

# Inhibition of Onion Germination and Root Growth by Cattail Extracts Involves Reduced Cell Proliferation and Disruption of Cell Wall Integrity

MARIA T. GALLARDO-WILLIAMS<sup>1,2</sup>, REBECCA L. BARTON<sup>1</sup>, PATRICIA M. DOORIS<sup>3</sup> AND DEAN F. MARTIN<sup>1,4</sup>

## ABSTRACT

The effects of cattail (*Typha domingensis* P.) aqueous extracts on germination of onion (*Allium cepa* L.) seeds and development of onion roots were studied using a standard germination bioassay and microscopic examination. Cattail extracts delayed and inhibited growth of onion roots and induced inversion in a significant percentage of germinating seedlings. Inhibitory effects correlated well with organic carbon content of the extracts. Aqueous extracts of cattail roots were more inhibitory than aqueous extracts of stem materials, which in turn were more inhibitory than extracts of cattail leaves. When actively growing onion roots were exposed to cattail extracts changes observed included loss of root caps, intense desiccation, reduced number of mitotic figures, impaired cell proliferation, and disruption of the cell wall integrity.

**Key words:** Bioassays, colchicine, mitotic figures, nocodazole, phytotoxins, *Allium cepa*, *Typha domingensis*.

## INTRODUCTION

In Florida public waters, cattails including *Typha domingensis*, are the most dominant emergent aquatic plant species (Schardt 1997). Several factors allow cattails to accomplish this opportunistic expansion, including size, growth habit, adaptability to changes in their surroundings, and the release of compounds that can prevent the growth of other species.

We have been concerned with the effects of cattail on other wetland species and with the determination of the chemical compounds that cattails release into surrounding waters. Cattail phytotoxins include fatty acids (linoleic and  $\alpha$ -linolenic acid) and phenolic compounds (caffeic, *p*-coumaric and gallic acid) (Gallardo-Williams et al. 2002). Both extracts and phytotoxins isolated from extracts have the potential to inhibit the growth and chlorophyll production of several species. Prindle and Martin (1996) found that aqueous extracts from different portions of cattail inhibited growth of lettuce

and radish seeds; the same extracts also inhibited oxygen production by *Lyngbya majuscula* (Prindle and Martin 1996). We have also shown that aqueous cattail extracts inhibit the growth of salvinia (*Salvinia minima* Baker) (Gallardo et al. 1998) and reduce its rate of oxygen production (Gallardo et al. 1999). A short-term exposure bioassay showed that oxygen production by *Valisneria americana* Michx., *Elodea canadensis* Rich., and *Myriophyllum spicatum* L. was also inhibited by exposure to aqueous cattail extracts (Ambrogio et al. 2000). A closely related cattail species (*T. latifolia*), which produces similar phytotoxins, has been shown to inhibit algal growth *in vitro* (Aliotta et al. 1990).

We have reached a certain understanding of the relationship between extract concentration and the toxicity of the resulting compounds. In order to further clarify the mode of action of cattail-derived substances on ecological targets, we investigated the cellular effects after exposing germinating roots to cattail extracts.

Microscopy studies have been a very useful tool to elucidate the mode of action of natural toxins at the cellular level. Comparison of affected and unaffected tissue can easily reveal subtle changes, including loss of membrane integrity, changes in starch allocation, degradation of ribosomes, and protrusion of mitochondrial membranes (Dooris et al. 1988, Kupidowska et al. 1994).

A limited number of sensitive, diagnostic bioassays are available to characterize and define the mode of action of compounds produced by aquatic weeds. In this study, we present our results of the inhibition of germination of onion seeds by cattail aqueous extracts, as well as the effects of cattail extracts on developing onion roots at the cellular level.

## MATERIALS AND METHODS

**Plant material.** Mature cattail samples were taken from a storm-water ditch near the University of South Florida campus in Tampa, FL and were identified by Dr. Richard Wunderlin, USF Herbarium, as *T. domingensis*. Samples were rinsed with deionized water and maintained in half-strength Hoagland's medium (Steward and Elliston, 1973) in a controlled environment Phytotron room (Environmental Growth Chambers, Chagrin Falls, OH) for 1.0 month prior to use. Conditions in the growth chamber were constant with a temperature of 26C, 12-hour photoperiod with a light intensity of 190  $\mu$ E per m<sup>2</sup> per sec at floor level, and 80% relative humidity. Plants were set in plastic trays with dimensions of 59 cm in length by 40.5 cm in width by 17 cm in height with acid-washed sand as a support substrate.

