ferrous sulfate.

These globules which are the subject of this thesis have not been reported in recent work on anatomy (Esau, 1977), plant cytology (Robards, 1970; Clowes and Juniper, 1968), or general cytology (Swanson, 1977).

III. MATERIAL AND METHODS

Evidence for the histological location and size distribution was assembled from representative samples of several shoot apices, stem, leaves, petioles, and roots taken from Kalanchoe blossfeldiana seedlings grown in a greenhouse under long day conditions. Root samples were obtained from cuttings rooted in a mist bed in pure sphagnum moss.

Tissue samples were fixed overnight in a solution of 5% glutaraldehyde and 2% calcium acetate. The tissues, in the fixing solution, were subjected to vacuum at 25 psi for 10-30 minutes depending on the size of the tissue pieces.

The size of the tissues varied greatly. Most samples were portions of stems and leaves 5 to 10 mm in length. It was found that a good overview of the leaf tissue could be obtained by using small leaves about 5 to 10 mm in length. In order to trace the location of the globules throughout the entire stem, the stems were fixed and infiltrated with paraffin in one piece and cut into smaller pieces just before placing them in the paraffin molds. This procedure insured that the sections could be examined in the proper sequence according to anatomical location. These large stem pieces were fixed satisfactorily unless the stem was greater than 7 mm in diameter. It was found to be important in these stem pieces to completely evacuate all of the air as the tissue would otherwise float in the dehydrating alcohols and paraffin.

The samples were then dehydrated in a 12 step series of ascending alcohol mixtures of tertiary butyl alcohol (TBA) and absolute ethyl

alcohol to a rinse of pure TBA (Jensen, 1962). From the TBA the tissue was either infiltrated with paraffin or diethylene glycol disterate wax. The tissue in wax was allowed to harden in small dishes. Small blocks of tissue and wax were removed and embedded in larger blocks of paraffin. Blocks of paraffin or paraffin and wax were ribboned sectioned on a rotary microtome at 5 microns.

After being mounted on bare glass slides, the tissue samples were deparaffinized in three washes of xylene and taken through three washes of absolute alcohol and rehydrated in a descending ethyl alcohol series to water. Samples were stained one minute in aqueous 0.1% safranin O and after a quick water rinse stained 10 minutes in aniline blue-orange G in a citrate buffer pH 3.5 (Jensen, 1962 and LaCour et al., 1958). After removal from the stain the slides were washed with water to remove excess stain and placed in a TBA rinse containing 5% isopropyl alcohol to prevent freezing of the TBA. This TBA rinse dehydrated the sections without leaching the stain as would occur in ethyl alcohol. The slides were transferred to xylene and covered-glassed with resin.

To check for tannin deposits, tissues fixed in ferric sulfate and formalin (Jensen, 1962) and in ferric sulfate in glutaraldehyde were dehydrated and embedded in paraffin in the same manner as the other samples. The tissue was not stained, but covered-glassed immediately after removal of the paraffin with xylene. Fresh hand sections treated with the ferric sulfate and formalin solution were also examined.

Material to be embedded in plastic was fixed in 5% glutaraldehyde in phosphate buffer as pH 6.8 and after rinsing in buffer solution was

post-fixed two hours in 2% osmium tetroxide and thirty minutes in uranyl acetate. After dehydration in alcohols, the tissue was embedded in Ladd low viscosity medium (Ladd Research Industries, data sheet) or in Spurr low viscosity medium (Spurr, 1968). Some tissue was prepared only with glutaraldehyde and no osmium tetroxide. Material embedded in plastic was sectioned at 3-4 microns on an ultramicrotome. The sections were floated on water in small closed polyethylene capsules at 75°C for one and a half hour, to decompress and flatten them and then floated onto glass slides. Plastic was removed by allowing prepared glass slides to stand one hour in a saturated solution of sodium hydroxide in absolute alcohol (Lane and Europa, 1965).

A variety of histochemical tests were used on both the paraffin and the plastic sections. The periodic acid-Schiff's reaction (PAS) was done as described by Jensen (1962) using 2,4 dinitrophenylhydrazine as a blockade and by itself to test for aldehydes according to Feder and O'Brien (1968). Also, sections were stained with Sudan black B in propylene glycol as a test for lipids (Chiffell and Putt, 1951). Aniline blue black in acetic acid solution was used to test for total proteins (Fisher, 1968). Some plastic sections were stained by floating them on 0.5% w/v toluidine Blue O in 0.1 M citrate buffer, pH 4.0. This floatation staining was done in closed polyethylene capsules in an oven at 75°C for one and a half hour. The stained sections were rinsed in water in a microchemical spot plate and then transferred to glass slides.

All microscopic work was done on a Wild microscope equipped with a floutar objectives and a Nikon 35mm photomicrographic light meter and camera unit.

IV. RESULTS AND DISCUSSION

Observations on the globules and inclusions in Kalanchoe blossfeldiana were found to be dependent on the fixation method used. They appear to be fixed best with noncoagulent type fixatives especially glutaraldehyde. Lillie (1965) suggested using a calcium acetate solution to replace the calcium chloride commonly used with formalin for fixation of animal tissue, thus combining the beneficial effects of the calcium and the effect of a buffer. This idea was modified for the purpose of this investigation by using glutaraldehyde instead of formalim with calcium acetate. The aqueous mixture of 5% glutaraldehyde and 2% calcium acetate at pH 7.0 was an effective fixative for Kalanchoe shoot tissues.

