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Leaf Composition of American Bur-Reed (*Sparganium americanum* Nutt.) to Determine Pesticide Mitigation Capability

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Abstract

American bur-reed (*Sparganium americanum* Nutt.), a common aquatic plant in the middle and eastern United States and Canada, is often located in water-retaining drainage areas. The purpose of this study was to determine the leaf composition of *S. americanum*, paying attention to the cuticular waxes and the epidermis, and its ability to sorb pesticides. *S. americanum* leaves (n = 100) were collected in both early (June) and late (August) summer. Transverse sections of *S. americanum* were stained and studied with brightfield and fluorescence microscopy to estimate the structural and chemical nature of the leaf tissues cross sections. Mean total lipid content in early summer leaf samples (1.47 ± 0.83 mg mL^{-1}) was significantly greater (alpha 0.05) than late summer leaves (0.15 ± 0.36 mg mL^{-1}). In vitro analysis of epidermal peel permeability exposed to atrazine and malathion determined little to no sorption by the plant. Therefore, the structure of *S. americanum* leaves suggest this species does not have the capacity of sorbing these pesticides from runoff water.

Keywords Epidermal peel permeability · Atrazine · Malathion · Plant

Pesticide pollution of water resources because of agricultural runoff has been a global concern for decades. One potential solution to this problem involves phytoremediation of runoff in drainage ditches surrounding agricultural fields. Recent studies have demonstrated the ability of various aquatic plants at removing pesticides from the water column (Anderson et al. 2011; Elsaesser et al. 2011; Locke et al. 2011).

Sparganium americanum Nutt., American bur-reed, is found in both the United States and Canada (Ito and Cota-Sánchez 2014). Kao et al. (2003) reported the plant's ability to remove nitrogen and phosphorus from agricultural field runoff. In mesocosm experiments, Moore et al. (2013) reported *S. americanum* decreased the load of atrazine in simulated runoff by 31% ± 4%, but it was not significantly different from unvegetated mesocosms (13% ± 20%).

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Because *S. americanum* is an emergent aquatic plant often found in flow-through drainage ditches, plant leaf surface would likely be the primary site of pesticide uptake in these systems, should exposure occur. This can occur in the outer part of the primary cell wall of the plant epidermis, which is covered with layers of lipids (Holloway 1994). Plant structure and functionality may be moderated by the surrounding environment, and these changes may influence pesticide permeability (Skoss 1955). It is therefore important to examine plant lipid content and structure. Properties of the plant surface can be changed, leading to an increase or decrease in solute transport of molecules into the plant or adherence to the cuticle (Kirkwood 1999). Principal leaf chemical components are waxes, lignin, suberin, and cutin. While waxes form the top cuticle layer, lignin is a hydrophobic complex found in the secondary cell wall, giving plants their strength. Suberin is found in cell walls of external and internal plant tissues of epidermis and endodermis roots (Gou et al. 2009), and it functions to select the solute absorbed by the plant and protect against fungal pathogens (Soler et al. 2007). Aquatic plants, such as *S. americanum*, depend on suberin to prevent water from entering tissues. Cutin is found in the epidermis (Taiz and Zeiger 2010), impermeable to water, and protects plants from pathogens (Kolattukudy 1984). Many factors can affect a pesticide's sorption ability to plants. Therefore,

it is important in phytoremediation studies to examine the leaf chemical components in order to better understand the plant's potential for pesticide sorption. The purpose of this study was to determine leaf composition of *S. americanum*, particularly epidermal lipids and waxes, as well as its dermal peel permeability to atrazine and malathion.

Materials and Methods

Sparganium americanum were collected from unamended ponds at the University of Mississippi Field Station (Abbeville, MS), transported to the USDA Agricultural Research Service's National Sedimentation Laboratory in Oxford, MS, and allowed to equilibrate in individual outdoor mesocosms (378 L) for 6 weeks before experimentation. To quantify various lipid contents, plantlets were collected in early summer (June; 25°C) and late summer (August; 38°C). Plants were sectioned to separate above- and below-ground tissue. Leaf trimmings were then chemically fixed according to a modified PEM fixation buffer (Cold Spring Harbor Laboratory 2009) in 3.7% (w/v) paraformaldehyde in buffer (3 mM egtazic acid, 50 mM 1,4-piperazinediethanesulfonic acid, 25 mM potassium chloride, 0.5 mM magnesium sulfate) and stored in 50 mL conical tubes at 5°C.

