Evaluation of Sacred Lotus (*Nelumbo nucifera* Gaertn.) as an Alternative Crop for Phyto-remediation

by

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Abstract

Lotus, *Nelumbo nucifera,* offers a wide diversity of uses as ornamental, edible and medicinal plant. An opportunity for growing lotus as a crop in Alabama also has the potential for phyto-remediation. Lotus was evaluated for remediation of trace elements focusing on manganese (Mn), organic compounds targeting *s*-metolachlor and filtering aquaculture waste water. Lotus was evaluated for filtering trace elements by establishing a base line for tissue composition and evaluating lotus capacity to grow in solutions with high levels of Mn (0, 5, 10, 15, or 50 mg/L). Increasing Mn concentrations in solution induced a linear increase in lotus Mn leaf concentrations. Hyper-accumulation of Al and Fe was detected in the rhizomes, while Na hyper-accumulated in the petioles, all without visible signs of toxicity. Mn treatments applied to lotus affected chlorophyll content. For example, chlorophyll *a* content increased linearly over time while chlorophyll *b* decreased. Radical scavenging activity (DPPH) did not change over time but correlated with total phenols content, showing a linear decrease after 6 weeks of treatment. Ascorbic acid levels increased in response to lower Mn concentrations but at 50 mg/L or higher Mn treatment level, ascorbic acid levels were reduced.

Lotus was evaluated for its potential as a phyto-remediator of organic compounds by growing plants in different concentrations of *s*-metolachlor. In seedlings, biomass accumulation was affected by the treatments. However, membrane integrity, respiration, and photosynthesis were not affected by the treatments. Mature plants did not show symptoms of toxicity but chlorophyll (Chl) changes in content were related to *s*-metolachlor concentrations (Chl *a* content

increased while Chl *b* decreased). Potential *s*-metolachlor metabolites were found in rhizomes from treated seedlings. However, levels detected were too low to be identified. In addition, traces of *s*-metolachlor applied to seedlings were found in rhizomes, as well as, in leaves from treated mature plants. Lotus proved to be an effective phyto-remediator of nutrient run-off (NH_4^+, NO_3, P) and suspended solids from waste water from intense aquaculture systems.

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CHAPTER 1

REVIEW OF LITERATURE

1.1. Extension Programming for Introducing Lotus as a New Crop in Alabama

The Alabama Cooperative Extension System focuses on helping people and communities improving their quality of life and economic well-being by providing educational opportunities and information based on research based science. Part of the role of Extension is to select and study economically depressed communities and regions and identify or develop opportunities to assist the people in reversing and overcoming their depressed conditions (Whatley, 2011). In Alabama, there is an area known as the Black Belt Region and the term bears a definite association with the dark prairie soil of south central Alabama. This region features a humid mesothermal climate with an abundant rainfall averaging about 127 cm annually (Gibson, 1941). In the early days (1820 to 1830), the Black Belt emerged as the core of a rapidly expanding plantation area which make this one of the richest regions in the antebellum United States (Tullos, 2004). The plantation system of cotton culture proved an appropriate adjustment to nature's resources (Gibson, 1941). Late in the 18th century, the abolition of slavery and the displacement of the plantation aristocracy, soil erosion, the cotton boll weevil (*Anthonomus grandis* Boheman) invasion, and the failure to construct a vigorous, diversified economy, all combined to mire the southern Black Belt in a seemingly irreversible decline (Ransom and Sutch, 1972; Tullos, 2004). Commercial aquaculture is performed in several regions of Alabama, with much of the production concentrated in the Blackland Prairie region (Pine, 2008). Soils, terrain, climate and adequate water resources had allowed the establishment and expansion of aquaculture farms which provided a much need source of employment,

opportunity and income (Cline, 2011). In addition, the area has the land/water resources to support an expansion of the industry 10 times its current size (Crews and Chappell, 2007). As aquaculture production intensifies, feed inputs increase, and waste materials (e.g. organic matter, nutrients and suspended solids) in ponds increase and reduces oxygen, leading to an increase in eutrophication and turbidity in receiving waters (Lin and Yi, 2003). To increase production and sustainability, aquaculture should be integrated with other production systems (Sleeper, 2009). In Asia, a wide range of aquaculture systems are implemented to minimize environmental impacts and reuse pond effluents and bottom mud (Lin and Yi, 2003). Aquatic macrophytes may utilize reserve nutrients in mud either by rotation between two crops or co-culture with fish (Yi et al., 2002). Although in actual practice, fish and aquatic plants are rarely raised together in the same system, co-culture and rotated culture of lotus (*Nelumbo nucifera*) and fish has been practiced in China and Thailand for centuries (Guo, 2009; Yi et al., 2002). The lotus plant is an aquatic emergent angiosperm cultivated for thousands of years (La-ongsri et al., 2009). Lotus produces roots that are very popular as a vegetable (Billing and Biles, 2007). In addition, leaves, stems, seeds and other parts are edible and many organs of the plant are thought to have multiple medicinal properties (Ono et al., 2006). The flowers of lotus are highly appreciated as religious ornaments, with hundreds of ornamental cultivars with different plant sizes, flowers forms and colors (Wang and Zhang, 2004).

1.1.2. Natural History of Lotus

1.1.2.1. Origin and Geographical Distribution of Lotus

The name "Lotus" can be confusing because several plants have been known by this name. *Nelumbo nucifera* (Syn. *Nelumbium speciosum* Willd.) is known as sacred lotus, Indian

lotus, or Asian lotus. Other names includes lotus root (rhizome), East Indian lotus, Egyptian lotus; Lian, Lin ngau-China, Taiwan; Hasu -Japanese name for the plant, Renkon-edible storage rhizome in Japanese (Yamaguchi, 1990). The name sacred lotus of India is an appellation also applied by Egyptians to some species of water lilies. For instance, *Nymphaea caerulea* Sav. is known as the blue lotus of the Nile (Slocum and Robinson, 1996). There have been suggestions that lotus may have originated in India. However, Hsu Jin, a noted paleontologist, failed to find any petrified lotus seeds in India. The suggestion that *Nelumbo* might have originated in India came from J. Gaertner, a European botanist, who described the plant morphologically and named it. The name *Nelumbo* is the name of a place located in Sri Lanka, south of India, which might account for the idea that this plant originated there, consequently the common name "Indian lotus" emerged (Wang and Zhang, 2004). Japanese scientists consider the origin of lotus to be China (Xueming, 1987). Lotus has been known and cultivated for more than 5000 years (La-ongsri et al., 2009) and China is considered the main distribution and cultivation center of the plant (Wang and Zhang, 2004). This was confirmed by an archeological study and the use of C^{14} analysis, which demonstrated that Chinese history has been related to lotus for 7,000 years at least years (Xueming, 1987).

Lotus belongs to the Nelumbolaceae family and the genus *Nelumbo*. There are only two species in this genus: *N. nucifera* Gaertn. distributed in Asia and Oceania; *N. lutea* Willd. Is distributed in North and South America (Wang and Zhang, 2004). *N. lutea* Willd. (Syn. *N. pentapetala* or *Nelumbium luteum*), is known as American lotus, water-chinquapin, or yellow lotus (Sayre, 2004) and is native to the eastern and central portions of the US ranging from Maine to Wisconsin, and in the south, from Florida to Texas. It is also possible to find native stands in Mexico (Hernández et al., 1991) and northern South America (Wang and Zhang, 2004).

Small native populations can still be found in the West Indian Archipelago and the extreme southeastern portion of Ontario, Canada. In some areas, the natural environment for lotus is being destroyed, and the plant populations have dramatically decreased. The plant is listed as endangered in New Jersey and Pennsylvania, and threatened in Michigan and Delaware (Sayre, 2004). Sacred lotus wild distribution occurs from the Caspian Sea in the west to Japan in the east and from the Soviet Union in the north to the northern part of Australia (Xueming, 1987). Sacred lotus is mainly grown in China, Japan, India, Thailand, Sri Lanka, Philippines, and Indonesia (Hicks, 2005; Nguyen, 2001; Wang and Zhang, 2004).

1.1.2.2. Biology of the Species

N. lutea Willd. is a long-lived, aquatic perennial with cylindrical, spongy rhizomes that produce thick tubers at the end of the growing cycle in the Fall (Sayre, 2004). Some populations are found in headwater lakes of riparian corridors preferring water depths from 0.6 to 2.5 meters. Most plants establish themselves in more shallow water and subsequently grow out into deeper water. *N. lutea* blooms from June to September and the cream to yellow colored flowers open on a single stalk and range from 8 to 20 cms in diameter when fully open (Sayre, 2004). A single bloom lasts from 3 to 4 days. The seeds are actually located in the woody receptacle that looks like a showerhead. If a flower was successfully pollinated, the majority of the ovaries in the torus will contain a single round, developing seed (Sayre, 2004). The hard, brown seeds, about 1 cm in diameter can survive for long periods of time (Shen-Miller et al., 2002). Leaves are orbicular in shape with crenulate margins. They range from 20 to 60 cms diameter with the petiole attached to the leaf at the center (peltate). Leaves are slightly concave, giving the

impression of a shallow bowl. Leaves arise directly from the rhizome and can either be floating on the water surface or rise 30 to 45 cms above the water (Sayre, 2004).

N. nucifera is a perennial water plant which grows from a submerged, segmented root system which is composed of a branching system of horizontal stems. These stems (runners) spread rapidly during the growing season (Billing and Biles, 2007) growing at the bottom of ponds or slow-moving streams (Magness et al., 1971). Leaves are nearly round and large in diameter and the petioles are long and thorny. In shallow waters, depending on the cultivar, the leaves raise high (up to 2 meters) (Billing and Biles, 2007). Usually, the plant takes 4 to 6 months to bud, leaf out, flower, fruit, form a rhizome, mature and reach a dormant period (Xueming, 1987).

Most of the cultivars found in *N. lutea* and its crosses with *N. nucifera* are only used as ornamentals. Limited reports on lotus uses for food/medicine do not specify particular cultivars, and therefore it is assumed that those references refer to the species only. Asian lotus has been classified in different ways based on use, flower-type, form, and size (Billing and Biles, 2007). There are cultivars that show one or more characteristics, however, they are classified according to their strongest feature: seeds, flowering and rhizome lotus (Guo, 2009). Often, cultivars that are used for the production of rhizomes yield few flowers. On the other hand, cultivars used to produce seeds have no appreciable rhizome (Nguyen and Hicks, 2004) and in some cases, disbudding is use to stimulate rhizome production (Tian, 2008). In China, cultivars are classified based on 4 characteristics: plant height, flower diameter, flower form (single petal, semi-double or double flowers) or flower color. The considerable variation in flower color and shape has made lotus one of the most popular ornamental and cultivated plants in Asia (Masuda et al., 2006). In China, for example, there are 400 to 600 cultivars (Wang and Zhang, 2004). Several

countries have breeding programs aimed at developing cultivars with better rhizomes and/or greater seed production. The Chinese cultivar 'Oulian', has been specifically developed for producing high quality rhizomes with few flowers or seeds. 'Zilian' is another Chinese cultivar grown for seed production (Follett et al., 2003). In Australia, the Germplasm inventory for UWSH and Gosford HRAS reported the following cultivars as rhizome producers: 'Paradise', 'Quangdong', 'Green Jade', 'Damaojie', 'Zhouou', 'Paozi', 'Big Lying Dragon' and 'Bitchu'(Nguyen, 2001). The Auburn University lotus research project has a collection of more than 130 ornamental cultivars. Most of the cultivars are dwarf ornamental type but also some are Chinese cultivars selected for the production of seed and edible rhizomes: 'Space 36', 'E#2', '04-R-31', '04-R-07', 'Hunan', 'White Stamen Hunan', 'Red Flower Fujian', 'White Hunan', 'Hubei # 5', 'White Flower Fujian', 'Wuzhi # 2', 'Hainan', 'Big Lying Dragon' and 'Jianzuihonghua' (Orozco-Obando et al., 2010). The National Garden of Aquatic Vegetables in Wuhan, China, grows the largest collection of edible lotus in the world with more than 90 cultivars. Within the collection, they have more than 310 rhizome lotus containing 201 landraces and 109 breeding lines. In addition, they have 33 seed lotus accessions and 19 cultivars (Guo, 2009; Qingdong, 2005).

1.1.2.3. Cultural Influences

American Indian tribes treated native lotus as a sacred plant with mystical powers, The Comanche, Dakota, Huron, Meskwaki, Omaha and Potawatomi tribes used various parts of the lotus plant as a source of supplemental food (Sayre, 2004). Native people of eastern Canada (Ojibwa) used dried roots cooked with venison, corn, and beans, and also ate the seed roasted (Arnason et al., 1981). The Chinese have developed a romance with lotus flowers (Xueming,

1987); artists painted lotus and poets and scholars wrote poems and essays about the plant. The ancient people of Tibet and Nepal held lotus in veneration. An ancient prayer heard in Tibet and other Himalayan regions included the phrase "*Om mani padme hum,*" which can be translated as "Oh, the jewel in the heart of the lotus" (Slocum and Robinson, 1996). Hindus believe that Buddha was born in the lotus heart. From Indian culture came the Brahmin claim that from a *Nelumbo* mystic blossom sprang the absolute creator of the universe – Bahma. In Japan, lotus is considered the symbol of life because of its edible parts. In Japanese legends and religious developments of the past, the plant represented sincerity and nobility due in part to its annual emergence from still waters (Slocum and Robinson, 1996).

1.1.2.4. Plant Uses

Several organs of *N. lutea* are edible. Leaves and young stems can be eaten cooked. The large tuberous roots can be baked as potatoes while the leaves can be eaten like spinach. The root is rich in starch and when baked (after having been steeped in water to remove any bitterness) it becomes sweet and mealy, somewhat like a sweet potato. The hard seeds are gathered and eaten like nuts, added as a thickening to soups, or roasted like chestnuts (Sayre, 2004, Arnason et al., 1981).

N. nucifera is cultivated for edible rhizomes in China, Japan, Hawaii, India, and Korea (Hanelt, 2001). In Mexico (Rogers and Redding, 2003) and Australia, lotus is considered or grown as a non-traditional vegetable for the export market (Nguyen, 2001). Different edible cultivars show compositional variation. Xueming (1987) evaluated roots from 33 cultivars and reported the following constituents: 8 to 23 % starch, 1 to 5 % total sugars, 0.2 to 2 % reducing sugars, 2 to 3 % protein, 0.7 to 1 % free amino acids, 26 to 35 mg vitamin C, and 0.2 mg vitamin

B_6 per 100 grams of tissue. For seeds, he reported: 38 to 58 % starch, 8 to 19 % total sugars, 1 to 6 % reducing sugars, 2 % lipids and 17 to 25 % protein. Seeds can be dried and ground into flour for making bread. Edible oil can be extracted from the seeds (Nguyen, 2001). Flowers are fragrant and a very expensive perfume is extracted from it (Billing and Biles, 2007). They are popular as a cut flower (La-ongsri et al., 2009).The pods are quite striking and dried pods are used in silk flower arrangements and may be painted (Billing and Biles, 2007).

The starchy rhizomes are eaten roasted, as pickles or as dried slices, fried as chips or used for starch production. The acorn-like seeds are also an oriental delicacy. These are eaten raw, roasted, boiled, pickled, candied or ground as meal. The young leaves, leaf stalks and flowers are consumed as vegetables. In China, young folded emerging leaves are harvested and cooked as a vegetable. The rhizomes, leaves and flowers are also used in savory dishes. Flowers are used for the production of perfume (Hanelt, 2001). In Thailand, the red flower petals as well as the white ones are used to make lotus wine. Furthermore, petals are used to roll tobacco and make cigarettes. In China, fresh petals are dipped in eggs and deep fried. In Thailand, the sun dried fruit or torus (enlarged receptacle containing many embedded seeds) are used as fuel, burned as a mosquito repellent or dried, grounded and used as an additive to mosquito repellents (Chomchalow, 2007).