The fixation of large stems provided a general overview of the distribution of the globules and other cellular inclusions. However, the quality and detail of the inner cells of the tissue was poor. There was a lot of destruction in the cells, probably due to very slow diffusion of the fixative into these large sample pieces. Whole leaves were embedded with considerable success. Leaves up to the size of the paraffin block were used, but leaves about 3 mm long gave the best results, as the larger and older ones had a lot of compression in the ribbon sections.

The use of the disterate wax did improve the quality of the cells because there was less shrinkage and compression of the block. However, the block formed by this method was hard to ribbon and the ribbon broke easily. Also, the wax block tended to separate from the paraffin upon

slicing. Modification of this technique needs to be done before it can be used effectively for extensive routine sectioning.

Much better results were obtained with plastic, although embedding in this medium required much smaller tissue pieces than the paraffin method. Specimens that were fixed in glutaraldehyde and osmium tetroxide were poor in quality. The large dense protoplasts of the petioles absorbed heavy amounts of osmium tetroxide and upon slicing, pieces flaked out contaminating the sections and making them difficult to interpret. The deposits of osmium were so dense that overstaining with toludine blue offered no advantage in interpreting structure. To obtain satisfactory results with Kalanchoe modification of the post-fixation with osmium is needed. The plastic sections that were not post-fixed with osmium tetroxide showed considerably more detail than was observed in the paraffin sections.

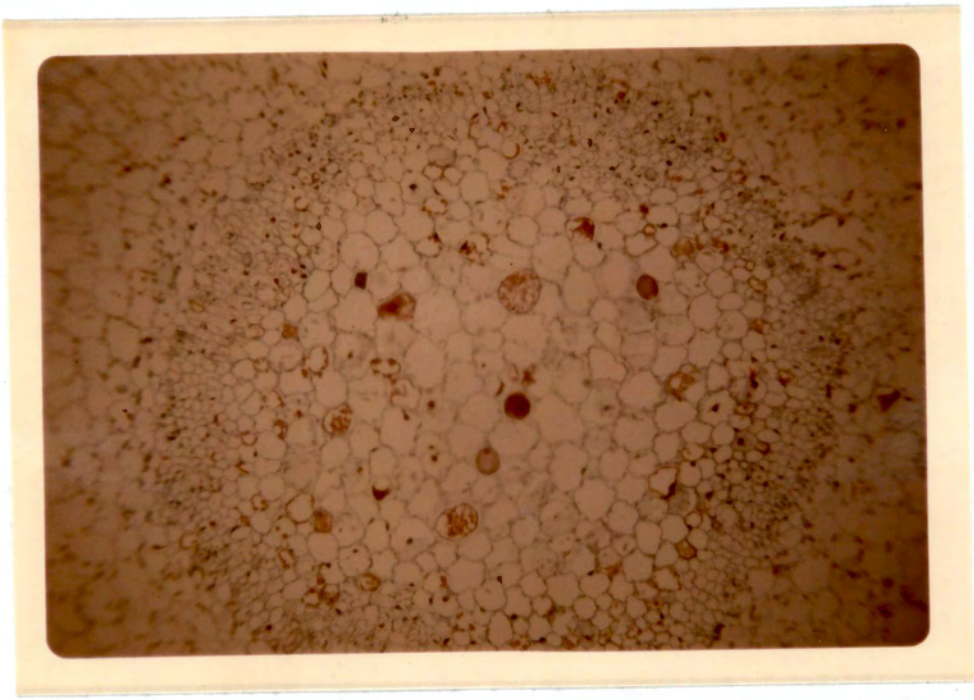
The globules and the inclusions were differentially stained by the use of safranin followed by the combination stain aniline blue-orange G. This stain was originally developed (LaCour, 1958) and later recommended (Jensen, 1962) as a specific histochemical stain for phospholipids, but has been adapted as general histological stain for the purpose of this study. If stained with safranin after aniline blue-orange G, there was no color differentiation in the cells. When safranin preceded the aniline blue-orange however, an effective tri-colored stain was obtained with the cytoplasmic glubules showing a pronounced attraction for the orange G dye. Using this stain, cellulose cell walls stained blue; interphase nucleus blue with a red nucleolus; prophase nucleus, red;

starch, blue gray often with a red hilum; plastids, red orange or red; and cytoplasm, blue (Figure 1). Both the globules and whole cell inclusions in Kalanchoe could be found in the tissue from must below the apical meristem to the crown. The bright orange globules varied considerably in size from filling almost the entire cell down to the limit of the resolution of the light microscope. Large globules occurred singly and could be sectioned serially while smaller ones often occurred in clusters.