Samples were hand-sectioned with a razor blade (1 mm thickness) and stained for light and fluorescence microscopy. Sudan IV, Nile Blue A, and phloroglucinol stain were used to determine the nature of lipids in different sections of *S. americanum* leaves. Identification of wax substances (e.g., cutin and suberin) was achieved by incubating sections in 0.1% (w/v) Sudan IV (Sigma, USA) and 60% (v/v) ethanol for 30 min in glass dishes (Buda et al. 2009). Sections then were rinsed in 50.0% (v/v) ethanol with no destaining period. To identify natural lipid components, sections were incubated in aqueous 1.0% (w/v) Nile Blue A for 30 s and rinsed in double distilled water with no destaining period (Gahan 1984). To stain lignin under light microscopy and suberin under fluorescent microscopy, sections were incubated in 1.0% (w/v) phloroglucinol in 20.0% (v/v) hydrochloric acid for 10 min and rinsed in double distilled water for 5 min. Suberin was visualized using fluorescent microscopy under phloroglucinol quenching. Excitation was at 340–380 nm (Biggs 1984; Rittinger et al. 1987). Sections were viewed on a model BX41 fluorescence microscope under ×400 magnification (Olympus, Tokyo), and images captured using Spot 4.0.9 software (Diagnostic Instruments, Sterling Heights).

Vanillin assays were used to quantify soluble lipids in leaves according to Bligh and Dyer (1959). Briefly, for the vanillin assay, 2 mL of leaves (measured using water displacement) that were completely submerged in the water column were cut and mixed in a blender with 40 mL methanol. Samples were then transferred to a beaker and 25 mL chloroform was added. The homogenate was separated into aliquots and dispensed into 100 replicate microcentrifuge tubes (500 µL each) and centrifuged at 10,000 rpm for 10 min at room temperature. Following a digestion step, samples were incubated in 3 mL vanillin reagent for 10 min. Absorbance was then measured using a UNICO S1000 spectrophotometer at 595 nm.

Epidermal peel permeability was determined by placing epidermal peels (GTAC 2018) directly between two 55 mm Teflon disks with a 6-mm opening. Each epidermal peel, 10 mm wide, was compressed between the two disks, allowing water to be pulled through the dermal peel with a vacuum pump. A 1 L combined pesticide stock solution of Atrazine 4L® (42.6% atrazine active ingredient; 10 µg L^{-1}) and Malathion 5EC® (57% malathion active ingredient; 27 µg L^{-1}) was established. From that atrazine–malathion stock solution, 50 mL were added to a glass filter bell and pulled through the epidermal peel via suction (14 kPa). Pesticide filtrates were collected in glass containers and immediately extracted and analyzed by gas chromatography according to Bennett et al. (2000) and Moore et al. (2013). Detection limits for atrazine and malathion were 1 µg L^{-1} and 17 mg kg^{-1} for water and plant, respectively.

Descriptive statistics were used to characterize total lipid, polar extractable, and no-polar extractable lipid concentrations. Student t tests were conducted using Microsoft Excel at an alpha level of 0.05.

Results and Discussion

Sudan IV binds to all lipids including oils, fats, and waxes on the plant (Refat et al. 2008), thus it specifically stains suberin (pink) and cutin (red) (Biggs 1984). Microscopy of *S. americanum* leaf sections revealed most were stained pink, indicating suberized tissue all the way to the epidermis (Fig. 1). Because lipids are sometimes difficult to detect with Sudan IV, other stains are necessary to study and confirm lipids in leaf components. Nile Blue A distinguishes between neutral lipids stained red such as triglycerides, cholesteryl esters, and steroids, while fatty acids, chromolipids, and phospholipids are stained blue (Cain 1947). Nile Blue A-stained *S. americanum* sections showed two different shades of blue, suggesting different kinds of acids in the leaf. Both cutin and suberin stained blue because of the fatty acids in their structures. Vascular tissue stained light blue with Nile Blue A, while similar tissue was darker blue in the cuticle and around the phloem and xylem (Fig. 2a). The same areas that stained suberin pink with Sudan IV were stained light blue with Nile Blue A, while the same cutin areas stained red by Sudan IV were stained dark blue with Nile Blue A (Fig. 2b).