1.1.2.4.1. Medicinal and Nutritional Values

All parts of the lotus plant, in one form or another are used in Chinese medicine (Xueming, 1987). *Nelumbo nucifera* is considered an important traditional Chinese herb and all parts of it are used in medicine. A rhizome extract showed anti-diabetic (Mukherjee et al., 1997) and anti-obesity attributes (Ono et al., 2006). The leaves are known for their refrigerant,

astringent and diuretic actions leading to diverse applications such as using the leaves for diarrhea, high fever, hemorrhoids and leprosy (Lee et al., 2005; Nguyen, 1999). Koreans prepare traditional liquor (lotus liquor) from the blossoms and leaves that they have found to have antioxidant activities useful for reducing oxidative stress and the risk of chronic diseases (Lee et al., 2005).

Ripe lotus seeds also provide a spleen tonic (Follett et al., 2003) and are used for their astringent action in the treatment of chronic diarrhea (Nguyen, 1999). In many Asian states the seeds are used as an antidepressant and to inhibit inflammation (Bi et al., 2006). Lotus plumele, also known as Lian Fang, Lien Tze Hsin, or Lian Xu, is the green germ of the mature lotus seed that is rich in compounds such as alkaloids (demethylcolaurine, isoliensinine, liensinine, lotusine, methylocrypalline, neferine, nuciferine, and pronuciferine), flavonoids (galuteolin, hyperine and rutin) and some microelements (Zn, Fe, Ca, and Mg) (Bi et al., 2006). Seed extracts have shown hepatoprotective and free radical scavenging effects (Ono et al., 2006). The lotus plumule, removed from the ripe seed and sun-dried, is primarily used for nervous disorders, insomnia, high fevers with restlessness and hypertension (Nguyen, 1999). In Korea, nelumbinis semen or lotus seed is one of the most well-known traditional herbal medicines used to treat cardiovascular symptoms (Kim et al., 2006). The flower receptacles contain proteins, carbohydrates, and a small amount of the alkaloid, nelumbine, used to stop bleeding and to eliminate stagnated blood. The flower itself contains alkaloids (isoliensinine, lotusine, methylcorypalline and demethylcoclaurine) with vasodilating effects (referine) and antihypertensive and antiarrhymic abilities [liensinine] (Lee et al., 2005). The stamens assist consolidation of kidney function and are particularly useful in the treatment of male sexual disorders and female leucorrhea (Nguyen, 1999).

1.1.3. Cultivation

1.1.3.1. Sexual and Asexual Propagation

Lotus can be propagated via seeds or via underground stem division. The lotus seeds may survive for long periods of time (1,000 to 2,000 years). Their longevity can be attributed to the hard shells around the seeds, a nearly impermeable seed coat, small changes in the inner gas composition during long periods of storage, as well as, slow changes in the concentration of high dehydroascorbic acid (200 mg/100 g) content (Xueming, 1987). Seed propagation is mainly used in breeding new cultivars and commercially is unusual because seeds are highly heterozygous. In Australia, imported seeds present problems due to low viability. In addition, germinated seedlings do not produce a crop until the following season (Nguyen, 1999). In Thailand, micro-propagation is used to determine and compare efficacy of different irradiation methods to produce variegated phenotypes of lotus (Arunyanart and Soontronyatara, 2002).

Rhizomes enlarged in the previous year are usually used for commercial cultivation (Masuda et al., 2006) which is considered the best and simplest method to ensure a harvestable crop in one season. The enlarged rhizome found in *N. nucifera* acts as a dormant organ to aid in the survival of the plant under unfavorable circumstances. In China, propagation material (rhizomes) is classified in 3 categories: parent, son and grandson rhizomes and farmers use the entire parent rhizome (Guo, 2009). Rhizomes with a top bud, free of pest or diseases are selected as seed rhizomes (Wang and Zhang, 2004). Asexual propagation allows the original characteristics of the mother plant to be preserved, flowers to be enjoyed, and the lotus "root" to be harvested in the same season (Xueming, 1987). The rhizomes used for propagation must have at least two segments sealed at either end by an intact node and planting should be done before rhizomes break dormancy, because plants transplanted after rhizomes have begun growth do not

establish as well (Nguyen, 2001)**.** Although, rhizomes seem to be the preferred propagation method, sometimes apical buds or running stems are also used **(**Wang and Zhang, 2004). According to Nguyen (2001), the rhizomes are planted in saturated media at an angle of 15^{o} with the shoot meristem buried under 5 cm of media. Propagation material should be grown in separate ponds. At the end of each growing season it is recommended to save 20 percent of the rhizomes for next season (Nguyen, 1999). To fully utilize the land, Chinese farmers use three types of planting patterns: lotus followed by rice and/or other crops in the fall/winter. Lotus rhizomes are left in the field and other crops planted in the fall or winter in the same field (*Brassica* spp, *Spinacia oleracea* L.) and the third system is intercropping aquatic animals (fish, shrimp, crab) which are raised in the lotus field (Guo, 2009).

1.1.3.2. Soil Conditions

For production in containers and ponds, several substrates have been suggested; however, lotus prefers rich fertile soil. Lake or pond bottoms containing large amounts of organic matter are the most suitable (Xueming, 1987). Some commercial stores suggest the use of a heavy clay loam or soil formulated for the growth of aquatic plants. Slocum and Robinson (1996) recommended the use of a soil so heavy that it would not float which limits the use of most potting soil mixes found at garden centers. Shen-Miller et al. (2002) germinated hundreds-of-years-old seed in a 3:1 soil mix of clay soil and greenhouse soil composed of sphagnum moss, washed sand, and sandy loam in equal proportions. If the soil is heavy clay, roots cannot penetrate and harvesting is more difficult. Sandy soils lack binding sites for nutrients, and have been reported to induce a undesirable flavor on edible rhizomes (Nguyen, 2001). Optimal soil is a soft silt loam, free-form particulate matter (Nguyen and Hicks, 2004) and those soils with

correct organic matter (manure or well-rotted mulches) provide nutrients over an extended period, a buoyancy to texture, and good binding sites for nutrients (Nguyen, 2001).

1.1.3.3. Fertilization

Fields, lakes, ponds, or paddies deficient in fertility should be supplemented with various organic supplements such as an oil-press cake, or composted and green manure (Xueming, 1987). Well-rotted and composted cow manure can be used in the bottom-half if mixed one-part composted manure to two- or three-parts topsoil (Slocum and Robinson, 1996). In India, growing media are enriched by incorporating well-decomposed cattle manure at a rate of 5 kg/m^2, Neem (*Azadirachta indica*) cake (100 g/m^2), di-ammonium phosphate (25 g/ m^2) and muriate of potash (25 g/ m^2) 15 days prior to planting (Goel et al., 2001). In China, farmers use different combinations to fertilize fields. In some cases, fields are enriched by adding 45,000 to 60,000 kg/ha of animal waste. In other cases, they combine 1,500 kg of bean manure plus 15,000 kg animal waste per hectare. In other instances, they use 600 kg of special lotus formulated fertilizer combined with 375 kg of NH_4HCO_3 / ha. The correct amount of fertilizer is dictated by the maturity stage of the crop. For example, plants reaching maturity and rhizome formation required more potassium and less nitrogen (Nguyen, 2001). Fertilization of young plants has to be carefully administered because they can be easily burned. It is recommended that the doses be split in 3 to 4 applications (Nguyen, 2001). If seedlings are held for longer than 1 yr. in pots, controlled-release fertilizer can be used after new leaves emerge in the spring (Sayre, 2004). Manure proved fatal to young seedlings (Shen-Miller et al., 2002).

1.1.3.4. Container Management

Large lotus perform at their best in large boxes, e.g., Aqualite pool (120 X 95 X 30 cm) or Super Tub (90 X 60 X 20 cm) (Slocum and Robinson, 1996). At the National Botanical Research Institute, Lucknow, India, the lotus germplasm collection is successfully grown in concrete tanks with a clay soil stratum up to 45 cm thick at the bottom of the tank (Goel et al., 2001). Round containers are preferable for lotus production because the tubers and runners can jam up in the corners of square planters (Slocum and Robinson, 1996). Seeds or seedlings can be planted in pots without holes (Wang and Zhang, 2004). Researchers at Auburn University evaluated the effects of container soil volume on 3 cultivars of lotus ('Embolene', 'Garton #98' and 'Garton #1') and Native American lotus (*N. lutea* Willd). They found that electrical conductivity (EC), pH, plant growth indices, and plant nutritional content were influenced by the soil volume in the container (¼, ½, or ¾). Lotus planted in 29 liter containers filled ¼ with natural sandy loam soil (8 cm) were found to produce the largest number of propagules when compared to the other two treatments (½ or ¾), but no significant difference in emerging leaf numbers. Flower number decreased as soil level increased (Tian et al., 2009b).

1.1.3.5. Pond Management

Water quality and availability play an important role in the success of lotus cultivation (Nguyen, 2001). Although lotus grows best in acidic soil of pH 4.6 (Shen-Miller et al., 2002), growth is not affected by water with pH values ranging from 5.5 to 8.0. During a survey in Samaspur Lake, India, it was observed that lotus was growing luxuriantly despite an alkaline pH of 9.0 to 9.3 (Goel et al., 2001). Maintaining EC in a range of 2.8 to 3.1 mS cm, plants show a tolerance for some levels of salinity in the water (Nguyen, 2001).

Water management issues (acquisition, movement and storage) are primary considerations. This is a critical step in commercial production because once the pond is constructed; it can be difficult to change. Commercial producers should consider a deep reservoir on higher ground to supply the ponds by gravity, a water irrigation system, and a draining system (Nguyen, 2001). The site for a pond needs to be relatively flat, expansive, and close to a reliable source of a large volume of fresh water (Nguyen and Hicks, 2004). Water availability should be seriously considered because the size of the pond is predicated on the amount of water available. As a rule of thumb, a lotus crop requires 60,000,000 L of water per ha (Hicks and Haigh, 2001). The soil capacity to retain water is also very important. If the soil cannot retain water, the use of liners should be considered. The deepness of the pond should be carefully designed to prevent restriction of the rhizome's growth and the consequently rhizome deformation (Nguyen, 2001). Lotus is optimally grown in shallow ponds with a soil depth range of 20 to 30 cm to 1 meter (Nguyen and Hicks, 2004; Xueming, 1987) with a pond depth of 30 to 50 cm and the water level recommended to be set at 10 cm (Nguyen, 2001).

1.1.4. Vegetable Production

1.1.4.1. Field Production

Lotus growth can be affected by temperature, photoperiod, and altitude (Wang and Zhang, 2004). In China, large quantities of lotus are produced throughout the country from 19^{o}N to 47 ^{o}N and the growing area extends from the east coast to the Tian Mountains. In some areas, lotus is found growing as high as 2000 m above sea level (Follett et al., 2003). From rhizome to maturity, lotus requires at least 6 months with temperatures greater than 15 ^{o}C, but the maximum rhizome growth occurs at temperatures over 20 ^{o}C (Follett et al., 2003). At high cultivation

temperatures, more rhizome branches are produced (Nguyen, 2001) and flower development is stimulated (Follett et al., 2003). Long-day-lengths accelerate rhizome elongation and upright leaf production, and short-day-lengths promote rhizome enlargement and inhibits upright leaf production (Masuda et al., 2006).

Lotus grown in Europe and the United States are mainly used for ornamental purposes and rarely for food (Nguyen and Hicks, 2004). However, lotus has been grown in the Imperial valley of California and Yamaguchi (1990) believes that it can be successfully grown in the southeastern United States. In New Zealand, researchers have evaluated the commercial production of cultivars in an area south of Whangarei that is located at similar latitude to that of Ibaragi, the most important Japanese production area (Follett et al., 2003). In Europe, temperatures are too cold for lotus to bloom. However, blooms are produced in southern France, Spain, Portugal, Italy, Greece and some areas of former Yugoslavia (Slocum and Robinson, 1996). In China, Japan, Hawaii, India, and Korea, lotus is cultivated for its rhizomes (Hanelt, 2001). In Mexico (Rogers and Redding, 2003) and Australia (Nguyen, 2001), the crop has been explored to be grown as a new root crop for export markets.

1.1.4.2. Planting and Harvest

In Australia, certified rhizomes with 2 to 3 segments are planted early in the spring. The propagules are placed in the mud at an angle. Rhizomes are planted in rows spaced 1.8 to 2.7 m apart with 0.7 to 0.9 m between plants in a row. Seed rhizomes should be planted in a grid with an orientation so that the meristems are in the same direction of the pond within a row (Nguyen, 2001). In China, early maturing varieties are planted closer together than those that produce later

(Xueming, 1987). Plant density varies from 4115 to 7936 plants per hectare with an average of 4500 (Guo, 2009).

Harvesting time and methods depend on the environmental conditions, which vary depending on the cultivar and country where the crop is cultivated. In China, rhizomes are harvested from August to March. In Taiwan, harvest starts in June to November. In South Korea, the rhizomes are collected from August to December (Nguyen, 2001). In Japan, rhizomes are harvested from late-summer to the following spring (Masuda et al., 2006). At harvest, the water is drained and the rhizomes are dug. In Japan, harvesting is done by hand and the muddy roots are cleaned using water from wells. Some Japanese farmers practice mechanical harvesting using a backhoe with a specialized fork. This practice results in high wastage and damage to rhizomes. Also in Japan, high pressure water is used to wash away the mud and expose the rhizomes (Yamaguchi, 1990). The edible lotus rhizomes (60 to 120 cm long and 6 to 9 cm in diameter) have cream, light brown, or buff colored skin (Nguyen, 1999). They can be harvested after 120 days in warm climates and after 150 to 180 days or after the leaves die in cold climates (Yamaguchi, 1990).

Yields vary depending on the cultivar, location and cultivation practices. In Australia, yields from 8 to 40 tons/ha have been reported (Nguyen, 2001; Nguyen and Hicks, 2004). The Chinese have average productions of 22.5 to 45.0 tons/ha, and the South Korean reach productions of 31.8 tons/ha (Guo 2009; Nguyen, 2001).

1.1.4.3. Packaging and Postharvest Handling

After harvest rhizomes are washed and trimmed, soil residues, as well as lateral shoots removed. The highest grade rhizomes are cut in lengths of at least three segments. There are

four grades or classes including large, medium, small and second grade (Nguyen, 2001). The destiny of the product determines the type of packaging used. Japanese markets require the rhizomes to be packed in cartons of 1, 2, 4, 5, or 10 kg. Lotus rhizomes must be handled with care because they are easily bruised and physical damage results in an immediate purple discoloration. In Japan, market quality rhizomes have to meet specific color, size and flesh characteristics: skin color must be milky white, and 3 rhizome segments have to have a diameter larger than 4 cm, and the flesh water concentration must be high. Also, the texture has to be soft and crunchy (Nguyen, 1999).

Nguyen (2001) recommended the use of Styrofoam boxes with sealable lids to maintain high humidity and avoid bruising, and suggested the potential use of modified atmospheric packaging. At room-temperature the shelf-life of the rhizome can reach a maximum of 14 days (Tian, 2008). In Australia, fresh lotus rhizomes for the market that kept in a non-refrigerated environment only last 2 to 3 weeks (Nguyen, 2001). Lotus can be store at temperatures between 3 and 7 ºC. Lower temperatures induce chilling injury and surface damage. *Acacia* gum, sphagnum moss and Terra-Sorb® hydrogel have been tested to extend the shelf-life of the rhizomes. In his study, shelf life of the roots can be maintained for 45 days when stored at 5 ºC and 95 % relative humidity (Tian et al., 2008). High temperatures (>15 ºC) accelerate disease development (*Pseudomonas, Botrytis* and *Colletotrichum*), weight lost, and rhizome dormancy resulting in an increase in carbohydrate degradation (Nguyen, 2001). In addition, enzymatic activity accelerates with a consequent flesh-browning (Xin et al., 2002) and sugar degradation (Xiong et al., 2000).