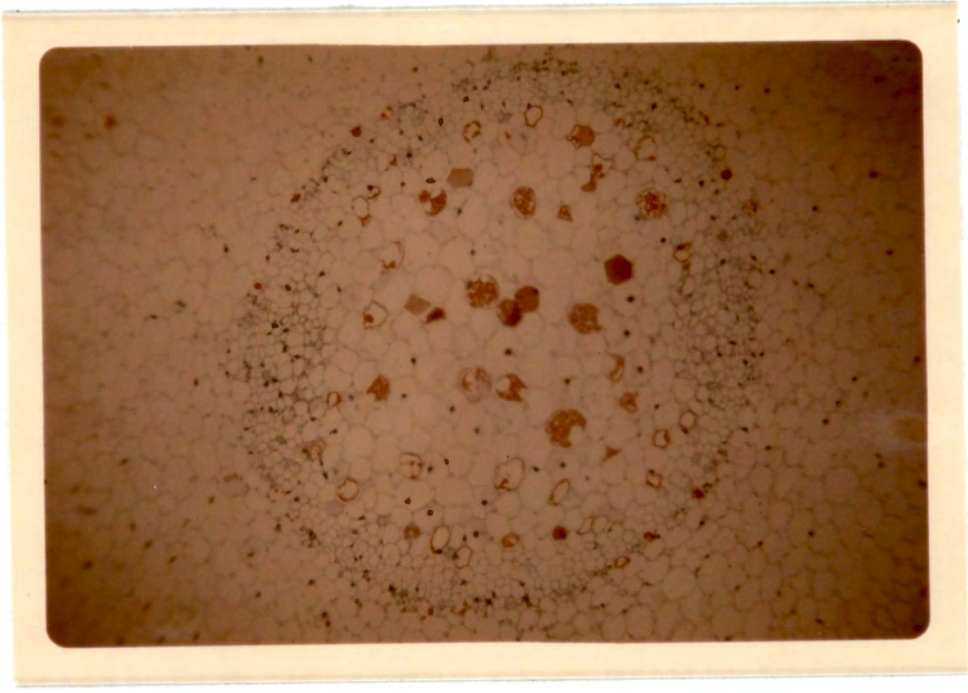
Globules are present in the pith parenchyma cells just below the apical meristem, but not in the actively dividing layers of the apical meristem. Globules occur in the pith parenchyma cells throughout the stem, especially in the layer of cortex just outside the vascular tissue (Figures 1 and 2). At the nodes the globules also were observed in the parenchyma cells surrounding the vascular tissue that branches into the petioles. In the petioles, globules were also found in the parenchyma cells, but they were not as numerous. In both stems and petioles, globules were occasionally found near the epidermis and also in randomly distributed cells in the cortex.

Within the leaves globules were found in the mesophyll cells surrounding veins and occasionally in the vascular tissue itself (Figure 3a). As in the stem and petiole, apparently random cells throughout the mesophyll and around the edges of the leaves contained globules (Figure 3b).

Samples of the roots were examined and were brightly colored in blue, red, purple, and orange. However, the glutaraldehyde calcium acetate

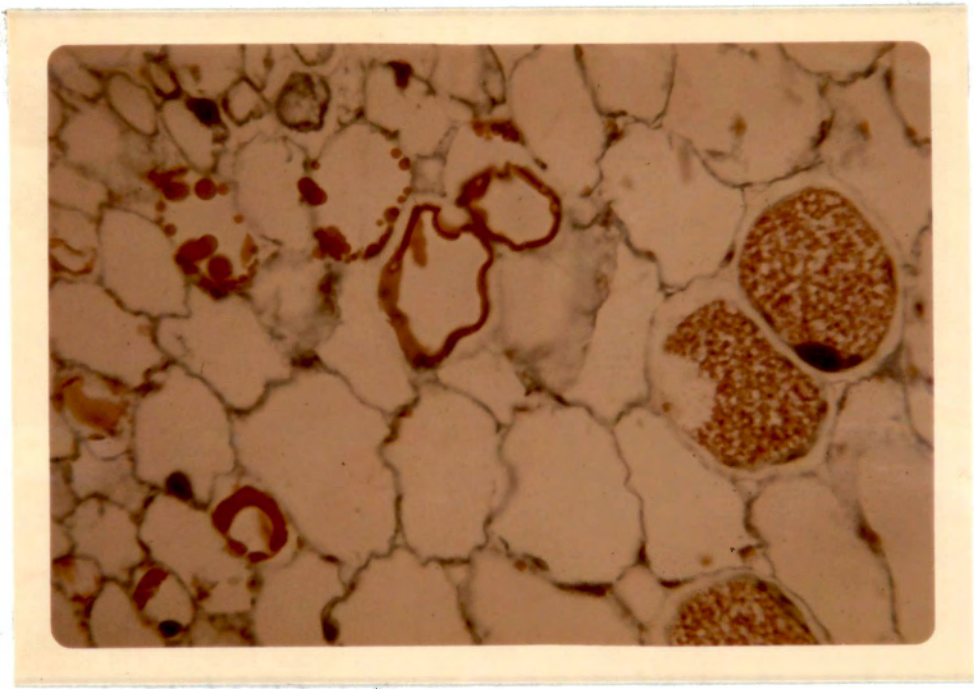


a. Stained in safranin-aniline blue-orange G, embedded in paraffin, X25 enlarged X3