<sup>1</sup>Institute for Environmental Studies, Department of Chemistry, University of South Florida, Tampa, FL 33620.

<sup>2</sup>Current address: Laboratory of Molecular Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.

<sup>3</sup>Environmental Section, Southwest Florida Water Management District, 2379 Broad Street, Brooksville, FL 34604.

<sup>4</sup>Corresponding author. Received for publication March 16, 2002 and in revised form May 23, 2002.

*Cattail extracts.* Mature cattail plants were harvested from the growth chamber and divided into three sections of roots, stems (from immediately above the root to where the leaves began to separate), and leaves. Sections were weighed and extracted with deionized water (10 ml per 1.0 g of fresh material) for 3 minutes at room temperature using a Hamilton Beach blender. The resulting crude extracts were then filtered using Whatman no. 1 filter paper, filter-sterilized, placed in a refrigerator at 0 to 4C, and used within 2 weeks.

*Determination of Organic Carbon.* Organic carbon analyses of the aqueous root, stem, and leaf extracts were performed using a Dohrmann (model DC-180) automated carbon analyzer (Rosemount Analytical, Inc., Cincinnati, OH). Calibration was accomplished by using a 2000-ppm stock solution of potassium hydrogen phthalate (J.T. Baker Chemical Co., Phillipsburg, NJ) in deionized water. Calibration standards were made from serial dilutions of the stock solution, and then extracts were analyzed for organic carbon concentration.

*Bioassays.* Germination inhibition bioassays were performed using onion seeds (var. White Lisbon Bunching, NK Lawn & Garden Co., Chattanooga, TN). Seeds were disinfected by rinsing in a 5% solution of commercial bleach for 1 minute, followed by 5% Alconox powder detergent (Alconox, Inc., New York, NY) in water for 1 minute. Seeds were rinsed with distilled water until no residual foam was observed. Ten seeds were placed on each well of a 3-cm diameter well tray fitted with sterile disks of Whatman no. 1 filter paper. Aseptic conditions were maintained at all times. Each well of the well tray received 1.5 ml of distilled water dispensed with a sterile pipette; 0.5 ml of the cattail extract to be tested was then added to the well. Each extract was replicated twice in a randomized design. Each of three control wells in each tray received 0.5 ml of distilled water. Trays were then wrapped in clear plastic and kept under constant cool white light at 170  $\mu$ E per m<sup>2</sup> per sec. Trays were aerated and moisture was replenished every other day. The number of germinated seeds and orientation of the seedlings was recorded on a daily basis.

*Onion root preparation.* The dried outer layer and one fleshy layer of onion bulblets (Carolina Biological Supply, Burlington, NC) were removed and the dried roots were trimmed. Prepared bulblets were placed on filter paper moistened with distilled water in a small plastic cup and maintained in the dark. Containers were left undisturbed for 24 hours for control roots, and then the roots were harvested and stained. Treated roots received 2 ml of a concentrated (approx. 600 mg/L total organic carbon) cattail root extract after 12 hours of pre-sprouting time and maintained for an additional 12 hours. Reference samples were pre-sprouted for 12 hours and then treated with 10-ppm solutions of either

colchicine or nocodazole (Sigma Chemical Co., St. Louis, MO). Harvested roots were observed using a dissection microscope to assess the presence of root caps, and then roots were fixed as a squash preparation (Gray 1954). Number of mitotic figures for each preparation was determined utilizing a light microscope (five fields counted per slide). Control and treatments were done in triplicate.

*Determination of mitotic figures.* Onion root tips were transferred with forceps to a small beaker containing 5 ml of 18% (6 M) HCl for 4 minutes, then were transferred to another small beaker containing 5 ml of Carnoy fixative (1:3 acetic acid:ethanol) for 4 minutes. Root tips were transferred to a microscope slide and 2 mm of the root tip was sectioned using a dissection knife. The remainder of the root was then discarded. The root tip was treated with a drop of toluidine blue solution (Carolina Biological Supply, Burlington, NC) for 2.0 minutes. Excess stain was then blotted with a paper towel and the root tip was treated with a drop of deionized water. A coverslip was then lowered onto the root tip and firmly pressed in order to spread the cells into a single layer. Three fields were counted for each slide using a Vanox Olympus microscope at 40 $\times$  magnification. The total number of cells and the number of cells in any state of mitosis (Clowes and Juniper 1968) was determined, then the percentage of cells undergoing mitosis was calculated.