1.1.4.4. Markets

Rhizomes are a food used extensively in China and Japan, sold whole or in cut pieces, fresh, frozen, or canned. Rhizome consumption in Japan is about 1% of all vegetables consumed annually. Although Japan grows its own, it still has to import 18,000 tons of lotus rhizomes each year, of which China provides 15,000 tons (Dharmananda, 2002). In 1995, Japan imported 1,347 tons of fresh and 14,887 tons of salted lotus rhizomes (Nguyen and Hicks, 2004). Commercial production in Australia is very small and Australian quarantine regulations do not permit the importation of fresh roots. Consequently, only frozen and dried lotus is available (Nguyen, 2001). In Taiwan, lotus is traded as both rhizomes and seeds. Seed trade is 5% of the entire industry, but the price is higher than that of rhizomes (Nguyen, 2001).

In the USA, despite the large Asian and Asian-American population, the potential demand for lotus is unknown. There have been reports of lotus imports from China to San Francisco. In 2001, a USDA study cleared the import of rhizomes from El Salvador, Honduras, and Nicaragua (Kahn and Lima, 2001). In 2003, fresh roots from Guatemala where allowed into the country (Rogers and Redding, 2003). Of the several countries in Asia where lotus is cultivated and consumed, the Japanese market seems to offer the best opportunities for U.S. export. Japan produces 70,000 tons annually valued at approximately $800 million (Nguyen and Hicks, 2004).

1.1.5. Cultivation of Lotus in Controlled Environments

Lotus is grown in greenhouses, raised beds, containers and ponds (Guo, 2009). Growing in glasshouses and in hot tunnels can ensure a year-round supply. Usually, the tunnels and glasshouses are erected over existing beds (Follett et al., 2003). Japanese farmers use

greenhouses for early production of rhizomes (Nguyen, 2001). In China, greenhouses are used to grow lotus and to produce blooms out of season (Wang and Zhang, 2004). Lotus is not used as much in the United States by gardeners as they are in many other countries however, it has a lot of potential (Tian et al., 2009a). Growth and performance of lotus is affected by genotype, media, water depth, light, planting time, and propagation methods (Tian et al., 2009a; 2009b). Japanese growers use greenhouses to force early commercial vegetable production (Nguyen, 2001) and the Chinese use greenhouses to force ornamental lotus to bloom on special occasions (Wang and Zhang, 2004). In addition, tunnel houses and under-glass production techniques are used to ensure year-round supply (Guo, 2009).

Nelumbo is classified as a long-day plant, thus long days or night-interrupted lighting affects growth and flowering. Long-day-lengths accelerate rhizome elongation and upright leaf production, and short-day-lengths promote rhizome enlargement and inhibits upright leaf production (Masuda et al., 2006). Chinese producers use 200 watt lamps to break dormancy (Zhen-Ming et al., 2006). In addition, Temperatures 22 to 28 ^{o}C stimulated bud germination and maintained vegetative and reproductive growth (Zhen-Ming et al., 2006). For proper development, lotus generally requires at least 6 months with temperatures higher than 15 ^{o}C for growth and flowering (Tian, 2008).

1.1.6. Evaluating How Plants Can Help to Clean Up Polluted Environments

A growing environmental concern in areas of intense agricultural production is the occurrence of soil and water contaminated with high concentrations of pesticides and nutrient runoff. In Western Europe, agriculture accounts for 37 to 82% of N and 27 to 38% of phosphorus (P) emissions into surface waters. In Denmark, studies indicated that 94% of the N

loading and 52% of the P loading arose primarily from agricultural activities (Smith et al., 2001), pesticides run-off used in urban areas used in urban , and from water treatment plants (Gerechke et al., 2002).

Plants have a natural ability to take up organic chemicals and metals from soil, sediment and water. Some of these materials are essential plant nutrients, while others have no known physiological function in plants (Arthur et al., 2005). This ability of plants to take up inorganic contaminants from soil (phyto-extraction) is becoming a widely-used remediation technology (Arthur et al., 2000). Depending on the degree of exposure and the physiological role of different parts of the plant, responses can differ (Ferrat et al., 2003). One possible solution for minimizing agricultural runoff impact is through the development of constructed wetlands (CWs) to replace lost edge-of-field and serve as buffers for runoff (Moore et al., 2001). These infrastructures can be enhanced using adequate vegetation (Brisson and Chazarenc, 2009). However, to find plant species that can tolerate mixtures and greater concentrations of pesticides in the soil is difficult (Arthur et al., 2000).

Plant species can be used as bio-indicators to determine the impact and progression of human activity on ecosystem vitality. Bio-indicators have the advantage of being very sensitive to environmental variations and react rapidly to the presence of pollutants (Ferrat et al., 2003). Macrophytes are used frequently to monitoring contamination and determine their capacity to accumulate a wide range of organic contaminants (Lovett-Doust et al., 1997) and trace metals (Paczkowska et al., 2007). Several of these compounds might be essential while others have no known physiological function in plants (Arthur et al., 2005). However, when herbicides, metals, or organic contaminants enter aquatic ecosystems, and eventually plant tissues, their accumulation might induce plant stress. A combination of plant and stress marker (biomarkers)

are helping scientists evaluate the pollution impact on ecosystems during light, thermal, herbicides, metals, and/or organic contaminant stress. Some of the most common biomarkers are based on measurable responses that occur in photosynthetic activity, secondary metabolite synthesis, enzymatic processes, oxidative stress and/or detoxification mechanisms (Ferrat et al., 2003).

1.1.6.1. Metolachlor as an Environmental Pollutant

Different studies evaluating US and Europe's ground water indicated that metolachlor concentrations are higher than those permitted by environmental legislation (Batista et al., 2002; Cerejeira et al., 2003; Guzzella et al., 2006; Tesoriero et al., 2007). In the US, estimated usage reaches 60 to 65 million pounds active ingredient per year (Rivard, 2003) and, metolachlor represents one of the most frequently detected herbicides contaminating shallow ground water beneath both agricultural and urban areas in concentrations of 0.05 µg/L (Kolpin et al., 2002). In 1991, approximately 56 tons of metolachlor were transported in the Mississippi River to the gulf of Mexico as runoff (Moore et al., 2001). In the Northern Chesapeake Bay, the annual mass loads for metolachlor were estimated at 1100 Kg/year, and it has been found in concentrations of 330 ng/L (Liu et al., 2002). Pesticides with solubilities higher than 10 mg/L can be transported in the water phase of runoff (Buttle, 1990). Metolachlor has a solubility of 530 mg/L (Wehtje, 2004), and is very mobile and its relatively high solubility allows it to move primarily in solution in surface run-off, with potential to leach to ground water (Rivard, 2003). In Greece, metolachlor presence in underground water was detected at concentrations ranging from 0.1 to 680 µg/ml (Patakioutas and Albanis, 2002). In Spain, concentrations of metolachlor (20 to 249 ng/L) were frequently found contaminating surface waters (Claver et al., 2006). The Swiss

quality goal for pesticides in surface waters is < 0.1 µg/L, and the concentration of metolachlor exceeds this quality goal (Gerechke et al., 2002).

Metolachlor is a member of the chloroacetanilide herbicide chemical family (i.e. acetochlor, alachlor, butachlor, butenachlor, metazachlor, propachlor, etc.). Metolachlor is used as a pre-emergent herbicide to control broadleaf weed species and annual grassy weeds (*Cyperus esculentus*, *Digitaria spp*, *Panicum dichotomiflorum*). Usually metolachlor is applied as a diluted emulsion to the soil surface to control weeds in variety of crops (corn, soybean, beans, potatoes, peanuts, sorghum, cotton, safflower, and woody ornamentals (Rivard, 2003). Metolachlor has been sold under different trade names (Codal, Dual, Dual 8E, Meteliachlor, Ontrack 8E, Pennant, Pennant 5G, Milocep), and it may be used in formulations with other pesticides (atrazine, cyanazine, and fluometruon). The Stalwart line of products from Sipcam Agro USA (Durham, NC, U.S.) are some of the more popular of these products in Missouri, but Me-Too-Lachlor and Me-Too-Lachlor II are also generic metolachlor products from Drexler. Stalwart is registered for use in cotton and soybean while Stalwart C and Stalwart Xtra (metolachlor + atrazine) are registered for use in corn. Me-Too-Lachlor is registered for use in cotton and soybeans while Me-Too-Lachlor II is registered for use in corn (Bradley and Kendig, 2005). These products behave differently and the doses used to control weeds differ according to the type of soil. For example, 2.12 L/ha Dual is recommended on coarse textured soils with less than 3% organic matter (OM), whereas 1.5L/ha of Dual II Magnum is recommended for the same soil type (Hartzler, 2000).

In the manufacturing process, chemists produce a product that contains only the active isomers, which allows farmers to reduce the amount of product required for weed control (Hartzler, 2000). Commercial brands containing the active ingredient metolachlor technically

contain a mixture of equal parts of two isomer pairs of metolachlor, commonly referred to as the R and S isomers. This was also the case with the metolachlor within the older Dual and Dual II products from Syngenta (Greensboro, NC, U.S.) but is not the case with Dual Magnum and Dual II Magnum. Dual Magnum and Dual II Magnum are enriched, or resolved, with the S-isomer of metolachlor, which has been demonstrated to be more biologically active than the earlier "mixed isomer" formulations (Bradley and Kendig, 2005). Unresolved formulations would contain approximately equal ratios of the S and R-isomers (Hartzler, 2004). Within the chloro acetanilide herbicides, metolachlor is the most persistence (Buttle, 1990).

1.1.7. Phyto-remediation of Organic Compounds (*S*-Metolachlor)

When *s*-metolachlor is sprayed on plants both root and shoot absorption occurs (Wehtje, 2004). Shoot tissue is generally the more sorptive, and it is also the site of herbicidal activity. Translocation occurs in both the xylem and phloem. When absorbed, metolachlor suppresses chlorophyll, protein, fatty acids and lipids, isoprenoids (including gibberellins) and flavonoids (including anthocyanins) synthesis (Rivard, 2003). Metolachlor metabolism in plants and animals proceeds through common Phase 1 intermediates and involves conjugation of the chloroacetyl side chain with glutathione, with subsequent conversion to the cysteine and thiolactic acid conjugates. Oxidation to the corresponding sulfoxide derivatives occurs with cleavage of the side chain ether group, followed by conjugation with glucose (EPA, 1998).

Lotus' massive biomass production, growth habit and the fast growth of lotus are attributes that can be used for phyto-remediation purposes (Goel, et al., 2001; Hicks, 2005; Tian, 2008). In a study at Edosakiiri Bay (Lake Kasumigaura, Japan), concentrations of the triazine herbicide Simetryn (Zhejiang Province Changxing First Chemical Co., Zhejiang, China) were

found in *N. nucifera* tissues. The simetryn residue concentration was highest in the lamina (300 µg/kg) among the various plant organs (Nohara and Iwakuma, 1996). Moore et al. (2001) evaluated the fate of metolachlor associated with simulated cropland runoff in constructed wetlands (CWs). Target concentrations were 73 µg/L and 147 µg/L. Total absorption of the total mass metolachlor to plant materials (primarily *Juncus sp*.) was estimated at 10 % and the absorption coefficients reached their greatest coefficient on day 35. Additionally, in wetlands with targeted concentrations of 73 µg/L, approximately 91 % of the aqueous metolachlor was taken up by the plant and transformed or degradated, while 16 % of the plant-associated pesticide was taken up. In wetlands targeted with metolachlor concentrations of 147 µg/ L, 87 % of the aqueous metolachlor was taken up by the plant. Additionally, 67 % of the plant-associated metolachlor was taken up.

1.1.8. Phyto-remediation of Heavy Metals and Nutrient Run-Off

The inherent remediation potential of plants could help nursery owners (Polomski et al., 2009; White et al., 2011) and small fish producers to transform constructed wetlands or retention ponds areas into viable production spaces in which marketable commodities could be produced (Yi et al., 2002), while maintaining the basic premise of using the CWs for improving water quality (Brisson and Chazarenc et al., 2009; Yang et al., 2008; Hong et al., 2001). Intensive aquaculture requires high fish stocking densities, large energy inputs, and intensive water quality monitoring and treatment (Lin et al., 2002). Recirculating systems are semi-closed systems in which water flowing through a series of tanks or raceways is captured, treated, and reused (Sindilariu et al., 2007). In intensive and semi-intensive aquaculture, high doses of fertilizers and feed are used in the confined waters of ponds and lakes. In the Southern United States,

catfish are fed during spring, summer, and early fall. Some farmers apply fertilizers to promote phytoplankton blooms to increase turbidity and prevent underwater macrophyte infestations. Fish assimilate less than 30% of the nitrogen supplied through feeding. Large quantities of nitrogen, phosphorus, and other elements accumulate in ponds. In Alabama most catfish ponds are not drained and the pond assimilates most of the nutrients (Boyd et al., 2000). It is common in other parts of the world for production ponds to be drained at harvest. This culture technique is a large constituent of water pollution in many developing countries (Lin et al., 2002). Discharged water from aquaculture production can lead to eutrophication of surrounding watersheds, ammonia toxicity, and increased BOD - Biological Oxygen Demand (Boyd and Queiroz, 2001; Ferdoushi et al., 2008; Neori et al., 1996). Discharged water from intensive systems has higher concentrations of nutrients and other pollutants than traditional culture techniques (Ferdoushi et al., 2008).

Today, the removal of nutrients before the water is discharged to streams in some cases is regulated by the government through the national pollutant discharge elimination system permit program - NPDES (Boyd et al., 2000). Bio and phyto-remediation are reasonable and economic ways to improve water quality and reduce the volume of pond effluents (Boyd, 2003). Bio-filtration can be accomplished in combination with other methods including physical filtration, chemical filtration and biological filtration. In aquaculture, biological filtration is very important because aquaculture waste water contains high amounts of ammonium (NH_4^+). This nitrogen (N) form is a product of protein catabolism excreted as NH_3 through fish gills and is also produced by aerobic and anaerobic transformations (ammonification) of fish feces and uneaten food. Ammonium is also converted to nitrites (NO_2^-) and further to nitrate (NO_3^-) by aerobic bacteria (nitrification). Nitrite toxicity is due to its diffusion across the fish gills and reacting

with iron or copper in the haemoglobin creating methaemoglobin that is unable to bind and transport oxygen, killing the fish (Snow and Ghaly, 2008). Increasing water quality parameters creates conditions favorable for nitrification, allows less water exchange and increased production efficiency. Vegetated CWs can potentially return water to the system for re-use and decrease the amount of discharged water entering surrounding watersheds. Plants produced can add extra income to the farmer's bottom line and also help to maintain desirable water quality for fish production.

In Alabama, pond culture of channel catfish (*Ictalurus punctatus*) results in effluent loss mainly from storm overflow in winter and early spring. Treatment of storm overflow by sedimentation is not feasible because settling basins with enough capacity to provide a hydraulic retention time of 8 hours would often be larger than production areas (Boyd and Queiroz, 2001). Development of CWs planted with macrophytes might allow filtration of large volumes of water. However, lotus potential for phytoremediation should be further evaluated (Goel et al., 2001; Hicks, 2005; Nguyen, 2001; Tian, 2008).

1.2. Heavy Metal Effect on Antioxidant Capacity of Lotus Leaves

Phyto-remediation of nutrients and heavy metals by lotus has been documented (Kumar et al., 2002; Kumar et al., 2008; Yi et al., 2002). For instance, high accumulations of cooper (Cu) and zinc (Zn) in lotus rhizomes (4,743 µg Cu and 519 µg Zn); fruiting torus (4,387 µg Cu and 612.5 µg Zn) and carpels (5,325 µg Cu and 600 µg Zn) per grams of dry weight were found (Kumar et al., 2002; Kumar et al., 2008). Furthermore, *Nelumbo* seedlings removed and accumulated large amounts of cadmium (Cd) and Cu as metal concentration (1, 2, 5, and 10 mg/L of Cd) increased and the exposure of the plants to the polluted solution increased (Mishra

et al., 2009). Lotus is a fast growing plant which has a high bio-mass production capacity (215 leaves/season per plant and a root/rhizome production of 40 t/ha (Nguyen, 2001), rhizomes of some cultivars are very rich in starch (Liangjun et al., 2006), and it has been reported to grow in contaminated areas.