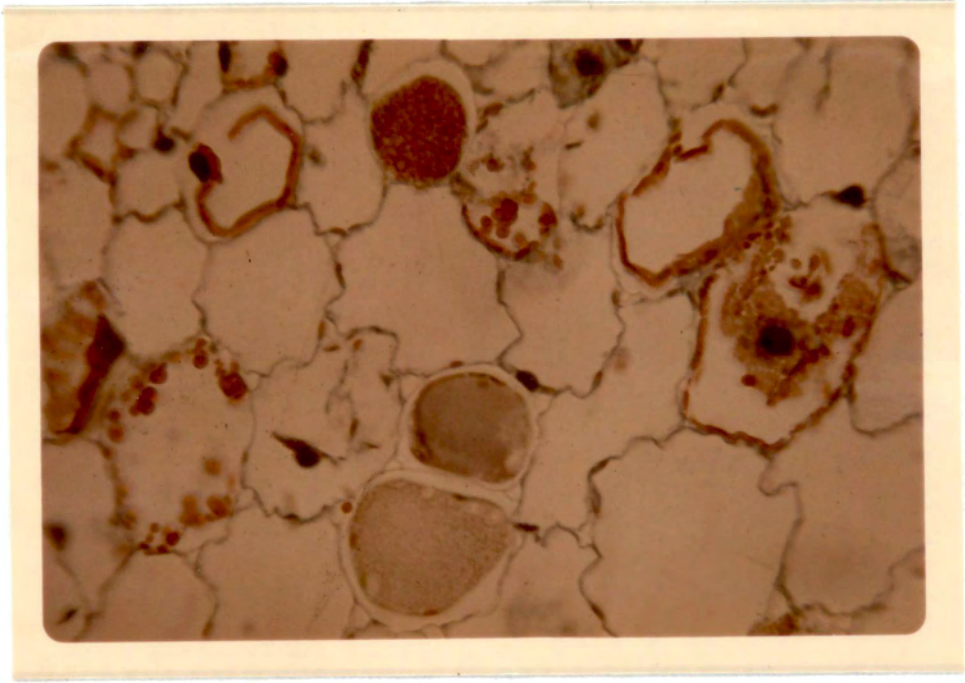


b. Embedded in paraffin, stained in safranin-aniline blue-orange G, X25 enlarged X3

Figure 1. Two cross sections of Kalanchoe stem showing orange globules and other cellular inclusions in the pith parenchyma cells.

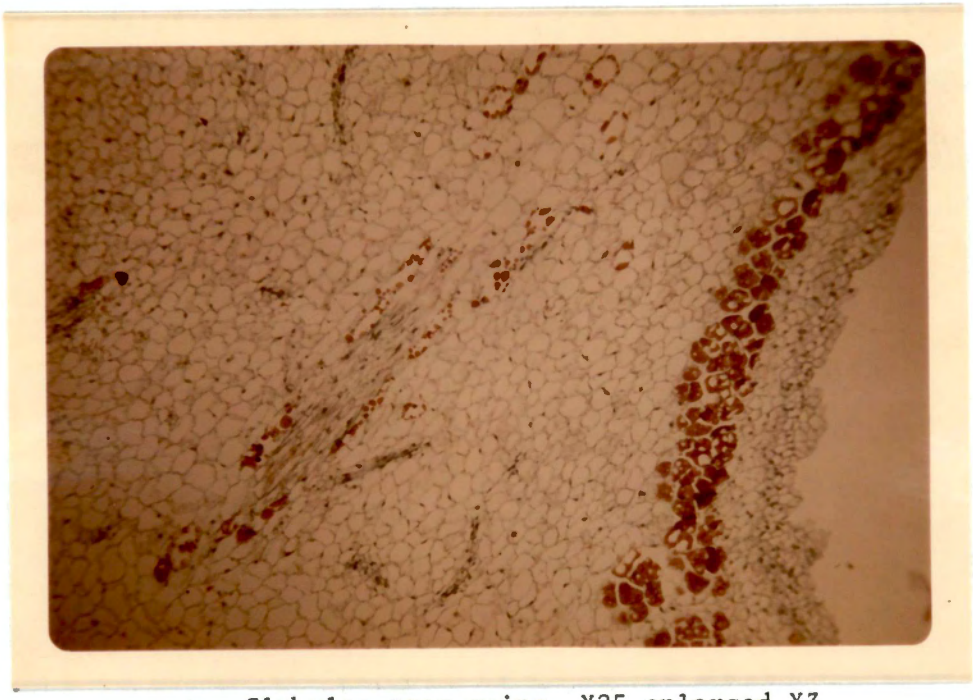


a. Embedded in paraffin, stained in safranin-aniline blue-orange G, X125 enlarged X3



b. Embedded in paraffin, stained in safranin-aniline blue-orange G, X125 enlarged X3

Figure 2. Cross sections of stem showing orange globules in clusters and the honeycomb appearance of cellular inclusions.