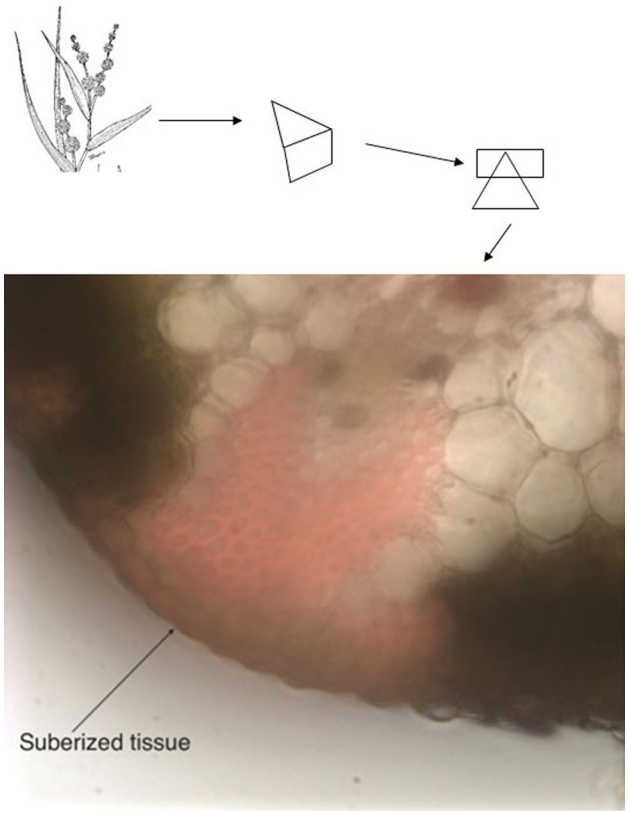

Fig. 1 Suberized tissue from leaf section collected in June of *S. americanum* Nutt. after staining with Sudan IV. Image is shown at ×400 magnification. Original plant sketch courtesy of USDA-NRCS *Wetland Flora: Field Office Illustrated Guide to Plant Species*

The definitive test for suberin uses epifluorescence microscopy to visualize leaf sections stained with phloroglucinol. Phloroglucinol stains lignin red under brightfield microscopy, and it stains suberin white blue under the fluorescent microscopy (Rittinger et al. 1987). *S. americanum* leaf sections did not stain on brightfield with phloroglucinol, suggesting the absence of lignin. Microscopy also revealed *S. americanum* leaf sections stained with phloroglucinol have large amounts of suberin in all the layers of the leaf (Fig. 3).

Sparganium americanum leaf sections had less cutin and large amounts of suberin. Both cutin and suberin increase the ability of the plant to control water and gaseous movement (Pollard et al. 2008). Suberin and cutin both have fatty acids and they bound to Nile Blue A. Phloroglucinol showed *S. americanum* leaves have many tissues suberized around the parenchyma, vascular, and epidermis cell walls. These suberized cells play an important role as a barrier to diffusion and to harmful solutes externally (Kolattukudy 1984).

Because plant composition may change along with environmental conditions, comparison of lipids during the season may detect the best condition for the plant to sorb pesticides. Suberin is nonpolar and gives the epidermis the ability to control the permeability of outside materials. Since *S. americanum* is an aquatic plant, it needs suberin to prevent water from entering the tissue. Suberin in the cell wall of the cuticle works as a diffusion barrier to the high concentration of water (Zee and O'Brien 1970). Suberin also controls transportation of water and nutrient in plant roots, protects a plant from pathogens, and alters the permeability of seeds and roots (Gou et al. 2009). The large amount of suberin in *S. americanum* may prevent plant leaves from sorbing and taking up various pesticides in water.