Plants can absorb and accumulate inorganic contaminants, primarily metals, from polluted soil and water. Mature plants capable of accumulating high levels of metals can be harvested and a fraction of the metal contamination is reduced (Lasat, 2002). However, higher concentrations of heavy metals can induce abiotic stress (Guo et al., 2004). Most heavy metals are strong oxidants and may disturb cell metabolism by increasing the production of reactive oxygen species (ROS) (Gwóźdź et al., 1997). ROS include superoxide radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) which cause oxidative damage to lipids, DNA and disrupt cellular metabolism (Guo et al., 2004). H_2O_2 has a weak toxicity, however, in the presence of transition metals produces the hydroxyl radical (OH^-) which it is the most reactive active oxygen. Therefore, scavenging of H_2O_2 is essential to avoid oxidative damage of plant tissues (Sakihama et al., 2002).

Plants under heavy metal stress invest energy and other resources in their protective enzymatic and non-enzymatic antioxidant defenses (Matés and Sánchez-Jiménez, 1999; Posmyk et al., 2008). Oxidative stress results because the balance between the production of ROS and plant detoxification by the antioxidative system is altered (Kachout et al., 2009). Plant phenolic compounds such as flavonoids and vitamin C (ascorbate) are potent antioxidants. H_2O_2 is detoxified to water by the ascorbate-glutathione cycle. In the scavenging system ascorbate acts as the electron donor for ascorbate peroxidase to remove H_2O_2, and ascorbate is regenerated by monodehydro ascorbate reductase (Sakihama et al., 2002).

Manganese (Mn) when present at abnormally high levels in the substrate becomes cytotoxic and can cause injury to plants (Boojar and Goodarzi, 2007). The most well documented role of Mn is water splitting and O_2 evolution system in photosynthesis (Mengel and Kirkby, 1987; Lidon et al., 2004). Mn also takes part in respiration, protein synthesis, acyl lipids and carbohydrates (Boojar and Goodarzi, 2007). Absorption of Mn by plant organs depends on the ability of the plants to transfer the metal across the soil-root interface, and on the total and available amount of Mn in the soil (Boojar and Goodarzi, 2007). Mn generally tends to accumulate predominantly in the plant shoots, not in the roots (Page and Feller, 2005). At subcellular levels, excess Mn might be stored in vacuoles, cell walls and in chloroplast thylakoids. Mn toxicity results in oxidative stress and stimulates the production of reactive oxygen species (ROS) (Boojar and Goodarzi, 2007) as well as the production of free radicals (Gonzales et al., 1998). Polyphenols scavenge reactive free radicals such ROS and DPPH radicals (Cho et al., 2003). Manganese in excess can damage the photosynthetic apparatus (Millaleo et al., 2010) and can significantly decrease chlorophyll contents (Lei et al., 2007). In addition, excessive Mn concentrations in plant tissues can alter absorption, translocation and utilization of other elements (Ca, Mg, Fe and P) also causing oxidative stress (Millaleo et al., 2010). Several assays have been used to estimate antioxidant capacities in fruits and vegetables including 2,2-diphenyl-1-picrylhydrazyl -DPPH (Cho et al., 2003); total phenol content –TPC (Hu et al., 2002), ferric reducing ability of plasma –FRAP (Thaipong et al., 2006), and lipid peroxidation - MDA (Lei et al., 2007).

1.3. Central Theme of Research

Based on a review of the literature and research into production practices required for lotus, the environmental conditions for growing lotus in the southeastern USA are suitable. The Black Belt Region of Alabama has several advantages: soil, climate, water, and a consolidated aquaculture industry with big expansion potential, all of which offer great incentives for further exploration of lotus as an alternative crop. The natural occurring species (*N. lutea*) is a good illustration of the potential for growing related species and cultivars. In addition, the capacity of the land and water resources of the State of Alabama for supporting a potential expansion of 10 times the current aquaculture industry opens the possibility for economic development of alternative aquatic crops. Lotus' wide diversity of use as vegetable, medicinal, or ornamental purposes would suggest that there may be opportunities for growing this plant and supplying local demand, as well as, providing export opportunities. Therefore, lotus is considered a great candidate which should be evaluated. However, more research is needed on harvesting techniques, cultural practices, cultivar development, evaluation of the native species, marketing potential, economic analysis and development of alternative uses. In addition, it is important to assess bio-markers for lotus which will help to determine physiological stress induced by environmental pollution. Specific research should be implemented to determine the capacity of the lotus to absorb, accumulate and metabolize organic compounds and heavy metals.

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CHAPTER 2

PHYTO-REMEDIATION OF *S*-METOLACHLOR USING LOTUS (*NELUMBO NUCIFERA* GAERTN.)

2.1. ABSTRACT

Due to extensive use, solubility, and persistence, *s*-metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide] has the potential of accumulating and impacting surface and ground waters. Asian lotus might be a suitable candidate for the phytoremediation of *s*-metolachlor. Various bio-markers were evaluated to determine if the compound was taken up by the plant, how the compound affected the plant, and if it was absorbed and transformed. Lotus seedlings and mature plants were grown in concentrations of *s*-metolachlor ranging from 2 to more than 20 times the recommended rate for field applications. In seedlings, traces of *s*-metolachlor were found in the rhizomes. In one experiment, biomass was significantly reduced (24%) by the highest concentration. Despite those results, membrane integrity, respiration, and photosynthesis were not affected by the treatments. Mature plants did not show symptoms of toxicity. Chlorophyll (Chl) accumulation did behave contrary to what usually is observed in plants treated with *s*-metolachlor. However, the trend suggests that the changes were related to herbicide levels in solution. Vitamin C content responded quadratically to time and, in most cases, increased linearly in response to concentration. In the case of lipid peroxidation, simple effects of concentration showed an increase in MDA levels, especially as the exposure time increased (4 - 6 weeks). MDA showed a positive linear accumulation over concentration. MS/LC/HPLC analyses detected traces of the active ingredient in leaves and potential metabolites were detected in the rhizomes, but levels were too low to be conclusive.

2.2. INTRODUCTION

Landscape and agricultural operations use pesticides and fertilizers throughout the year. Due to overhead irrigation (the most widely used system in this industry), approximately 75% of irrigation water runs off carrying high concentrations of pesticide and nutrients (Stearman et al., 2003). Production conditions at container plant nurseries encourage the potential movement of herbicides in runoff (Briggs et al., 2002). Agrochemicals have been detected in surface waters and ground waters, and their toxic effects on aquatic organisms have been documented (Abrantes et al., 2006). *S*-metolachlor is a member of the chloroacetanilide herbicide chemical family and is one of the most extensively used chloroacetanilides for selective weed control in maize, sorghum, soybeans, peanuts, sugarcane, cotton, potatoes, vegetables, and woody ornamentals (Coleman et al., 2002). Current formulations of commercial *s*-metolachlor has different trade names (Codal, Dual, Dual 8E, Meteliachlor, Ontrack 8E, Pennant, Pennant 5G, Milocep) which are more biologically active than earlier "mixed isomer" formulations (Bradley and Kendig, 2005). *S*-metolachlor is absorbed by roots and shoots (Wehtje, 2004). Shoot tissues are generally more sorptive and it is also the site of herbicidal activity. Translocation occurs in both the xylem and phloem. However, the exact mode of action has not been clearly established as compared to other pesticides. When absorbed, *s*-metolachlor suppresses chlorophyll and protein synthesis, fatty acids, lipids, isoprenoids (e.g. gibberellins), and flavonoid (including anthocyanins) metabolism (Rivard, 2003).

S-metolachlor has the potential to leach into groundwater because of its high water solubility that reaches 550 mg/L at 20 ^{o}C (Dimou et al., 2005). Although *s*-metolachlor is not registered as an aquatic herbicide, it is capable of infiltrating ground and surface waters (Dimou et al., 2005; Jayasundera et al., 1999; Kolpin et al., 2002; Liu et al., 2002). In Switzerland, the

concentration of *s*-metolachlor in water exceeded the quality goal of < 0.1 µg/L (Gerechke et al., 2002). In the US, the current regulatory limit for metolachlor is 0.1 mg/L (MassDEP, 1993).

Presence of *s*-metolachlor in soil, water, and plant tissue depends on temperature, chemistry of this compound, absorption and metabolism within the plant (Rivard, 2003), and varies with exposed tissue, plant age, species, solar exposure, and time in solution (Graham et al., 1999). In corn (*Zea mays*) and soybean (*Glycine max*) seedlings, herbicide uptake is rapid, and detoxication results in small residues of the parent molecule at 12 days after application (Scarponi, 1992). In field trials, the half-life of *s*-metolachlor in maize seedlings is approximately 4.8 to 6.7 days in, and 13 to15 days in soil producing residues as low as 0.005 to 0.045 mg/Kg in the soil and may not be detectable in the seedling tissue (Cao et al., 2008). In aquatic systems, *s*-metolachlor half-life ranged from 33 to 42 days (Graham et al., 1999).

One possible solution to minimize *s*-metolachlor runoff impact is through the development of constructed wetlands (CWs) to replace lost edge-of-field and to serve as buffers for agricultural runoff (Moore et al., 2001; Rogers and Dunn, 1992). Phyto-remediation is a technology suitable for sites with limited contamination and moderately hydrophobic pollutants or log octanol/water partition coefficient (K_{ow}). Phyto-remediation is also suitable for short-chain aliphatic chemicals and nutrients. The octanol/water partition coefficient (Kow) is defined as the ratio of a chemical's concentration in the octanol phase to its concentration in the aqueous phase of a two-phase octanol/water system. The log K_{ow} value of *s*-metolachlor is 3.45 (Zheng and Cooper, 1996) at 25 ^{o}C and its water solubility is 488 mg/L which make it a good candidate for phyto-remediation (Kawahigashi, et al., 2006). To enhance CWs efficiency, underutilized plants should be evaluated. However, identification of plant species that can tolerate mixtures and greater concentrations of pesticides in the soil is difficult (Arthur et al., 2000).

Asian Lotus (*N. nucifera*) and its American counterpart (*N. lutea*) are aquatic, herbaceous perennials that are sturdy, easy to grow, and with ornamental value. Some cultivars have the capacity of producing up to 40 tons /ha of rhizomes (Nguyen and Hicks, 2004) and 1.5 tons/ha of seeds. The massive biomass production and growth habit are attributes of lotus that make it useful for phyto-remediation purposes (Goel, et al., 2001; Hicks, 2005). In Edosakiiri Bay (Lake Kasumigaura, Japan), the concentrations of the herbicide Simetryn (Shanghai Skyblue Chemical Co. LTD., Shanghai, China) in lotus leaves (300 μg Kg) were similar to those in water (Nohara and Iwakuma, 1996).

To use lotus or other potential plants for phytoremediation, a detailed knowledge concerning plant physiology, acceptable doses, and metabolism in vivo is required (Trapp and Karlson, 2001). Today, stress markers (biomarkers) are used by scientists to evaluate pollution impacts on ecosystems associated with herbicides, metals, and/or organic contaminants stress. Some of the most common biomarkers are based on measurable responses that occur in photosynthetic activity, secondary metabolite, oxidative stress and/or detoxification mechanisms (Ferrat et al., 2003). The environmental stress can trigger a cascade of dysfunctional physiological responses in plants like lipid peroxidation, cell membrane instability, a reduction of photosynthetic pigments, and a reduction of growth. These responses have to be quickly compensated for by repair mechanisms to ensure plasticity and development of tolerance against the stress factors. Oxidative stress stimulates the synthesis of reducing compounds (e.g. ascorbate, tocopherol, glutathione), and several enzymes that are crucial components of tolerance development to environmental stresses (Fodorpataki et al., 2009). Reduced forms of ascorbic acid, partly oxidized monodehydro-ascorbate, and oxidized dehydro-ascorbate play an important role in anti-oxidative defense (Fodorpataki et al., 2009) and are particularly effective against

toxic oxygen (O_2) species (Cakmak and Marschner, 1992). It has been proposed that the stimulation of ascorbic acid synthesis may enable some wetland plants used in phytoremediation to cope with herbicides.

The objectives of this study were to determine the effects of various *s*-metolachlor concentrations in the photosynthetic response (chlorophyll content, net photosynthesis, stomatal conductance), radical scavenging activity (DPPH, ABTS), ferric reducing capacity (FRAP), membrane integrity (electrolyte leakage, lipid peroxidation), and biomass accumulation (FW).

2.3. MATERIALS AND METHODS

Experiment I: On September 15, 2008, individual 3 week-old lotus seedlings with two floating fully developed leaves were randomly selected, rinsed, and planted in a mix of pine bark and sandy-clay-loam soil (50:50). Three liter black plastic containers without holes were used for the experiment. Less than a third of the container were covered with the substrate and the containers were located at the Paterson Greenhouse complex, Auburn, AL (Latitude=34.18 N, Longitude = 86.85 W, Elevation = 242 m, USDA Zone 8b). The volume of the solution was kept constant by adding water to compensate for water lost through plant transpiration and evaporation. Every 3 weeks, plants were fertilized with a 150 mg/L Nitrogen solution of soluble fertilizer. The fertilizer was 20-20-20 TotalGro™ (STD Industries, Winnsboro, La) and contained 44% P (P_2O_5), 83% K (K_2O), and the micronutrients content for B, Cu, Fe, Mn, Mb and Zn ranged from 0.001, to 0.1 mg/L. Temperature set points maintained the greenhouse environment at 25 °C (day) and 20 °C (night). After 10 days of acclimatization, seedlings were exposed (1 per container) to four treatment solutions of 0.0, 1.0, 2.0, or 4.0 mg/L of *s*-metolachlor (DualMagnum® 83.7% i.a. Syngenta, Greensboro, NC). These solutions were

higher than those applied at the recommended rate of 0.5 mg/L (Wehtje, G., personal communication). Containers were arranged in a completely randomized design experiment with 3 replications per treatment plus a control (no herbicide). Treatment solutions were replaced every 2 weeks. Plants were grown from September 15 to December 13 when plant growth ceased and plants became dormant. At harvest, rhizomes were hand harvested, rinsed with water and stored in a -85°C freezer for later analysis by gas chromatography (Agilent 6890 gas chromatograph [GC], Agilent Technologies, Inc. Wilmington, DE), HPLC/MS (Ultra Performance LC Systems [ACQUITY UPLC™, Waters Corp., Milford, MA, USA] coupled with a quadruple time-of-flight mass spectrometer [Q-TOF Premier, Waters] with electro spray ionization [ESI]), to determine tissue levels of *s*-metolachlor residues and its metabolites.