a. Globules near veins, X25 enlarged X3



b. Globules and other inclusions near epidermis, X25 enlarged X3

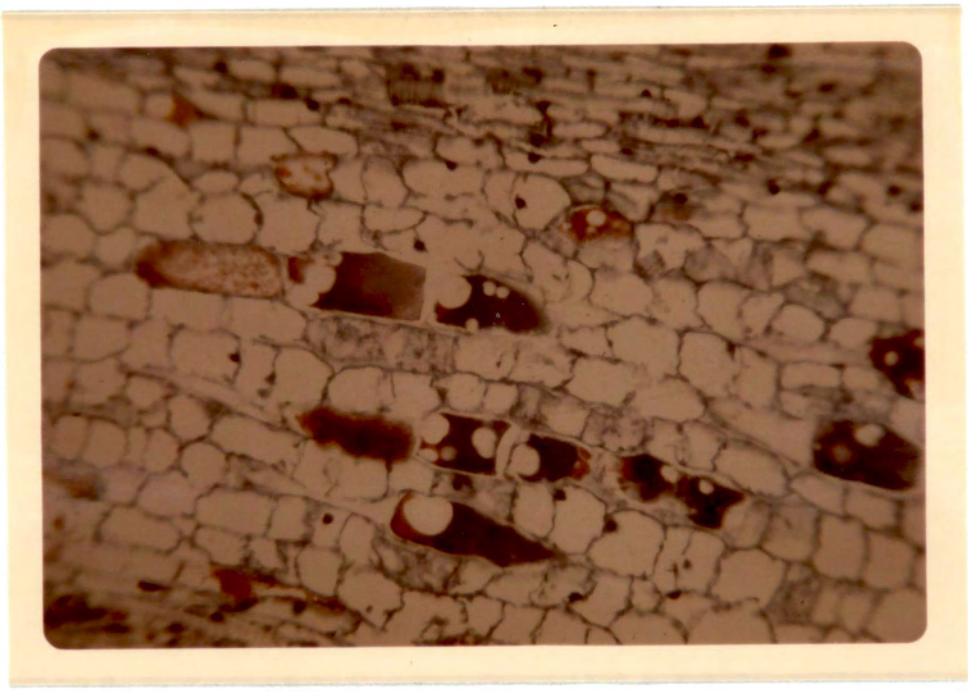
Figure 3. Sections of whole leaf showing orange globules and inclusions stained with safranin-aniline blue-orange G, embedded in paraffin.

fixative was much less effective for root than for shoot tissue, allowing severe compression and disruption during sectioning and mounting, and will require modification for further investigation of roots.

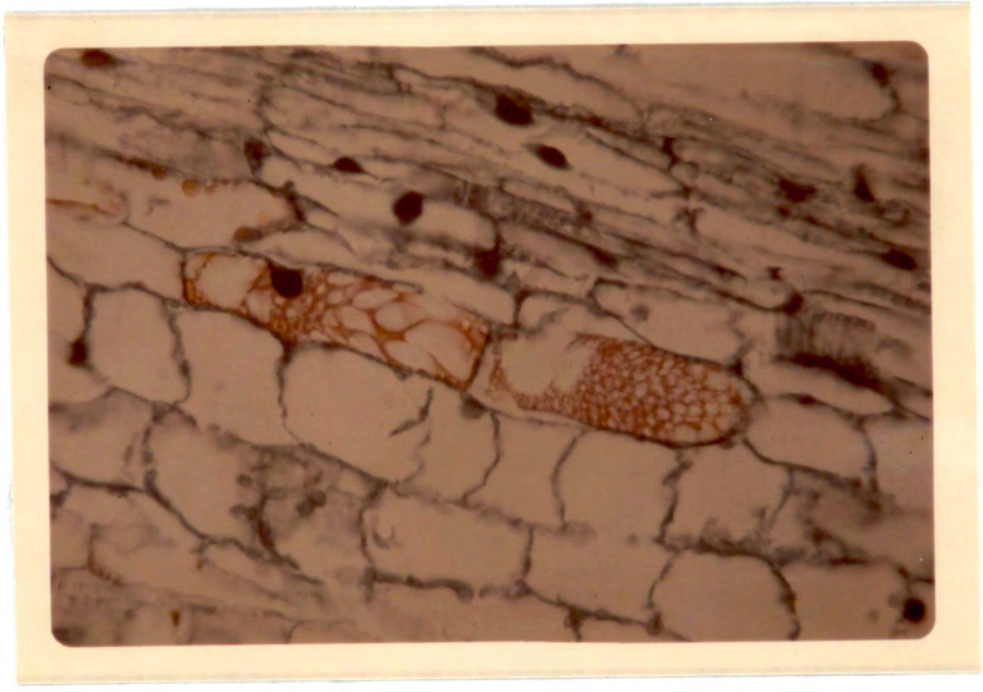
Certain cellular inclusions involving entire protoplasts also appeared throughout the shoot from immediately subjacent to the apical meristem to the crown. All subsequent usage of the word "inclusion" refers to the entire protoplasts or "whole cell" inclusion involving the cytoplasm. Frequently there was an almost continuous layer of cells with very dense protoplasts just under the epidermis. These inclusions often filled the entire cell and some appeared frothy of like a honeycomb Figures 2 and 4). These inclusions varied in color considerably from pink through red to purple when stained in safranin followed by aniline blue-orange G. The different colors appeared in groups, but the groups were not found in one specific location.