## RESULTS AND DISCUSSION

When aqueous extracts of cattail fractions were used to imbibe onion seeds, inhibition of the germination process was obvious within 24 hours. Seeds in the control wells swelled and the sheaths broke open, but seeds in the presence of cattail extracts remained undisturbed during this critical period and did not begin germination until 48 h later. Hindrance of normal development was also observed; browning of the root tips and abnormal (inverted) cotyledon orientation were present in most seedlings exposed to cattail fraction extracts (Table 1). Inhibitory effects were most noticeable when seedlings were treated with root extract; only 30% overall germination was observed, and 67% of the seedlings were inverted. Stem and leaves extracts also inhibited growth and produced inversion in some seedlings.

In order to unify the criteria for bioassays, it is important to use a standardized measurement of the concentration of the materials being tested. Organic carbon content was used throughout the study as a reference for extract concentration. The organic carbon content of cattail extracts correlated in a general way with the observed inhibitory effects. The root fraction was the most inhibitory of the extracts and had

TABLE 1. ONION GERMINATION BIOASSAY RESULTS USING CATTAIL (*TYPHA DOMINGENSIS*) EXTRACTS.

Fraction	Total organic carbon (ppm)	% Germination (168 h) <sup>a</sup>	Observation
Distilled water (control)		96.7 $\pm$ 3.3	normal (expected coloration and orientation)
Cattail root	616.5 $\pm$ 0.7	30.0 $\pm$ 6.7 <sup>b</sup>	brown root tips, 66.7% inverted hypocotyls
Cattail stem	289.9 $\pm$ 1.7	46.7 $\pm$ 8.8 <sup>b</sup>	brown root tips, 62.5% inverted hypocotyls
Cattail leaves	129.4 $\pm$ 1.3	76.7 $\pm$ 3.3 <sup>b</sup>	brown root tips, 71.4% inverted hypocotyls

<sup>a</sup>Mean value  $\pm$  standard deviation.

<sup>b</sup>Germination values are statistically different from the control at the 95% confidence level according to Student's *t*-test, unless otherwise indicated.