Experiment II: On September 23, 2009, 3 week-old lotus seedlings were planted in the same type of containers and grown hydroponically in the same fertilizer solution used in Experiment I. The pH of the solution ranged from 6.8 to 7.0. After 2 weeks acclimation period, the seedlings were exposed to 4 concentrations of 0, 1, 3 or 5 mg/L of *s*-metolachlor (October 8). Those concentrations were chosen to try to determine if higher levels of *s*-metolachlor in solution would induce a phyto-toxic response. Containers were arranged in a completely randomized design, and treatments were applied in a 2 x 4 factorial arrangement (2 harvest dates, 4 solution concentrations with 3 replications per treatment combination). Four leaves from each replication were randomly chosen and chlorophyll meter readings (CMR) were conducted (4n) with a transmittance-based chlorophyll content meter (SPAD-502 chlorophyll meter, Konica Minolta Sensing Corp. Japan) one week before each harvest. The day before harvest, light-saturated net CO_2 assimilation rate per unit leaf area (photosynthetic capacity, [P*n*]) and stomatal conductance to water (G_s) were measured with a portable infra-red gas analyzer (LI-6400, Li-

COR, Lincoln, Nebraska, U.S.) following procedures described by others (Kitajima et al., 2002). The CO_2 concentration of the reference air entering the leaf chamber was adjusted with a CO_2 mixer control unit so that the sample air exiting the chamber contained 400 mg/L of CO_2. This resulted in a CO_2 concentration of the reference air close to 380 mg/L. All gas exchange data were collected between 10:00 AM and 2:00 PM. Chamber temperature was set at ambient temperature (25 to 28 °C). Relative humidity of the reference air was kept as close to ambient as possible. Air-flow rate was 500 mL/min. Light with a photon flux density (PFD) of 1500 μmol · m^{-2} · s^{-1} was supplied with a red light emitting diodes (LI-6400-02). At the first harvest (October 22), leaf composite tissue samples were collected for electrolyte leakage (EL) measurements. Three samples from each replication (approximately 0.5 to 1.0 g) were taken with a cork borer and immerse in Mili-Q water to remove surface adhered electrolytes. Samples were gently blotted on paper towels to remove excess water. Excised leaf tissues were transferred to 20 mL scintillation vials and 25 mL of Mili-Q water was added. Tissues were incubated at 25 °C for 6h on a shaker (Barnstead Lab-Line Max-Q 2508, Thermo Fisher Scientific Inc. Pittsburgh, PA) and subsequent electrical conductivity was measured (C_1). Samples were then incubated at 90 °C for 2h in a water bath (Fisher Scientific model ISOTEMP 210. Dubuque, Iowa). Electrical conductivity was obtained after equilibrium at 25 °C (C_2). Percent electrolyte leakage was calculated using the following equation: (C_1/C_2) x 100. At harvest 2, biomass accumulation in terms of fresh weight (FW) was determined.

Experiment III: Dormant rhizomes from lotus cultivar 'Camellia Red' were harvested on February 18 and planted on March 03, 2010. A substrate blend consisting on 50:50 pine bark and top soil (47% sand, 40% silt and 13% clay with a loam texture) was used to fill (0.7 L) individual hole-less containers (10.7 L) for planting the rhizomes. Four weeks later plants were

fertilized on a weekly basis with 150 mg/L N solution of soluble fertilizer as in Experiment I. On June 25, plants were placed inside the greenhouse and the conditions were the same as experiment I. Plants were treated with 0, 5, 15, or 35 mg/L of *s*-metolachlor. Containers were arranged in a completely randomized experimental design and the treatments were applied in a 3 x 4 factorial treatment arrangement (3 destructive harvests, 4 treatments, 3 replications per treatment combination). At harvest, plant tissue samples (>10g) were collected (leaf, stems, root, and rhizomes) and stored at -85 °C for further analysis. Additionally, 25 mL of the treatment solution was collected and stored at -20 °C for further analysis. CMR readings were taken before each harvest following evaluations until the experiment was completed (October 24, 2010).

Chlorophyll analysis: Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total chlorophyll (Chl *total*) were estimated according to the method given by Hipkins et al., 1986. Approximately 100 mg of leaf tissue was homogenized with a mortar, pestle and liquid N, followed by the addition of 6.0 mL of 100 % HPLC grade methanol. The homogenized sample was transferred to a glass Corex centrifuge tube and incubated in the dark for 2 hours. Samples were centrifuged at 10,000g for 10 minutes. The supernatant was filtered with Miracloth (Calbiochem, La Jolla, CA). Final volume was raised to 10 mL with methanol and used to measure the pigments in the spectrophotometer using visible microplate # 3370.

The calculation of chlorophyll a, chlorophyll b and total chlorophyll was conducted as follows:

Chlorophyll *a* (µg/mL) = 16.5 x A_{665} – 8.3 X A_{650}

Chlorophyll *b* (µg/mL) = 33.8 x A_{650} – 12.5 X A_{655}

Total Chlorophyll (µg/mL) = 25.8 x A_{650} + 4.0 X A_{655}

Note: A stands for absorbance. One sample per rep (3n) was used for the extraction and 4 subsamples were analyzed in the spectrophotometer. Chlorophyll concentration was converted to micrograms of chlorophyll per gram of fresh weight by the following formula:

(µg chlorophyll/mL methanol) x 6 mL methanol / (g FW tissue)

Lipid peroxidation: The level of lipid peroxidation was measured in terms of malondialdehyde (MDA), a product of lipid peroxidation in the plant samples estimated by thiobarbituric acid (TBA) reaction as described by Hodges et al. (1999). Leaf samples (100mg) were homogenized using liquid N, in a mortar and pestle, followed by the addition of 5.0 mL of 80% (v/v) HPLC grade ethanol and vortexed for 1 minute. The combined extract was centrifuged at 10,000*g* at 4 °C for 10 minutes. The clarified homogenate was filtered with Miracloth. Final volume was raised to 10.0 mL using 80% (v/v) HPLC grade ethanol and vortexed for 1 minute. To 1.0 mL of the combined clarified supernatant, 1.0 ml of a solution of 20% (Trichloroacetic acid) TCA and 0.01% butylated hydroxyl toluene, BHT was added. This solution represented the -TBA solution. To another series of sample tubes, a solution of 20% TCA (v/v), 0.01% BHT (w/v) and 0.065% (w/v) thiobarbituric acid (TBA) was added. This solution represented the +TBA solution. Samples were carefully vortexed and heated at 95 ^{o}C for 25 minutes in water bath under the hood. The samples were cooled to room temperature and centrifuged at 10,000g at 4 ^{o}C for 10 minutes. Samples were transferred to a microplate, 200 µL of each sample (4 n) and the absorbance was read at 440, 532 and 600 nm. MDA content was calculated using the coefficient of extinction of 155 $mmol^{-1}$ cm^{-1} as follows:

MDA content (nmol/ mL) =

$$A = [(Abs\ 532_{[+TBA\ solution]}) - [(Abs\ 532_{[-TBA\ solution]} - Abs\ 600_{[-TBA\ solution]})]$$

$$B = [(Abs\ 440_{[+TBA\ solution]}) - [(Abs\ 600_{[+TBA\ solution]}) \times 0.0571]$$

MDA content nmol/ mL= (A – B / 157,000) x 10^6

Reduced ascorbic acid (VIT C): Vit C was extracted based on the solvent system (Wall, 2006). Frozen leaf tissue (100mg) was macerated with liquid nitrogen in a mortar and pestle, followed by the addition of 6.0 mL of cold *m*-phosphoric acid-acetic acid solution. The homogenate was transferred to Oak Ridge Centrifuge Tubes (Nalge Nunc International Corporation, Rochester, NY), followed by a 10 minute sonication (Branson, model 5510 Branson Ultrasonic Corporation, Danbury, CT.). Sonicated samples were clarified by centrifugation (Beckman Centrifuge, model J2-21, San Antonio, TX) at 13,000g for 15 min at 4 ^oC, filtered with Miracloth, and diluted to a final volume of 10.0 mL. Vitamin C was determined according to a procedure reported by Gossett et al., 1994, with modifications (Hodges et al., 1996) which permits adaptations for micro-plate determinations. A standard curve was generated by constructing six different concentrations of L-ascorbic acid (0, 20, 40, 60, 80, and 100 μM). The dilution factor was 4:1. Extracts and standards were immediately transferred to a multichannel pipette reservoir and 200μl was pipetted into 96 well flat bottom plates (Costar cat # 3370, Corning Inc., Corning, NY). The absorbance was measured at 525 nm in a micro plate reader (Synergy HT, BIO-TEK Instruments, Inc., Winooski, VT) maintained at 25 ^oC for the duration of sample determination. For the microplate blank, the mix was used as described in the micro-Eppendorf method for Vit C analysis. Results were expressed as mg/100g FW. Although several dilutions were conducted, our concentration readings were out of range. We presumed that the heavy concentrations of manganese interfered with the assay.

GC/LC/MS analysis: Water solution, soil, and composite tissue samples (leaves, stems, and rhizomes) from Experiment III were analyzed at the USDA Pesticide Lab, Auburn, AL to determine *s*-metolachlor residues using Gas Chromatography analysis (GC). An Agilent 6890

gas chromatograph (Agilent Technologies, Inc. Wilmington, DE) equipped with electron capture detectors was used. The columns were RTX – 5 and RTX – 1701, 30 meters in length, 0.53 mm. in diameter and 0.25 micron film thickness; LOD was 0.1 mg/L and the methodology was based on Luke et al., 1981. Rhizome extraction samples from Experiment I and leaf extraction samples from Experiment III from all treatments and harvests were analyzed using LC/MS (Liquid Chromatography/Mass Spectroscopy) at the Mass Spectrometry Center in the Department of Chemistry and Biochemistry, College of Sciences and Mathematics, Auburn University. An Ultra Performance LC Systems (ACQUITY UPLC™, Waters Corp., Milford, MA, USA) coupled with a quadruple time-of-flight mass spectrometer (Q-TOF Premier, Waters) with electro spray ionization (ESI) in both ESI-MS and ESI-MS/MS modes operated by the Masslynx software (V4.1) was utilized for the analysis. Each sample, in acetonitrile, was directly injected into the ESI source at a flow rate of 50 µL/min with mobile phase of 100% acetonitrile (ACN) or 50% ACN in water. The ion source voltages were set at 3 KV for positive and negative ion mode acquisitions, respectively. The sampling cone was set at 37 V and the extraction cone was at 3 V. In both modes, the source and desolvation temperature were maintained at 120 °C and 225 °C, respectively, with the desolvation gas flow at 200 L/h. The TOF MS scanning was from 50 to 800 *m*/*z* at 1 s with 0.1 s inter-scan delay using extended dynamic range acquisition with centriod data format. The Q-Tof was calibrated at multiple points with essentially linear to residue error <0.01 Dalton. Also, individual data files were calibrated by infusing Na Formate using a single point that was close to the mass of the analyte. For real time mass calibration, direct infusion of sodium formate solution (10% formic Acid/0.1M NaOH/ACN) at a ratio of 1:1:8 at 1 sec/10 sec to ion source at 2 µL/min were used for a single point mass calibration. Ions of interest were analyzed for elemental composition using accurate mass (less than 5 to 10 mg/L error) and

isotope modeling to identify the formula (Table 2.1). Collision-induced dissociation (CID) by argon on precursor ions resulted in structural fragments that further assisted the identification. For this analysis, samples of frozen tissue were macerated using a mortar/pestle in liquid nitrogen (1g of roots from Experiment I and 3g from rhizomes or leaves from Experiment III). Powdered sample was homogenized in 10 mL HPLC methanol extraction solution (95:5 methanol/deionized water [v/v]). The homogenate was transferred to centrifuge tubes (Oak Ridge, Nalge Nunc International Corporation, Rochester, NY), followed by a 10 minute sonication (Branson, model 5510 Branson Ultrasonic Corporation, Danbury, CT). Sonicated samples were clarified by centrifugation (Beckman Centrifuge, model J2-21, San Antonio, TX) at 10,000g for 15 min at 4 °C, filtered with Miracloth,and final volume was adjusted to 10 mL. *S*-metolachlor (83.7%, Dual Magnum®. Syngenta, Greensboro, NC) samples were analyzed to determine the ion's peak of the active ingredient, to estimate its molecular structure and to calculate the percentage of active ingredient in the sample. For this analysis, s-metolachlor was diluted in MeOH (1:200 MeOH), and injected to the DB-5 column, in a step temperature gradient (10 °C/min, 5 °C/min) to 300 °C, in EI^+ mode. Peaks from the spectrums with similar molecular weights to those from *s*-metolachlor standard and its metabolites (Table 2.1) were used for isotope modeling. From the experimental spectrums, peaks were used to calculate molecular mass and match it to the National Institute of Standards and Technology (NIST) Virtual Library samples to estimate how much active ingredient was in the sample.

Analysis of variance was performed on all response data using PROC MIXED and PROC CORR in SAS version 9.1 (SAS Institute, Cary, NC). Orthogonal contrasts were used to test linear and quadratic trends over treatments and harvest time simple effects at $\alpha=0.05$.

2.4. RESULTS

Experiments I and III determined the accumulation and translocation of *s*-metolachlor in the rhizome and leaf, respectively (see GS/MS/HPLC section). Tissues from Experiment II were not used for this analysis. Considering the accumulation of metolachlor, Experiment II determined the effects of herbicide on biomass accumulation, photosynthetic capability (P*n*), stomatal conductance (G*s*), and the percentage of electrolyte leakage (EL). Treatments did not affect P*n*, G*s*, and CRM readings during the experiment or EL in the first harvest (Table 2.1). A limited amount of tissue did not allow determination of EL at the second harvest. After 4 weeks of exposure to the herbicide (0, 1, 3, or 5 mg/L *s*-metolachlor), biomass accumulation (FW) exhibited a quadratic response (P=0.0189) with average FW increasing from 12 to 13 g, and then decreasing in seedlings treated with the highest *s*-metolachlor concentration (5.0 mg/L).

During all the experiments, plants did not show visual symptoms of herbicide damage such as distorted leaves, stunted growth, or loss of pigmentation (data not shown). Reduction of seedling growth and inhibition of protein synthesis has been referred to as the first signs of metolachlor injury (Scarponi et al., 1992); so chlorophyll contents were checked. In Experiment III, Chl *a*, Chl *b*, and Chl *total* levels were determined. In addition, lipid peroxidation (MDA) was measured, and CRM data were taken. Evaluating the simple effects (mg/L of *s*-metolachlor in solution) over time (harvest 1, 2, and 3) for each concentration, there was an interaction between Trt* harvest for chlorophyll levels (*a*, *b*, and *total*). There was a linear increase in non-treated and treated lotus chlorophyll levels (Table 2.2.). The exception was 15 mg/L, where trends were quadratic with levels of chlorophyll *a*, *b*, or *total* increasing in the second week and decreasing at 6 weeks. With the simple effects (each harvest) over concentrations, at harvest 1 and 2, Chl *a* increased linearly as herbicide levels in solution increased. Later in the study (6

weeks), the trend shifted to quadratic (Table 2.2). Chl *b* and Chl *total* at 2 weeks, both pigment concentrations increased linearly as *s*-metolachlor concentration in solution increased. At the second and third harvests (4 and 6 weeks) the response shifted to quadratic. A Pearson Correlation Coefficient between Chl *total* and CRM was very low (r = 0.0001). CRM measurements were not affected by time or concentration treatments and values ranged 38.4 ± 7.5.

Lipid peroxidation was determined by measuring the accumulation of malondialdehyde (MDA). MDA accumulation was influenced by time and *s*-metolachlor (interaction) concentrations ($P < 0.0251$). Analyzing simple effects over time, MDA accumulation in non-treated and treated plants increased linearly (Table 2.3). However, at 35 mg/L the trend was quadratic where at 4 weeks there was a drop in the MDA levels. In an analysis of the changes in concentrations due to simple effects over concentrations, MDA accumulation responded quadratically (Table 2.3). In the following two harvests, the MDA levels increased linearly.

Ascorbic acid (vit C) has been suggested as a promising biomarker of estuarine plants exposed to herbicide runoff (Lytle and Lytle, 1997). The method used for Experiment III measured the reduced form of ascorbic acid, expressed as mg/100g FW (Table 2.3). There was an interaction between treatment and harvest time. Assessing simple effects over time, in non-treated plants, vit C levels increased linearly as well as for plants treated with 5 and 35 mg/l of *s*-metolachlor. Treatment with 15 mg/L induced a quadratic response. With simple effects over concentration, in the first harvest, no significant trend could be fitted for the response of vit C to concentrations of the *s*-metolachlor herbicide. However, in the following harvest, vit C responded quadratically to the concentrations of *s*-metolachlor.