In the stem and petioles, the inclusions were found in the pith parenchyma cell and in the cortex near the epidermis. As with the globules, cells scattered throughout the cortex contained the inclusions. This was most apparent in the sections embedded in plastic. There was a wide band of cells with densely stained cytoplasm near the outer edges of the leaves (Figure 5). From cross sections of the leaves it was found that the inclusions were present under both surfaces. Such whole cell inclusions occurred only infrequently in the inner mesophyll tissue.

The brief look at the roots showed some material that was similar to these inclusions, but, more precise interpretation of the root will require improved fixation of the root tissue.

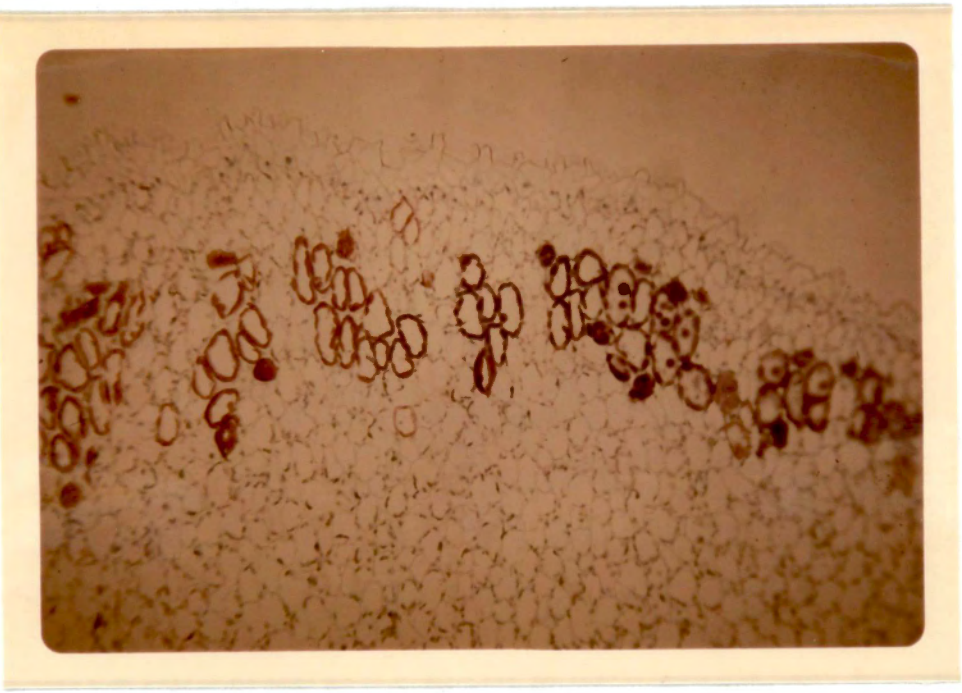


a. X25 enlarged X3

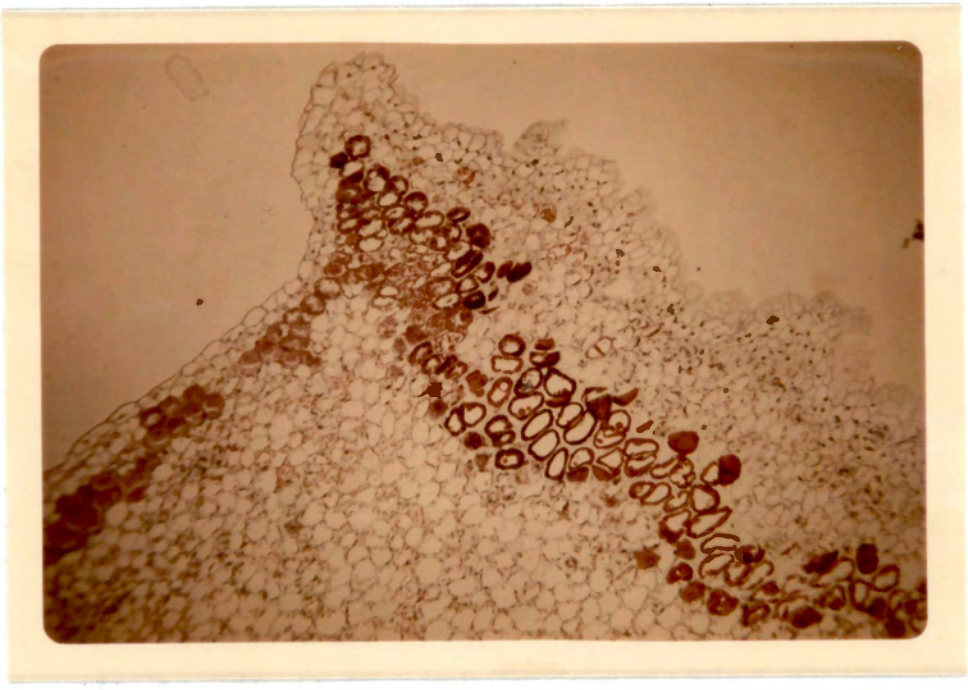


b. X125 enlarged X3

Figure 4. Longitudinal sections of stem embedded in paraffin and stained with safranin-aniline blue-orange G showing whole cell inclusions.