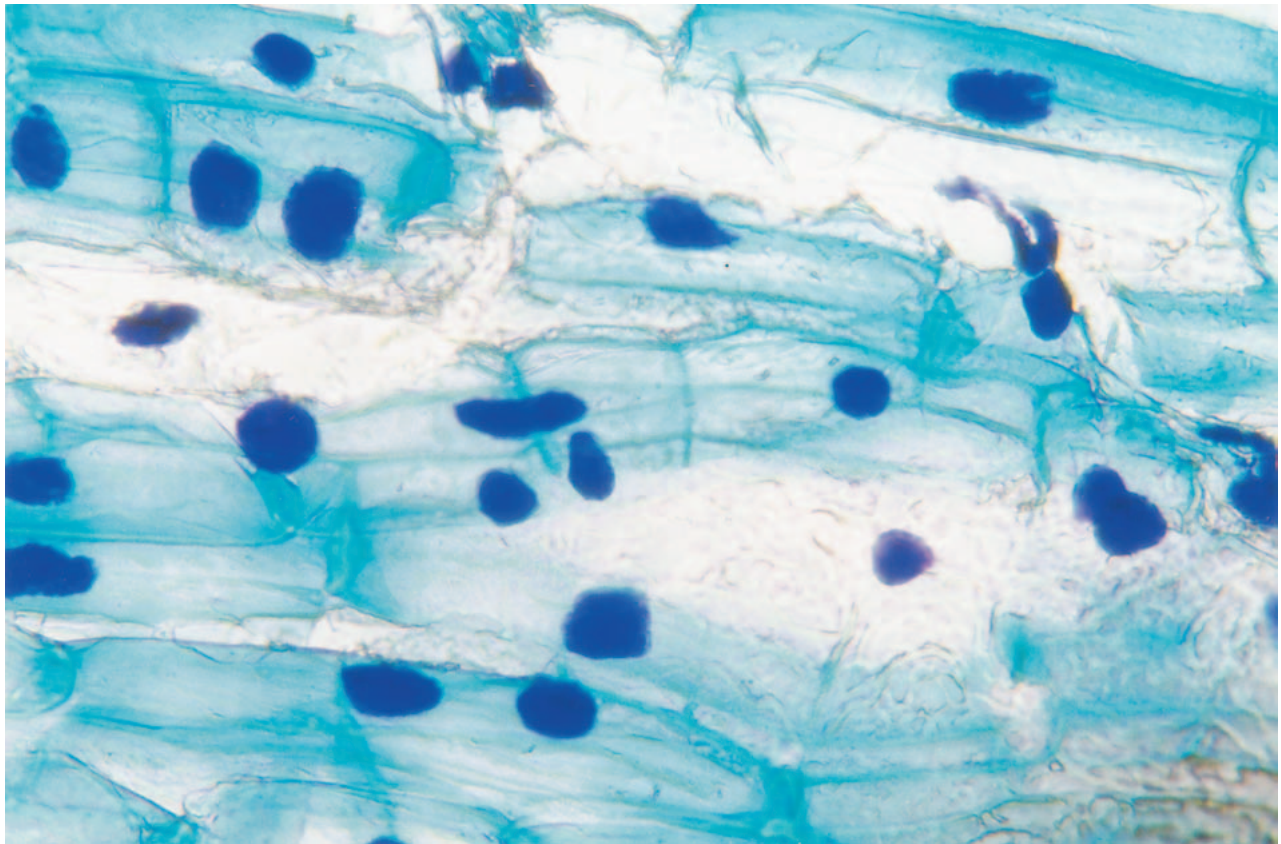
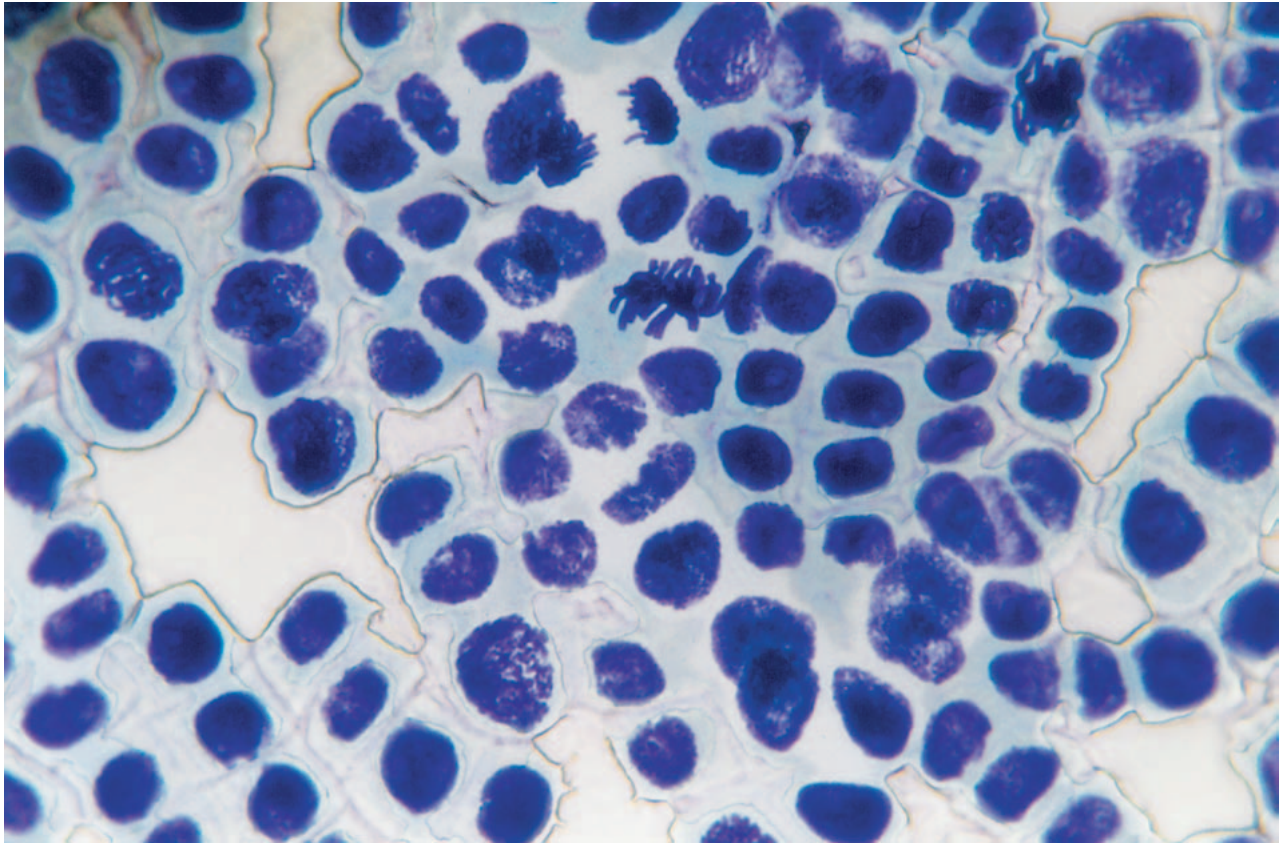


Figure 1a (top). Onion root tip squash preparation after 24 h in deionized water. 1b (bottom). Onion root tip squash preparation after 12 h in deionized water and 12 h exposed to cattail root extract (518 ppm).