Mass Spectroscopy (MS), Liquid and Gas Chromatography (LC/GC) analysis: MS analysis from *s*-metolachlor samples helped to identify the experimental mass of the active ingredient. We found a spectrum generated by the MS at 28.8 minutes peak with the 238 base peak ion (Fig. 2.1). This spectrum matched that from the NIST library (Fig. 2.2a). Isotope modeling the entry from the NIST library matched that from *s*-metolachlor (Fig. 2.2b). The active ingredient concentration was estimated to be 85.5%. MS spectrum and proposed fragmentation ions for *s*-metolachlor from rhizomes extracts (Experiment I) were analyzed (Fig. 2.3a). Comparing the spectra from treated plants vs. control allowed identification of a potential metabolite with a 285 MW which only appeared in treated plants (Fig. 2.3b). Further analysis of tissues (leaves) from Experiment III which were treated with 35 mg/L *s*-metolachlor was conducted (Fig. 2.4a). A compound with a molecular weight similar to that of the herbicide active ingredient MW = 283.1339 was found (Fig. 2.4b). Isotope modeling of $C_{15}H_{22}ClNO_2$ determined that this compound could not be *s*-metolachlor (Fig. 2.4c). The spectra were used for further isotope modeling in the search of *s*-metolachlor metabolites (Table 2.4). However, no matches were found. A further analysis with LC using a wax column (DB-5- column) allowed determination of a spectrum of 28.8 min peak with 233 base peak ion which matched that from *s*-metolachlor (Fig. 2.5a). The same peaks were noted on the spectrums from leaf tissue from plants in Experiment III, harvest 3 treated with 35 mg/L of *s*-metolachlor (Fig. 2.4b) Further analysis displayed a peak of 238.07 at 0.05 Dalton. However, the retention time and signal did not allow conclusive determination of presence and amounts of the compound (Fig. 2.5c).

Water solution, soil, and a composite tissue sample (leaf, rhizome and petioles) from Experiment III were sent to the USDA Auburn Pesticide Residue lab. GC analysis did not detect *s*-metolachlor residues. But two potential metabolites were noticed (data not shown).

2.5. DISCUSSION

Extensive use of certain synthetic organic chemicals has led to numerous instances of contamination of farm soils, surface, and ground water. The regulation to prevent further release of hazardous chemicals into the environment has become stricter over the years, and some countries (e.g. Europe) have adopted zero-tolerance approaches towards contaminated soils (Chaudhry et al., 2005). There is a growing interest in the use of plants to increase microbial degradation of hazardous organic chemicals in soil. Plants have a natural ability to sequester organic and inorganic chemicals (including metals) from soil, sediment, and water. The most common route of chemical uptake into plants is through the roots via the aqueous soil phase. Ions and organic molecules move to roots from soil and sediment through plant transpiration and diffusive transport (Arthur et al., 2005). The use of plants to concentrate elements and compounds from the environment and to metabolize various molecules in their tissues is an emerging cost-effective technology for phytoremediation (Hernández-Allica et al., 2002). Phyto-extraction is limited to a number of plant species that can tolerate mixtures and greater concentrations of pesticides in the soil (Arthur et al., 2000), and aquatic plants have been commonly recommended for use in regulatory testing of pollution sites (Fairchild et al., 1998). Sacred Lotus (*N. nucifera*) is capable of producing high quantities of leaves, rhizomes and seeds (Nguyen and Hicks, 2004). Massive biomass production, its growth habit, and fast growth are attributes that can be used for phyto-remediation purposes (Goel, et al., 2001; Hicks, 2005). Aquatic plants like lotus can adapt themselves to contaminated environments (Vajpayee et al., 1999). However, the physiological effects of some of these compounds are not well documented.

Biochemically and physiologically, *s*-metolachlor affects protein and lipid synthesis, gibberellic acid induced reactions, respiration, and photosynthesis (Singh et al., 1997). Different studies on vascular plants have documented the damage to membrane structures induced by environmental stress that resulted in an increase in electrolyte leakage and reduction of photosynthesis (Talbert and Camper, 1983; Levitt, 1980). In lotus seedlings, we did not observe differences in EL from leaf tissues of the treated plants. A comparison of our data to published information indicates that our results were similar to those reported in the literature. Ketchersid et al. (1982) did not detect EL increases on *s*-metolachlor labeled carbon from roots of Sorghum (*Sorghum bicolor*) as compared to the control. EL also can be influenced by plant species (Mellis et al., 1982), leaf age (Lutts et al., 1996), concentration, and exposure time (Graham et al., 1993).

S-metolachlor can have a negative effect on biomass accumulation, nutrient uptake and carbohydrate partitioning rates on some aquatic plants (Krieger, et al., 1988). Day and Hodges (1996) determined the effects of *s*-metolachlor on a macrophyte (*Lemna gibba*) and found a reduction in frond production and dry weight. In seedlings, the responses of some biochemical parameters such as reduction of seedling growth and inhibition of protein synthesis have often been referred to as the first signs of metolachlor injury (Scarponi et al., 1992). Results from this study showed that after 2 weeks of exposure at low concentrations of *s*-metolachlor (0 to 3 mg/mL), lotus did not substantially alter total biomass accumulation. However, 5 mg/L induced a substantial reduction (23.8%) in fresh weight compared to the control treatment ($P = 0.0189$).

Experiment II: After two weeks of seedling exposure to the herbicide concentration treatments (0, 1, 3 or 5 mg/L *s*-metolachlor), fresh weight (FW) was affected by *s*-metolachlor concentrations with a decrease at the higher treatment level (5.0 mg/L). These results are in

agreement with the observations reported by Fleming et al. (1988), where plants sensitive to metolachlor showed reductions of more than 35% in dry weigh.

Various aquatic species exposed for short time periods to herbicide stress can show a rapid but reversible reduction in photochemical efficiency. In Experiment II, photosynthetic capacity (P*n*) and stomatal conductance (G*s*) of treated lotus seedlings did not show differences when compared with the non treated seedlings. This was not reported for beans. In 10-d-old hypocotyl-epicotyl cells isolated from *Phaseolus coccineus* var. *albonanus* and *P. vulgaris* photosynthesis was stimulated 328% while respiration increased 39% (Rensburg et al., 1990). Higher concentrations and longer exposure times might result in a sub lethal dose to the organisms (Jones et al., 2003). For example, *s*-metolachlor was determined to affect photosynthetic activity on plants 48 h after application (Juneau et al., 2001). In this study, the measurements were taken 2 weeks after application and the concentrations were two times, 6 times, and 10 times those recommended. During that time, the plant might have the opportunity to adapt to the stress induced by the compound, or in the case of the doses, be too small for seedlings to be affected.

Experiment III: In this study, the effects of *s*-metolachlor on photosynthetic pigment concentration, lipid peroxidation, and vitamin C content were evaluated. *S*-metolachlor can inhibit photosynthesis by affecting the state of membrane structural components through the impairment of lipid and protein synthesis (Juneau et al., 2001). In studies using lettuce (*Lactuca sativa* L.), beans (*Phaseolus vulgaris* L.), and peas (*Pisum sativum* L.) *s*-metolachlor was found to affect photosynthesis by altering components of the photosynthetic apparatus and reported decreases in Chl *a* and Chl *b* (Štajner et al., 2003). In our experiment, lotus leaves exposed for short periods of time (2 weeks) to increasing concentrations of *s*-metolachlor showed an increase

in chlorophyll levels (*a*, *b*, and *total*), and the data confirmed a general effect of *s*-metolachlor on chlorophyll content.

Ascorbic acid is considered a suitable stress biomarker for plants exposed to herbicide runoff (Lytle and Lytle, 1997). Under stress conditions, an ascorbic acid synthesis is stimulated, and its accumulation enables some plants to cope with low levels of herbicides (Shaaltiel et al., 1988). In laboratory exposure of *Hibiscus moscheutos* for 14 days at 0.1 and 1 μg/mL metolachlor in fresh and brackish water, to low herbicide levels (<0.1 μg/mL), some plants showed increased total ascorbic acid content suggesting a stimulatory effect on ascorbic acid synthesis. At higher herbicide concentrations (≥ 0.1 μg/mL), a notable decline was documented (Lytle and Lytle, 1997). At harvest 1, ascorbic acid (reduced form) concentrations were not affected by the different treatments. After 4 and 6 weeks of exposure, vit C levels showed a quadratic trend. Over time, vit C contents increased linearly in most of the treatments with the exception of 15 mg/L (quadratic). The trend suggests that high levels of *s*-metolachlor had a negative effect on vit C levels. This is an indication that lotus leaves under herbicide stress might stimulate the antioxidant mechanism. At harvest 2, the levels of vit C might had been depleted because of its use by the antioxidant protective mechanism. However, with longer time of exposure to the herbicide, the compound and/or its metabolites might affect the system.

Environmental stress causes the generation of ROS and free radicals and if the accumulation of them is not controlled, oxidative chain reactions can occur (Gonzales et al., 1998; Mashhadi et al., 2007). Proteins, nucleic acids, and lipids can withstand oxidation to a certain extent; however, plants usually respond to this stress by increasing activities of the antioxidant system (Lyubenova and Schroder, 2011). Under normal conditions, production and scavenging of these activated O_2 species are regulated by the plant. However, the formation of

excessive amounts of ROS and the production of free radicals disturbs metabolic pathways and induces macromolecular damage (Mashhadi et al., 2007; Gill and Tuteja, 2010). This is an indication that lotus leaves under herbicide stress might stimulate the antioxidant mechanism. As time passed, the compound and/or its metabolites might affect the system.

Some herbicides enhanced the formation of superoxide radicals and hydrogen peroxide causing oxidative stress and inducing lipid peroxidation (Fodorpataki et al., 2009). In plants, *s*-metolachlor inhibits the biosynthesis of several plant components such as fatty acids and lipids (Senseman, 2007). Large amounts of malondialdehyde are formed during lipid peroxidation (Yen et al., 2005). Oxidative stress in lotus plants damage membranes and lipids, forms toxic alkanes, and aldehydes such as malondialdehyde (Xing et al., 2010). In this investigation, MDA content was low in lotus at the first harvest, and although the trend was quadratic ($P < 0.0001$), the regression had substantial variation ($r^2 = 0.13$). At 4 weeks, MDA concentrations were higher and plants also showed high concentrations of ascorbate and adequate photosynthetic pigments in plants that were exposed to lower concentrations of *s*-metolachlor. Chl *a* and ascorbic acid contents decreased, and MDA progressively increased parallel to increases in *s*-metolachlor concentrations. A similar response was noted with ascorbate in an evaluation of *Vigna luteola*, *Sesbania vesicaria*, and *Hibiscus moscheutos* treated with *s*-metolachlor (*Pennant*®). In this study, *s*-metolachlor treatments (<1 to 10 mg/L) stimulated the biosynthesis and accumulation of oxidized and reduced ascorbic acid (Lytle and Lytle, 2001).

Phyto-remediation utilizes the capabilities of plants to degrade, harvest, or biofix hazardous chemicals in the environment (Chaudhry et al., 2005). Phytoremediation of *s*-metolachlor runoff in CWs has been documented before. *Juncus sp*. can uptake up to 10 % of the material in its tissues and more than 90% of it was absorbed and transformed. However, the

authors did not specify what compounds were produced by the transformation of the mother compound (Moore et al., 2001). In our work, GC analysis of lotus' tissue, water, and soil from the study did not show traces of *s*-metolachlor, but showed unknown peaks (data not shown). In recent years, a considerable body of evidence has accumulated indicating that *s*-metolachlor in aquatic systems is metabolized into ethane sulfonic acid (ESA) and oxanillic acid (Aga et al., 1996; Krutz et al., 2004). However, some reports showed that a thermally labile compound such as *s*-metolachlor degrades to metabolites that are not easily amenable to analysis using GC (Huang et al., 2008). MS/LC analysis allowed us to determine some unknown metabolites in treated rhizomes (Experiment I) and traces of s-metolachlor in leaves (Experiment III). However, the levels were too low to offer a conclusive result. The time interval from application to first harvest should have given the plant enough time to metabolize the compound. This hypothesis is supported by another study using CWs and different hydraulic retention times – HRT (days) to remove metolachlor from container nursery runoff. In that study, absorption of *s*-metolachlor is considered a relatively rapid process, requiring only minutes to hours (Stearman et al., 2003). Furthermore, all the cells removed at least 50 % of the *s*-metolachlor ((*Pennant*®) applied in the first 2 to 3 HRTs and by day 13, almost 100 % was removed. Considering that the first sampling in Experiment III was done 15 days after application, the compound might have been absorbed and metabolized in the plant (Graham et al., 1999). To determine if those peaks were s-metolachlor metabolites, further analysis of the MS/LC spectrums should be considered and also the use of alternative methodologies (Ferrer et al., 1997).

2.6. LITERATURE CITED

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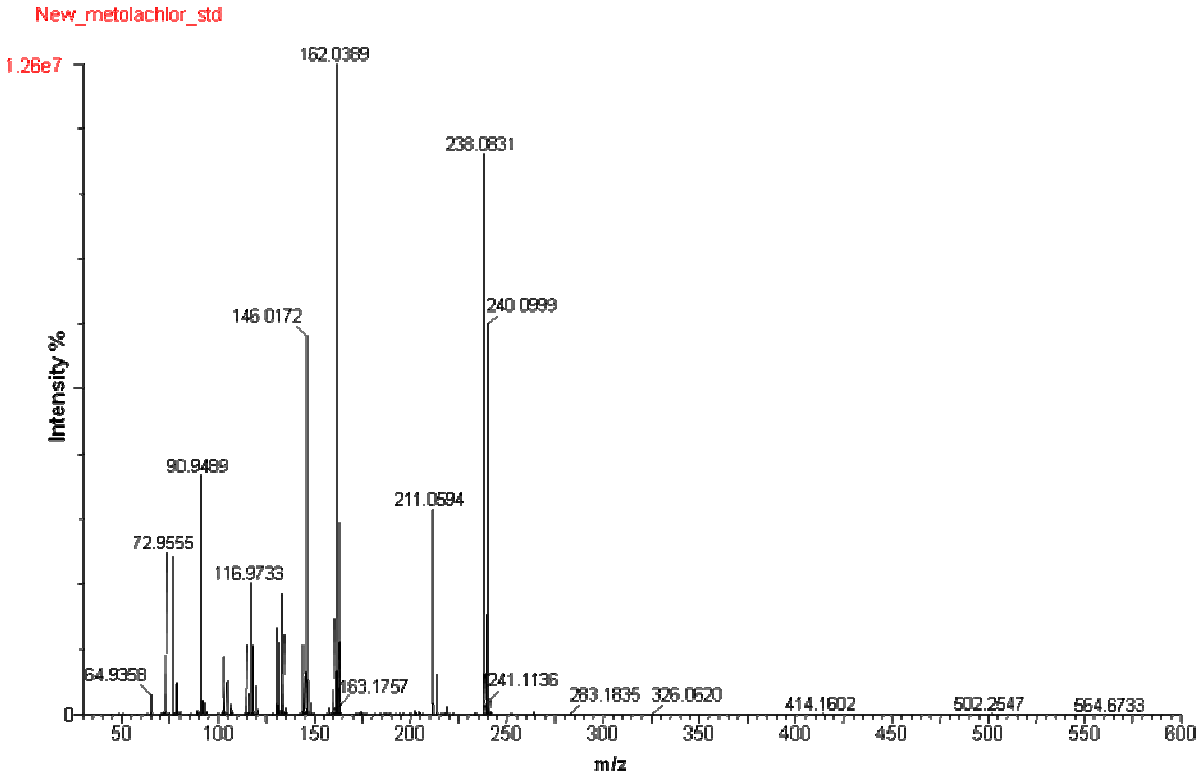
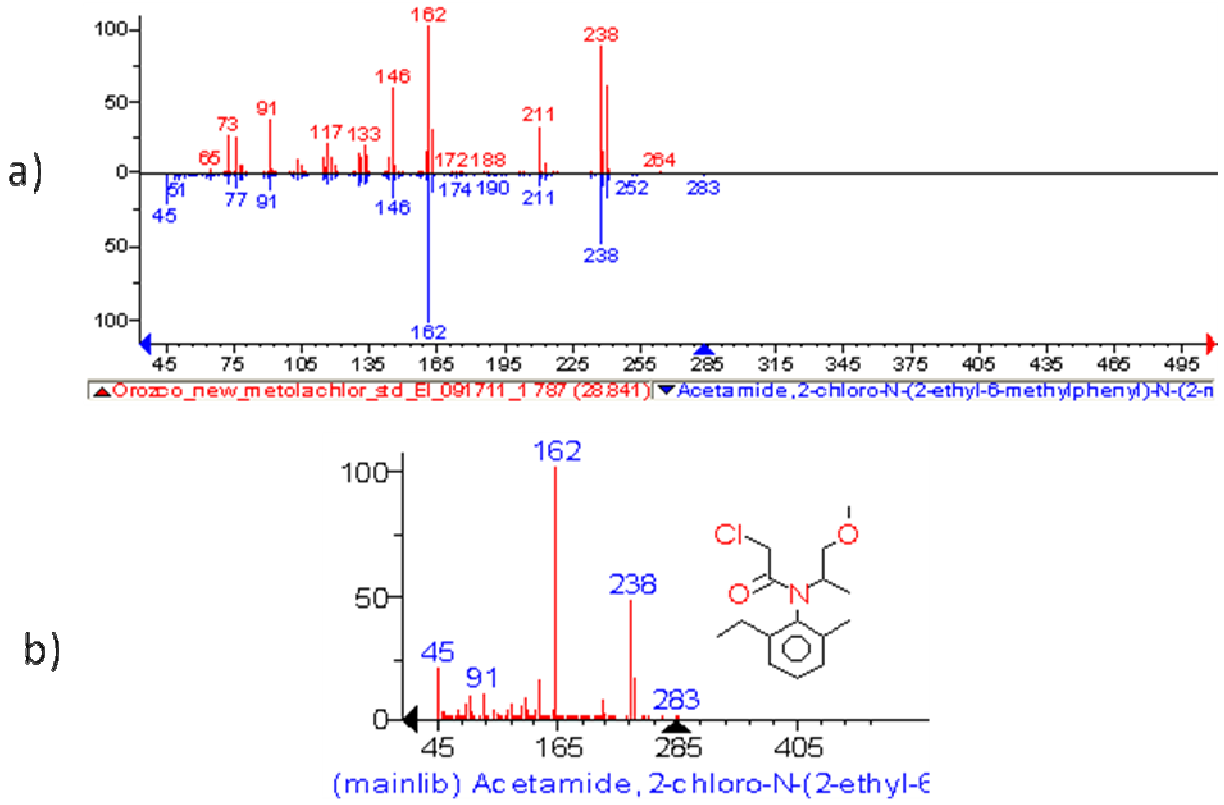