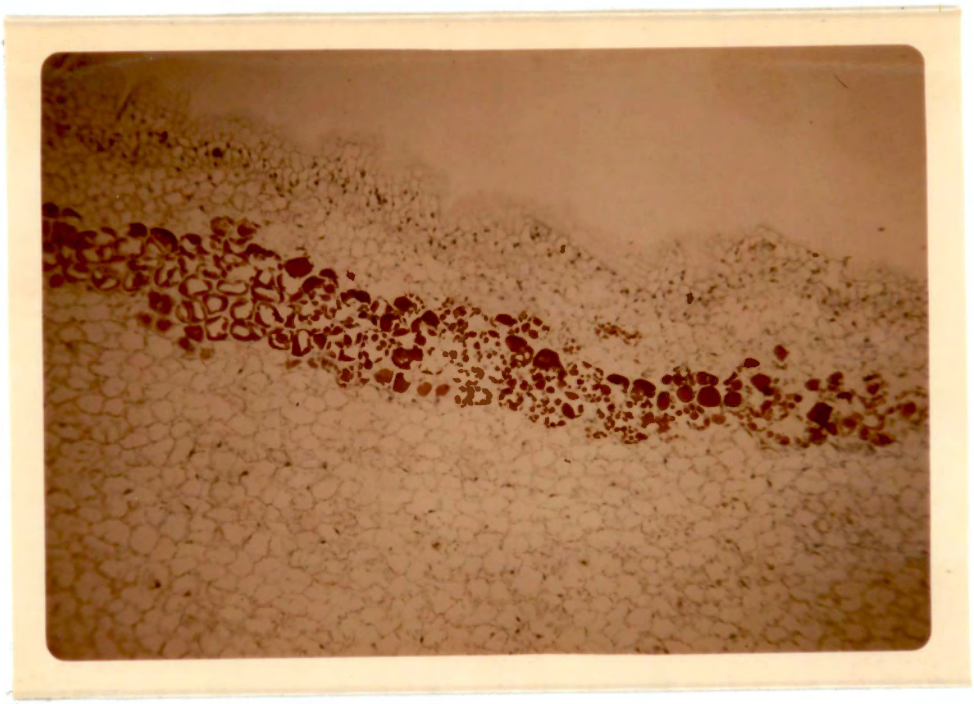


a. X25 enlarged X3

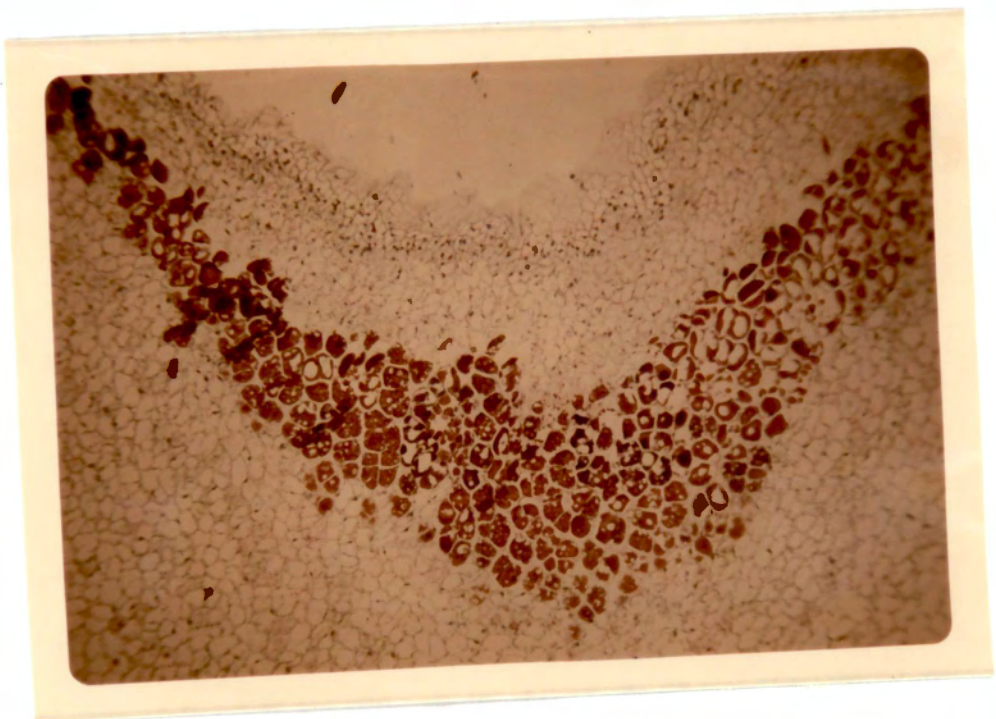


b. X25 enlarged X3

Figure 5. Four views of cellular inclusions located near the epidermis of leaf sections, showing the variety of colors, embedded in paraffin and stained with safranin-aniline blue-orange G.



c. X25 enlarged X3



d. X25 enlarged X3

Figure 5 (continued)

petioles of Kalanchoe, however, appeared the same yellow in the PAS test with or without treatment with 2,4 dinitrophenylhydrazine. Much more research is needed to explain these unusual results.