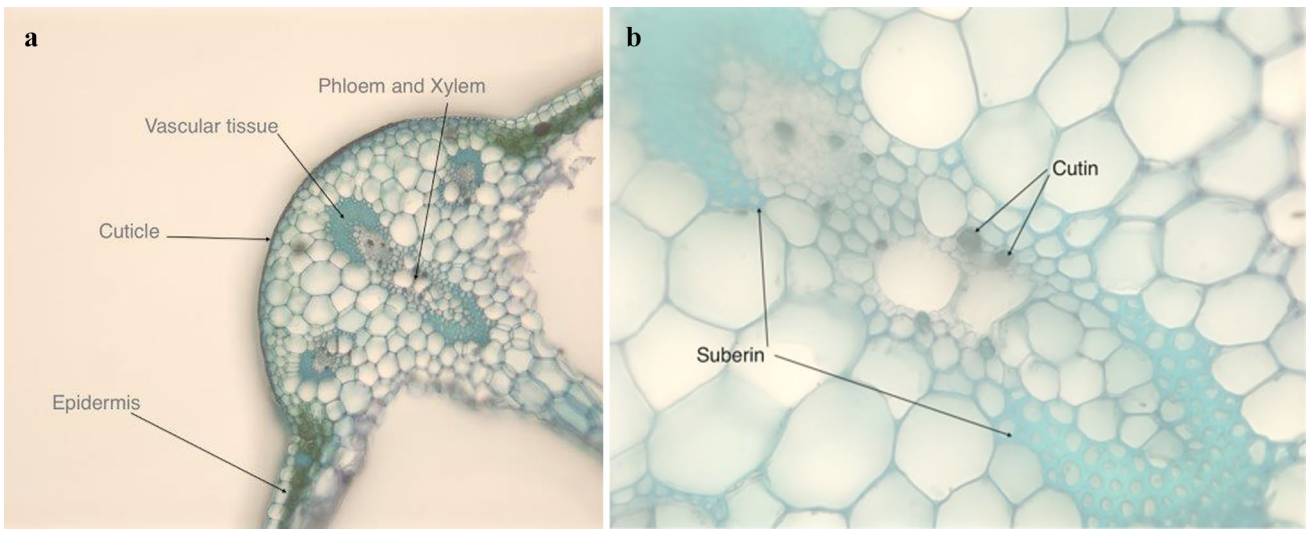

Fig. 2 *Sparganium americanum* Nutt. leaf section from early summer (June) stained with Nile Blue A. **a** Shown at ×100 magnification. **b** Shown at ×400 magnification

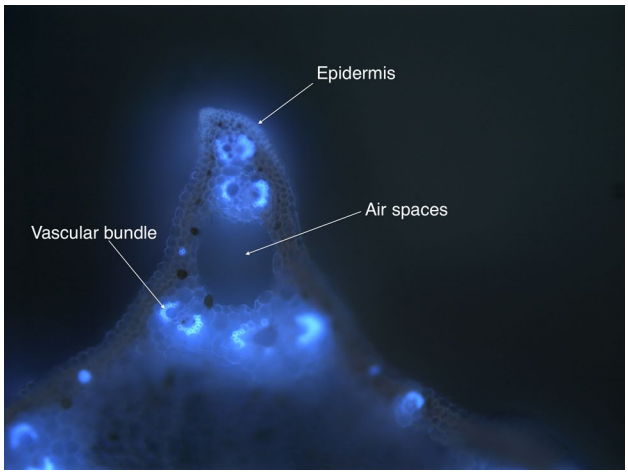

Fig. 3 *Sparganium americanum* Nutt. leaf section (collected in June) stained with phloroglucinol showing suberized vascular and epidermal tissue. Image is shown at ×100 magnification

Results of lipid analysis using the vanillin assay indicated the mean ± SD of total lipids in early summer (June) *S. americanum* leaves was 1.47 ± 0.83 mg mL^{-1}, which was significantly greater than that in late summer (August) (mean lipid content of 0.15 ± 0.36 mg mL^{-1}). Somerville (1991) found decreased total lipid content in *Arabidopsis* (rockcress) at high temperatures. With a decrease in total lipids likely due to heat stress in the leaves, further examination of lipid polarity was conducted. However, difficulty with materials used to establish standard curves prevented calculation of polar and non-polar extractable lipid content. Alfonso et al. (2001) found when wheat leaves were exposed to high temperature, polar lipids increased to 80%–90% among all lipid classes. Since polar lipids are more permeable to water, plants, including *S.* americanum, may increase the amount of polar lipids in higher temperatures to increase the permeability and absorption of water. Plant survival at high temperatures is enhanced with low levels of fatty acids (Grover et al. 2000). This suggests *S. americanum* lost some of the nonpolar lipids in heat stress to enhance survival. These lipid contents confirmed previously discussed microscopy data from *S. americanum* leaves indicating the non-polarity of most of the suberized tissue.

Epidermal peel permeability results indicated > 99% of applied atrazine concentrations were detected in the filtrate, and atrazine was above detection limits in only 3 of the 50 epidermal tissue samples. For malathion, although only 84% of the applied pesticide was measured in the filtrate, all 50 epidermal tissue samples were below detection limits. Because of the extremely small amount of tissue being analyzed in individual samples (2–10 mg), detection limits likely prevented any meaningful pesticide measurement in plant samples. Atrazine and malathion were not sorbed to the plant material most likely because of the abundance of suberin in the plant tissue. Therefore, the majority of their detection was in the filtrate.

This study highlights the need for plant chemical composition data to accompany phytoremediation studies. Based on the results of the presented study, *S. americanum* does not appear to be a strong candidate for atrazine and malathion sorption by leaf material, due to its structural and chemical composition. Pesticides may enter aquatic plants through different organs, and research such as that presented here, can help address potential problems with absorption. With improved knowledge of the chemical structure of aquatic plants, resource conservationists and farm managers can better plan mitigation strategies for pesticides in agricultural runoff.

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