Figure 2.1: Metolachlor (DualMagnum®) chromatogram on DB-5 column, in a step temperature gradient (10 °C/min, 5 °C/min) to 300 °C, in EI^+ mode. The figure shows the spectrum of 28.8 minutes peak (238.0831) from the active ingredient of the herbicide.



Name: Acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)-
Formula: $C_{15}H_{22}ClNO_2$
MW: 283 CAS#: 51218-45-2 NIST#: 125547 ID#: 104356 DB: main library
Other DBs: RTECS, EINECS, IRDB
Contributor: ACCUSTANDARD Chemicals / NIST MS Data Center.
10 largest peaks:

162	999	238	469	45	197	240	154	146	152
163	122	91	96	41	92	77	88	131	79

Figure 2.2: a) Spectrum of 28.8 min peak, with 238 base peak ion from the bottle of *s*-metolachlor. b) Matching of the spectrum to Metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide] in NIST library.

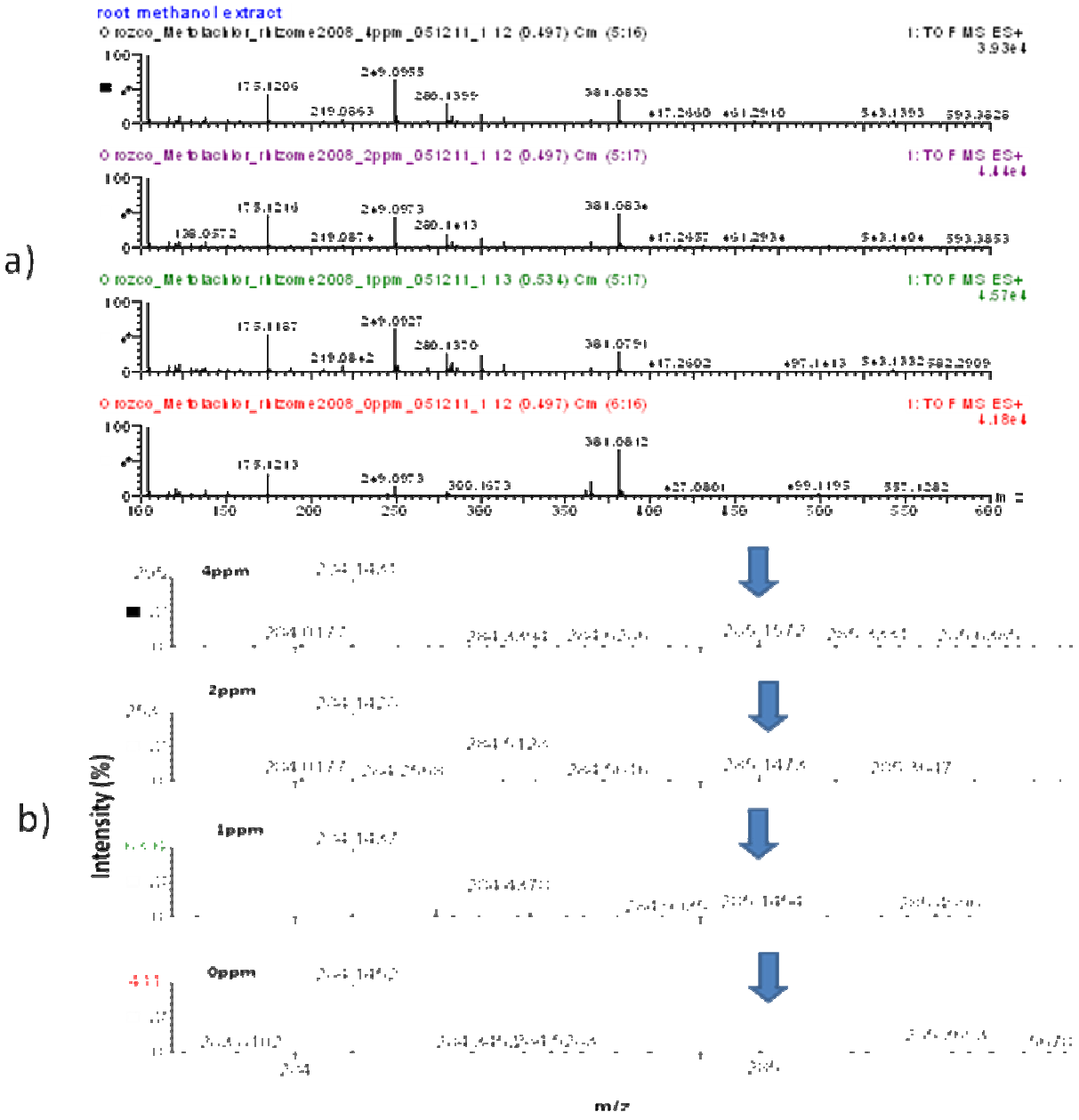


Figure 2.3: a) Mass spectrum and proposed fragmentation ions for metolachlor (spectrums in the top) in extracts from rhizomes from plants treated with *s*-metolachlor (DualMagnum®) at 4.0, 2.0, 1.0 and 0.0 in Experiment I. b) This spectrum point out (blue arrows) an isotope (MW 285.1) that is present in the rhizomes from treated plants (1.0, 2.0 and 4.0 mg/L) but is absent in the control (0.0 mg/L of *s*-metolachlor).

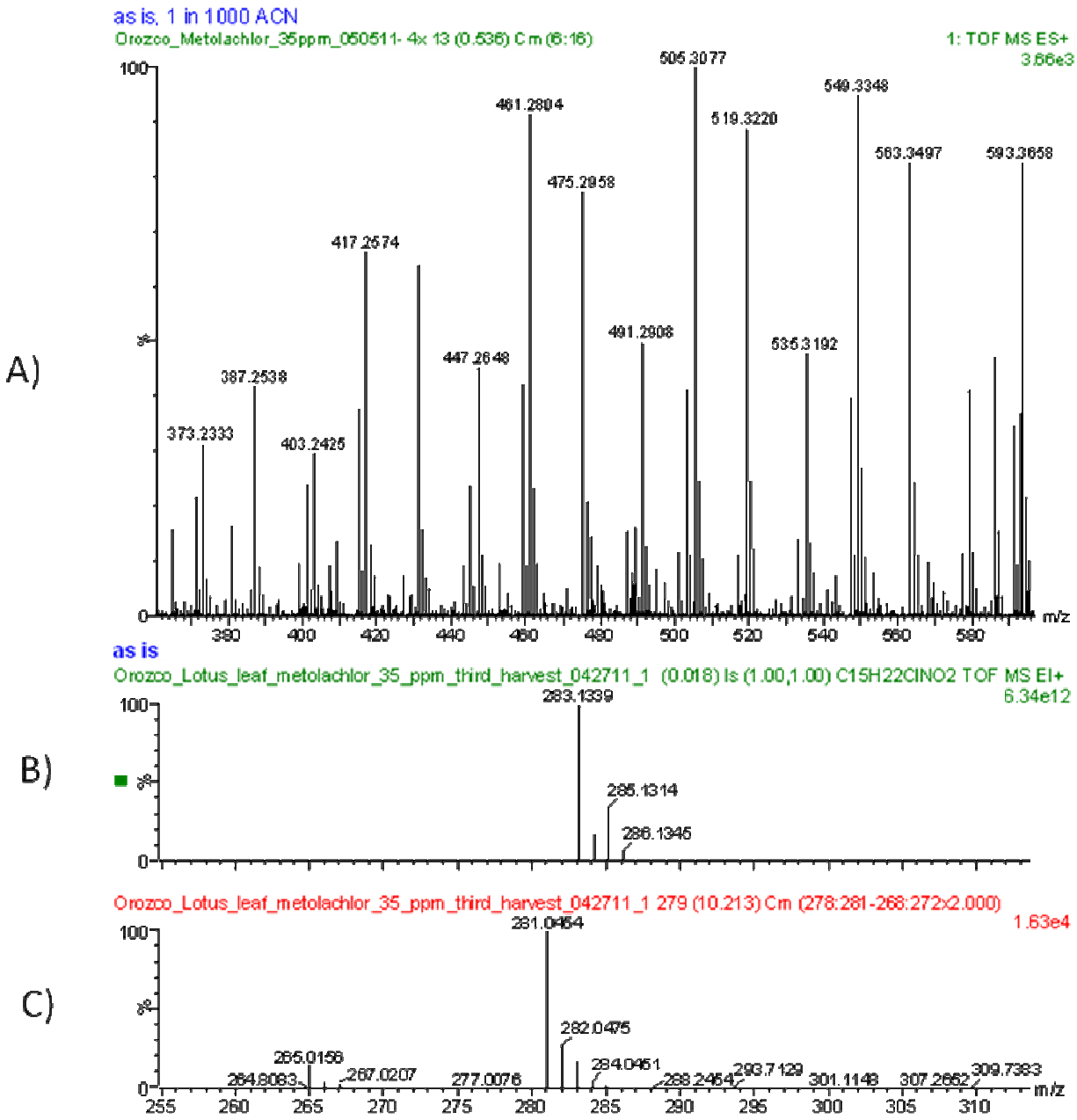


Figure 2.4: Mass Spectroscopy chromatograms (Waters® Acquity UPLC™ and Q-Tof Premier™) from leaf extracts from plants treated for 6 weeks with 35 mg/L of *s*-metolachlor (DualMagnum®). A) Mass spectrum and proposed fragmentation ions of compounds found in the treated leaves. B) Identification of a potential peak related to *s*-metolachlor. C) Magnification of the peaks which were used for isotope modeling and identification of potential *s*-metolachlor metabolites.

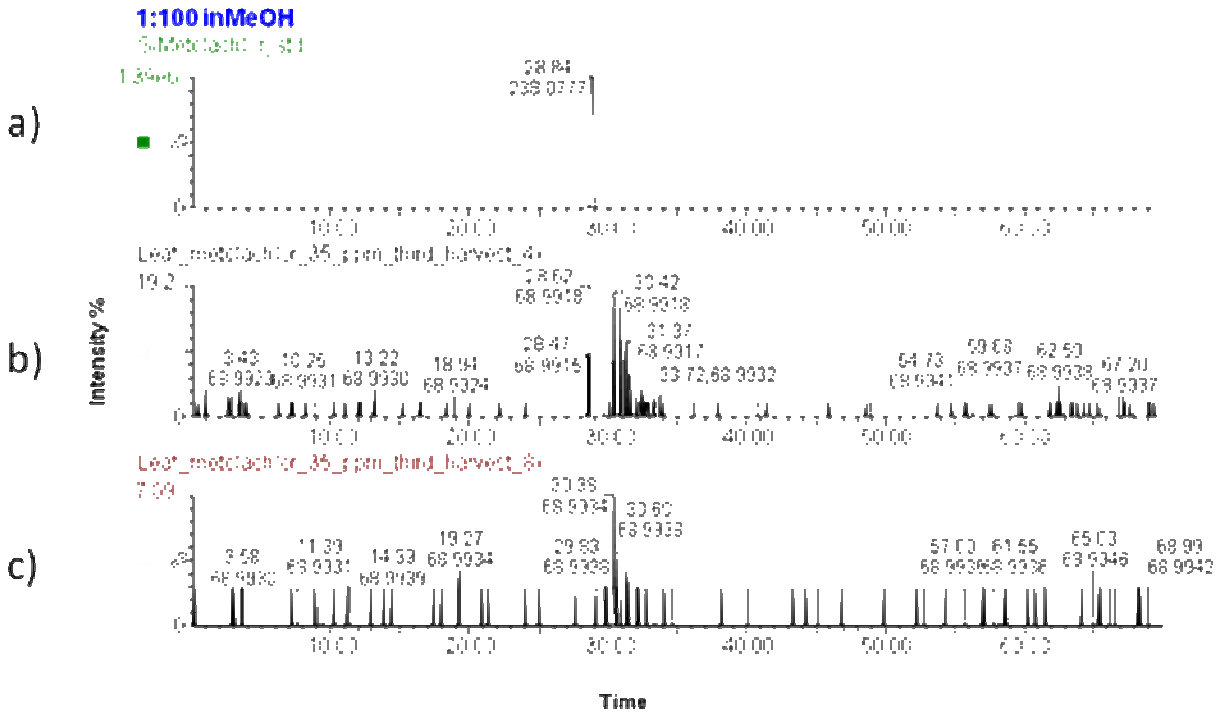


Figure 2.5: a) Spectrum displaying of 28.8 min peak with 238.07 base peak ion from *s*-metolachlor standard. b) Spectrum displaying traces of *s*-metolachlor in a tissue sample (4g of leaf tissue/10 ml of 89% ETOH) from plants exposed to 35 mg/L of *s*-metolachlor (Experiment III). c) Spectrum displaying traces of *s*-metolachlor in a tissue sample (4g of leaf tissue/20 ml of 89% ETOH) from plants exposed to 35 mg/L of *s*-metolachlor (Experiment III).

Table 2.1: Effect of S-Metolachlor on membrane plasticity, photosynthesis and biomass accumulation of lotus seedlings.