TABLE 2. EFFECT OF EXPOSURE OF ONION ROOTS TO CATTAIL EXTRACTS, COLCHICINE AND NOCODAZOLE. ROOT LENGTH AND MITOTIC FIGURES.<sup>a</sup>

Sample	Average root length (cm)	Average cells per field	Mitotic figures (%)
Control (d.i. water)	3.2 ± 0.7	167 ± 16 <sup>b</sup>	9.9 ± 2.5
Cattail root (518 ppm)	1.4 ± 0.3 <sup>b</sup>	69 ± 21 <sup>b</sup>	1.6 ± 1.1 <sup>b</sup>
Colchicine (10 ppm)	1.7 ± 0.6 <sup>b</sup>	150 ± 14	2.4 ± 0.9 <sup>b</sup>
Nocodazole (10 ppm)	1.5 ± 0.7 <sup>b</sup>	109 ± 13 <sup>b</sup>	1 ± 0.9 <sup>b</sup>

<sup>a</sup>Values refer to mean ± standard deviation.

<sup>b</sup>Values are statistically different from the control at the 95% confidence level according to Student's *t*-test unless otherwise indicated.

the highest organic carbon content. The stem fraction was less inhibitory and had a lower organic carbon content, while extracts from leaves had the lowest inhibitory activity, and the lowest organic carbon content.

Onion roots were soaked in deionized water for 12 hours then exposed to cattail root extracts for 12 hours to assess effects of cattail extracts at the cellular level. Roots were measured after 12 hours and the root tips were fixed as a squash preparation. The same procedure was followed for control samples (exposed to deionized water) and for reference samples (treated with 10-ppm solutions of either colchicine or nocodazole; (Alberts et al. 1989)). The number of cells undergoing mitosis in each treatment group was determined.

Colchicine is a well-known mitotic poison that prevents the formation of spindle fibers and inhibits the progress of the mitotic cycle (Clowes and Juniper 1968). Colchicine, nocodazole, and certain alkaloids are mitotic inhibitors for most animal and plant cells, and cause arrest during metaphase. Chromosomes condense to their metaphase configuration, but spindle fibers do not form due to the ability of these drugs to affect the polymerization of tubulin, the protein that forms microtubules in cells (Altman and Katz 1976). Inhibition of microtubule polymerization can also be induced by low temperature or high pressure and is reversible; once the depolymerizing agents are removed, normal spindle formation occurs.

The results obtained are summarized (Table 2). We used onion roots because of the availability of data concerning their cellular structure. Onion roots have been used as a model for the study of phytotoxic activity in other similar systems (Kupidlowska et al. 1994). We found that cattail root extracts stunted the growth and development of onion roots. In addition, the percentage of cells undergoing mitosis was lower in root tips that were exposed to cattail extracts than control root tips.

A representative section of control and treated root tips is depicted in Figure 1. The control preparation shows normal cell shape and size; cell walls are intact, nuclei are properly differentiated, and several cells can be seen undergoing mitosis. Root tips exposed to cattail root extract have distorted and elongated cells, cell walls ruptured, and abnormal nuclei. Cells are swollen and no mitotic activity is visible. Disruption of proper cell wall function is thought to be the mode of action for long-chain fatty acids (Kanai and Kondo 1979), which can be used as abnormal building blocks by the proteins that weave the cell walls. Once in place, unsaturated fatty acids (or their *in situ* oxidized derivatives) weaken the cell wall and cause ruptures (Aliotta et al. 1990).

Exposure to cattail root extracts also inhibited root cap development. The root cap is vital for proper root development and plays a protective role (Clowes and Juniper 1968). Root tips exposed to cattail root extracts lacked root caps and were stunted with brown, desiccated tips.

Differences at the macroscopic level were evident between the roots exposed to the cattail root extracts and roots exposed to mitotic inhibitors. Roots treated with colchicine or nocodazole had intact root and root tips with normal coloration. Root growth was inhibited, but no desiccation of the surface of the root was observed. Cells of root tips exposed to colchicine or nocodazole were comparable in size to the deionized water controls; however, cells were more rounded in the colchicine or nocodazole-grown root tips than in control roots. A comparable rounding of other cells in the presence of colchicine has been documented, implying that microtubules play a role in the development of cell shape (Dyson 1978).