The cellular inclusions showed negative results to the tests for proteins and lipids, but there was a positive reaction to some of the inclusions in the PAS test and in some tannin tests. There did not appear to be a uniformity among the inclusions in their reactions to the histochemical stain tests.

In summary, the orange G specific globules and other cytoplasmic inclusions can be observed throughout the shoot of K. blossfeldiana. When tissue is fixed in glutaraldehyde at near neutral pH and stained with safranin and aniline blue-orange G, bright orange globules and other brightly stained inclusions, are differentiated. Both the globules and the inclusions are present in the pith parenchyma cells, the cortex, and in the mesophyll of the leaves. Although both types of inclusions appear with regularity in certain tissues of the plant, they are not limited to one type of cell. There does not seem to be any relationship between the distribution of the globules and the whole dense protoplasts. The presence or absence of one type of inclusion does not indicate the presence or absence of the other.

BIBLIOGRAPHY

BIBLIOGRAPHY

Akers, S. W., Anderson, C., and Blum, U. 1977a. Characterization of vacuolar bodies in Spartina alterniflora: I. Formation, development, morphology, and ultrastructure. American Journal of Botany 64(6): 635-640.

Akers, S. W., Anderson C., and Blum U. 1977b. Characterization of vacuolar bodies in Spartina alterniflora: II. Some physical and chemical properties. American Journal of Botany 64(6):641-648.

Chafe, S. C. and Durzan, D. J. 1973. Tannin inclusions in cell suspension of cultures of White Spruce. Planta (Berl.) 113:251-262.

Chiffell, T. L. and Putt, F. A. 1951. Propylene and ethylene glycol solvents for Sudan IV and Sudan black B. Stain Technology 26:51-56.

Clowes, F. A. and Juniper, B. E. 1968. Plant Cells. Oxford: Scientific Publication.

Esau, K. 1977. Anatomy of Seed Plants. New York: John Wiley and Sons.

Fisher, D. B. 1968. Protein staining of ribboned epon sections for light microscopy. Histochemie 16:92-96.

Gifford, E. M. and Stewart, K. D. 1968. Inclusions of the proplastids and vacuoles in the shoot apices of Byrophyllum and Kalanchoe. American Journal of Botany 55(3):269-279.

Jensen, W. A. 1962. Botanical Histochemistry. San Francisco: W. H. Freeman Co.

LaCour, L. F., Chayen, J., and Gahen, P. S. 1958. Evidence for lipid material in chromosomes. Experimental Cell Research 14:469-485.

Lane, B. P. and Europa, D. L. 1965. Differential staining of ultrathin sections of epon-embedded tissues for light microscopy. Journal of Histochemistry Cytochemistry 13:579-582.

Lillie, R. D. 1965. Histopathologic Technic and Practical Histochemistry. New York: McGraw Hill.

Parhan, R. A. and Kaustinen, H. M. 1976. Differential staining of tannin in sections of epoxy-embedded plant cells. Stain Technology 51(4): 237-240.

Reeve, R. M. 1959. Histological and histochemical changes in developing and ripening peaches: I. The catechol tannins. American Journal of Botany 46(3):210-217.

Robard, A. W. 1970. Electron Microscopy and Plant Ultrastructure. London: McGraw Hill.

Rost, T. L. 1965. Comparative histological techniques and the foliar anatomy of Crassula argentea. Master's Thesis, Mankato State College, Mankato, Minnesota.

Rost, T. L. 1969. Vascular pattern and hythodes in leaves of Crassula argentea. Botanical Gazette 130(4):267-270.

Shumway, L. K., Vivian vie Yang, Ryan, C. A. 1976. Evidence for the presence of ptoteins inhibitor I in vacuolar protein bodies in plant cells. Planta (Berl.) 129:161-165.

Shumway, L. K., Cheng, V., Ryan, C. A. 1972. Vacuolar protein in apical and flower petal cells. Planta (Berl.) 106:279-290.

Shumway, L. K., Rancour, J. K., Ryan, C. A. 1970. Vacuolar protein bodies in tomato leaf cells and their relationship to storage of chymotrypsin inhibitor I protein. Planta (Berl.) 93:1-4.

Sidman, R. L., Mottla, P. A., Feder, N. 1961. Improved polyester was embedding for histology. Stain Technology 36:179-184.

Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research 26:31.

Swanson, C. P. and Webster, P. L. 1977. The Cell. New Jersey: Prentice-Hall.

VITA

Emily Robertson Whittier was born to Mr. and Mrs. Roy C. Robertson on October 8, 1954, in Knoxville, Tennessee. She attended Bearden Elementary and Bearden High School in Knoxville and graduated in June of 1972. In June, 1975, she was graduated with honors from the University of Tennessee with a Bachelor of Arts degree in Botany. She received her Master of Science in Ornamental Horticulture and Landscape Design in December, 1977, from the University of Tennessee.

She is married to Michael D. Whittier of Knoxville, Tennessee, who is a civil engineer.