The effect of cattail root extracts on the percentage of mitotic figures is very similar to that of colchicine or nocodazole treatments (Table 2), but the mechanism for inhibition of mitosis may follow a different pathway, as indicated by differences in the cell wall, shape, and size after exposure to the different agents.

The use of onion seeds and roots as part of the study of the phytotoxicity of an aquatic species may seem limited in its ecological relevance. However, its well-established end points make it useful as a predictive tool. The effects of cattail root extracts on other seeds and roots may follow the trends found using this standard bioassay: delayed germination, intense desiccation, reduced cell proliferation, and disruption of cell wall integrity. Future work in this area should concentrate on the mechanistic pathways involved in cattail phytotoxicity and their ecological consequences.

## ACKNOWLEDGMENTS

We thank to Dr. Bruce Cowell and Dr. Renee Brooks, USF Department of Biology, for their helpful comments.

## LITERATURE CITED

- Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J. D. Watson. 1989. *Molecular Biology of the Cell*, 2nd ed. Garland Publishing, Inc. New York, NY. 1219 pp.
- Aliotta, G., M. Della Greca, P. Monaco, G. Pinto, A. Pollio and L. Previtara. 1990. *In vitro* algal growth inhibition by phytotoxins of *Typha latifolia*. *J. Chem. Ecol.* 16: 2637-2646.

- Altman, P.L. and D.D. Katz. 1976. Cell Biology. Federation of American Societies for Experimental Biology. Bethesda, MD. 454 pp.
- Ambrogio, D. M., M. T. Gallardo, R. F. Benson and D. F. Martin. 2000. Use of a computer-interfaced system for determination of oxygen production by selected aquatic weeds in the presence of cattail (*Typha domingensis*) extracts. Florida Scient. 63: 118-122.
- Clowes, F. A. L. and B. E. Juniper. 1968. Plant Cells. Blackwell Scientific Publications LTD. Oxford, England. 546 pp.
- Dooris, G. M., P. M. Dooris and D. F. Martin. 1988. Effect of a naturally occurring growth inhibitor on the ultrastructure of hydrilla. J. Aquat. Plant Manage. 26: 72-73.
- Dyson, R. D. 1978. Cell Biology: A Molecular Approach. Allyn and Bacon, Boston, MA.
- Gallardo, M. T., B. B. Martin and D. F. Martin. 1998. Inhibition of water fern *Salvinia minima* by cattail (*Typha domingensis*) extracts and by 2-chlorophenol and salicylaldehyde. J. Chem. Ecol. 24: 1483-1490.
- Gallardo, M. T., J. R. Ascher, M. J. Collier, B. B. Martin and D. F. Martin. 2000. Effect of cattail (*Typha domingensis*) extracts, leachates, and selected phenolic compounds on rates of oxygen production by salvinia (*Salvinia minima*). J. Aquat. Plant Manage. 37: 80-82.
- Gallardo-Williams, M. T., C. L. Geiger, J. A. Pidala and D. F. Martin. 2002. Essential fatty acids and phenolic acids from extracts and leachates of southern cattail (*Typha domingensis*). Phytochem. 59: 305-308.
- Gray, P. 1954. The Microtome's Formulary and Guide. The Blakiston Company, Inc., New York, NY.
- Kanai, K. and E. Kondo. 1979. Antibacterial and cytotoxic aspects of long-chain fatty acids as cell surface events: Selected topics. Japan J. Med. Sci. Biol. 32: 135-174.
- Kuplidlowska, E., K. Dobrzynska, E. Parys and A. M. Zobel. 1994. Effect of coumarin and xantotoxin on mitochondrial structure, oxygen uptake, and succinate dehydrogenase activity in onion root cells. J. Chem. Ecol. 20: 2471-2480.
- Prindle, V. and D. F. Martin. 1996. Allelopathic properties of cattails, *Typha domingensis*, in Hillsborough County, Florida. Florida Scient. 59: 155-162.
- Schardt, J. D. 1997. 1994 Florida Aquatic Plant Survey. Dominant Plants in Public Waters. Tech. Report #972-CGA. Bureau of Aquatic Plant Management. Florida Department of Natural Resources. Tallahassee, FL. 85 pp.
- Steward, K. K. and R. A. Elliston. 1973. Growth of hydrilla in solution culture at various nutrient levels. Florida Scient. 36: 228-233.