Treatment (mg/L)	Electrolyte leakage (%)	Photosynthetic capability ($\mu mol\ CO_2.m^{-2}.s^{-1}.d^{-1}$)	Stomatal conductance	Fresh weight (g)
0	15	9	0.3	12
1	19	9	0.4	13
3	16	10	0.4	13
5	17	10	0.3	9
[y]Sign.	ns	ns	ns	[z]Q***

[y]Effect *s*-metolachlor concentrations (0.0, 1.0, 3.0, and 5.0 mg/L) were determined to be non-significant (ns) at $\alpha = 0.05$, or quadratic (Q) at $\alpha = 0.05$ or 0.001 (***) using orthogonal contrasts.

[z]Fresh weight: $y = 11.863 + 2.0221x - 0.5632x^2$, $r^2 = 0.3252$, and $p = 0.0189$.

Table 2.2: Effect of *s*-metolachlor concentrations (0, 5, 15, and 35 mg/L) on mature lotus plants photosynthetic pigments.

Parameter	Harvest	*s*-metolachlor treatments (mg/L) 0	5	15	35	Sign.[z]
Chl a	2 weeks	342	346	360	389	L***
(mg/g FW)	4 weeks	457	475	512	421	L***
	6 weeks	451	468	398	455	Q***
Sign.[y]		L***	L***	Q***	L***	
Chl b	2 weeks	218	221	224	240	L***
(mg/g FW)	4 weeks	285	225	325	263	Q***
	6 weeks	293	315	261	296	Q***
Sign.[y]		L***	L***	Q***	L***	
Chl total	2 weeks	410	414	424	455	L***
(mg/g FW)	4 weeks	541	585	613	498	Q***
	6 weeks	549	584	491	554	Q***
Sign.[y]		L***	L***	Q***	L***	

[z]Simple effect over concentration (rows) and simple effects over time (columns) were determined to be non-significant (ns), linear (L), or quadratic (Q) at $\alpha = 0.001$ (***) using orthogonal contrasts.

[y]Simple effect over time (columns):
Chlorophyll a: 0.0 mg/L (y= 330.91825 + 45.78413x, $r^2 = 0.19$, p = 0.0001); 5.0 mg/L (y= 325.04602 + 53.42445x, $r^2 = 0.24$, p = 0.0001); 15.0 mg/L (y= -59.24375 + 552.23958x - 133.31250x^2, $r^2 = 0.33$, p = 0.0001); and 35.0 mg/L (y= 354.49059 + 33.49601x, $r^2 = 0.09$, p = 0.0013).
Chlorophyll b: 0.0 mg/L (y= 201.63294 + 33.21786x, $r^2 = 0.26$, p = 0.0001); 5.0 mg/L (y= 190.56029 + 43.76029x, $r^2 = 0.37$, p = 0.0001); 15.0 mg/L (y= -41.47500 + 348.15417x -82.44583x^2, $r^2 = 0.30$, p = 0.0001); and 35.0 mg/L (y= 208.63608 +28.70756x, $r^2 = 0.15$, p = 0.0001).
Total chlorophyll: 0.0 mg/L (y= 385.06310 + 60.46627x, $r^2 = 0.24$, p = 0.0001); 5.0 mg/L (y= 364.32829 + 77.95770x, $r^2 = 0.34$, p = 0.0001); 15.0 mg/L (y= -75.44792 + 655.39792x -155.55833x^2, $r^2 = 0.30$, p = 0.0001); and 35.0 mg/L ($r^2 = 0.13$, p = 0.0005).
[z]Simple effect over concentration (rows):
Chlorophyll *a*: Harvest 1 (y= 340.54806 + 1.35682x, $r^2 = 0.07$, p = 0.0001); harvest 2 (y= 483.85639 - 0.83995x, $r^2 = 0.01$, p = 0.0001); and harvest 3 (y=456.91667 + 2.94618x + 0.16998x2, $r^2 = 0.16$, p = 0.0137).
Chlorophyll *b*: Harvest 1 (y= 217.43836 + 0.61542x, $r^2 = 0.05$, p = 0.0129); harvest 2 (y= 280.86839 + 5.24337x - 0.16417x^2, $r^2 = 0.16$), p = 0.0001); and harvest 3 (y= 307.54803 -2.92109x + 0.07258x^2, $r^2 = 0.02$, p = 0.0183).
Total chlorophyll: Harvest 1 (y = 408.58360 + 1.28987x, $r^2 = 0.06$, p = 0.0077); harvest 2 (y = 532.41109 + 9.45940x - 0.29691x^2, $r^2 = 0.15$, p= 0.0001); and harvest 3 (y= 573.63630 - 5.42920x + 0.13729x^2, $r^2 = 0.02$, p = 0.0241).

Table 2.3: Effect of *s*-metolachlor concentrations (0, 5, 15, 35 mg/L) on mature lotus plants lipid peroxidation (MDA) and vitamin C accumulation.

		S-metolachlor (mg/L)				
Parameter	Harvest	0	5	15	35	Sign.[z]
Lipid Peroxidation	2 weeks	- 0.33	0.23	- 0.04	-0.38	Q***
(µmol/mL)	4 weeks	0.68	0.39	0.24	-0.75	L***
	6 weeks	0.97	1.07	1.27	1.44	L***
Sign.		L***	L***	L***	Q***	
Vit C	2 weeks	32	46	42	40	ns
(mg/100g FW)	4 weeks	64	95	71	0	Q***
	6 weeks	128	116	81	97	Q***
[y]Sign.		L***	L***	Q***	L***	

[z]Simple effect over concentration (rows) and simple effects over time (columns) were determined to be linear (L), or quadratic (Q) at $\alpha = 0.001$ (***) using orthogonal contrasts.

[y]Simple effect over time (columns):

Lipid peroxidation (MDA): 0.0 mg/L ($y = 56.304x - 43.213$, $r^2 = 0.63$); 5.0 mg/L ($y = 0.4405x - 0.3042$, $r^2 = 0.48$); 15.0 mg/L ($y = 51.562x - 72.665$, $r^2 = 0.43$), and 35.0 mg/L ($y = -9.7835x^2 + 58.689x - 6.868$, $r^2 = 0.18$).

Vitamin C concentrations: 0.0 mg/L ($y = 56.304x - 43.213$, $r^2 = 0.63$); 5.0 mg/L ($y = 29.975x + 29.555$, $r^2 = 0.48$); 15.0 mg/L ($y = -9.7835x^2 + 58.689x - 6.868$, $r^2 = 0.18$); and 35.0 mg/L ($y = 51.562x - 72.665$, $r^2 = 0.43$).

[z]Simple effect over concentrations (rows):

Lipid peroxidation (MDA): Harvest 1 ($y = -0.001x^2 + 0.0263x - 0.125$, $r^2 = 0.13$, $p = 0.0251$); harvest 2 ($y = -0.0395x + 0.6851$, $r^2 = 0.48$, $p < 0.0001$); and harvest 3 ($y = 0.0128x + 1.0177$, $r^2 = 0.27$, $p = 0.0008$).

Vitamin C concentrations (rows): Harvest 2 ($y = -0.1224x^2 + 2.1494x + 73.732$, $r^2 = 0.65$, $p < 0.0001$); and harvest 3 ($y = 0.1067x^2 - 4.7091x + 130.88$, $r^2 = 0.30$, $p < 0.0001$).

Table 2.4: *S*-Metolachlor metabolites found in plants, soil and water.

Compound	Reference	Formula	Reported molecular mass	Calculated mass (M+1)
Metolachlor	Henderson et al., 2007	$C_{15}H_{22}NO_2Cl$	283	284.1417
	Hladik et al., 2005			
Metolachlor propanol	Hladik et al., 2005		269	
[y]Morpholine	Henderson et al., 2007	$C_{14}H_{19}NO_2$	233	234.1494
	Hladik et al., 2005	$C_{13}H_{19}NO_2$		204.1626
Deschlorometolachlor propanol	Xie et al., 2010			
Deschlorometolachlor	Hladik et al., 2005	$C_{15}H_{23}NO_2$	249	250.1807
	Xie et al., 2010			
Deschloroacetylmetolachlor	Hladik et al., 2005		207	
Deschloroacetylmetolachlor propanol	Hladik et al., 2005		193	
Carbinol	Henderson et al., 2007	$C_{15}H_{23}NO_3$	147	266.1756
[z]Hydroxymetolachlor	Hladik et al., 2005	$C_{15}H_{23}NO_3$	265	265.1678
	Xie et al., 2010		265	
Sulfonic acid	Aga et al., 1996	$C_{15}H_{22}NO_5S$	328	329.1297
	Ferrer et al., 1997			
Ethane Sulfonic acid (ESA)	Henderson et al., 2007	$C_{15}H_{24}NO_5S$		331.1453
	Aga et al., 1996			

Alternative names: [y]Morpholinome, [z]Metolachlor - 2 – hydroxyl.

CHAPTER 3

EVALUATION OF TRACE METAL PHYTOREMEDIATION POTENTIAL IN ORNAMENTAL LOTUS (*NELUMBO NUCIFERA* GAERTN.)

3.1. ABSTRACT

Proper selection of plant species plays an important role in the development of remediation efforts. Lotus (*Nelumbo nucifera*) is a sturdy, aquatic, herbaceous perennial capable of producing large quantities of biomass. Little work with lotus has been done regarding the pattern of accumulation of metals. A base line for lotus' tissue composition was determined and the pattern of Mn accumulation was assessed by exposing mature plants to different Mn concentrations (0, 5, 10, 15 and 50 mg/L). Despite high foliar levels of several elements (e.g. Mn, Ni, Na, and Zn) lotus plants showed minimal visual signs of toxicity. Increasing Mn concentration in solution treatments induced linear accumulation of Mn concentrations in leaves (>3000 mg/L). Mn treatments did not show an effect on accumulation of As, Cu, Fe, Cr, Ni, Pb, and Zn. But concentrations of As, Cu, Fe, Ni, and Zn in the rhizomes increased linearly with time. Cr concentrations were higher than the threshold of toxicity for many plants. Na was affected by Mn concentration in solution with longer exposure (4-6 weeks) yielding higher accumulation, predominantly in petioles. Over time, only leaves showed a linear increase in Pb levels. Lotus plants hyper-accumulated Al and Fe in the rhizomes (> 9500 and >6000 mg/L, respectively), and Na in the petioles (>13,000 mg/L) without visible signs of toxicity. This research demonstrates that lotus is a promising candidate for use in phyto-remediation.

3.2. INTRODUCTION

The presence of heavy metals (arsenic, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, nickel, zinc) in natural ecosystems has become a matter of concern in many countries. These pollutants are introduced into the aquatic systems as the result of various industrial, storm run-off and agricultural operations (Gupta et al., 2008). Some plants have a natural ability to take up higher levels of organic and inorganic chemicals and metals from soil, sediment and water (phyto-extraction), and can be classified as excluders (high tolerance); highly sensitive, accumulators; and hyper-accumulators (Fischerová et al., 2006). Hyper-accumulators can take up and concentrate more than 1 % of their dry matter in heavy metals (Dhir et al., 2009). Aquatic macrophytes are known to accumulate metals taken from the environment (Miretzky et al., 2004). Inclusion of these plant species plays an important part in the development of remediation methods. Uptake and accumulation of pollutants vary from plant to plant and also from species to species (Miretzky et al., 2004). Stearman et al. (2003) studied the use of constructed wetlands (CWs) planted with Bulrush (*Scirpus validus*) to remove organic pollutants from nursery run-off. Surrency et al. (2003) mentioned the use of yellow and red canna lilies (*Canna spp*.), summer orange daylily (*Hemerocallis fulva*), elephant ear (*Colacasia esculenta*), blue flag Iris (*Iris versicolor*), Louisiana Iris (*Iris hexagonae*), yellow flag Iris (*Iris pseudacorus*), Umbrella Palm (*Cyperus alterufolius*), and Powdery Thalia (*Thalia dealbata*) as phyto-remediation plants. Several aquatic macrophytes (*Pistia stratiotes*, *Spirodela intermedia* and *Lemna minor*) were used for the removal of cadmium (Cd), chromium (Cr), lead (Pb), manganese (Mn) and others (Kumar et al., 2008a, 2008b; Rai, 2009; Srivastava et al., 2008). Some species die-back during the fall and winter seasons thus releasing some stored pollutants back into the ecosystem. Some species are difficult to harvest and offer low foliage/root ratio, lack ornamental value, and have limited availability at local nurseries. Aquatic higher plants

such as water hyacinth (*Eichhornia crassipes*), *Hydrocotyle umbellate*, and water velvet (*Azolla pinnata*) have also been utilized for phyto-remediation of polluted waters. However, the efficiency of metal removal by these plants is low due to their small size and slow-growing roots, and their higher water content which complicates their drying, composting and incineration (Dushenkov et al., 1995). These disadvantages encourage the evaluation of alternatives.

Lotus (*N. nucifera*) is a sturdy, aquatic, herbaceous perennial plant. Some cultivars have the capacity of producing up to 40 tons/ha of rhizomes (Nguyen and Hicks, 2004) and 1.5 tons /ha of seeds. The massive biomass production and growth habit of lotus are attributes that can be beneficial for phyto-remediation purposes (Hicks, 2005).

Manganese toxicity is not restricted to a narrow critical concentration range. There are reports of concentrations of 200 mg/L affecting corn biomass accumulation and others as high as 5,300 mg/L for sunflowers (Mengel and Kirkby, 1987). In studies involving lotus, high concentrations of Mn were measured in leaves (8,360 mg/L) and petioles (2,511 mg/L) (Hicks, 2005; Nguyen 2001). A number of studies have been conducted to determine the effect of different industrial effluents on lotus (Arora et al., 2008; Aqeel-Ashraf et al., 2010), but little work has been done on the pattern of accumulation of metals in cultivated lotus (Hicks, 2005; Tian, 2008). The purpose of this study was to evaluate the chemical composition of visually healthy, commercially produced lotus plants, to establish a base line for tissue composition. In addition, lotus was evaluated for its potential as an accumulator of Mn and how Mn concentrations change among tissues with time.

3.3. MATERIALS AND METHODS

During the springs of 2008 and 2010, four ornamental lotus (*N. nucifera* Gaertn.) cultivars ('Ms. Slocum', 'Perry Superstar', 'Rosea Plena' and 'Camellia Red') were evaluated to determine a base line for tissue chemical composition. The first 3 cultivars were obtained from a commercial nursery (Mobjack Nurseries Inc. Mobjack, VA) where they were growing in an open field pond in 18 L containers. The substrate was sandy loam soil and no data concerning fertilization was provided. Three plants per cultivar were harvested, soil was removed and rhizomes, petioles and leaves were submitted for chemical analysis to the Auburn University ALFA Plant Diagnostic lab. 'Camellia Red' was obtained from the Auburn University lotus collection located at the Paterson Greenhouse complex, Auburn, AL (Latitude=34.18 N, Longitude = 86.85 W, Elevation = 242 m, USDA Zone 8b). Plants were grown in plastic containers without holes (29 L) in a mix of top soil (sandy clay-loam) and pine bark (1:1 by volume), pH 7.0, and EC = 0.13 mS cm^{-1}. Plants were fertilized using soluble fertilizer Pro·Sol (Frit Industries, Inc. Ozark, AL) 20-10-20 (20% total nitrogen, 10% P_2O_5, and 20% K_2O) with micronutrients (Table 3.1). Containers were placed under full sunlight with 25 cm spacing (Tian, 2008). In March 2010, dormant lotus rhizomes were harvested and planted in plastic containers (11.3 liters) in a mix of top soil (sandy clay-loam) and pine bark (1:1 by volume), and place in a greenhouse. Temperature set points maintained the greenhouse environment at 25 °C (day) and 20 °C (night). In May 2010, tissue samples from 3 plants were harvested and analyzed. The rest of the plants, were set up in the greenhouse. Every week, container water levels were refilled to the same height with a 150 mg/L N solution of soluble fertilizer TotalGro™ (STD Industries, Winnsboro, LA) 20-20-20 (6 % nitrate, 6% ammoniacal nitrogen, 8% soluble urea; 20% P_2O_5, and 20% K_2O) with micronutrients (Table 3.1). The volume of the solution was kept relatively constant by adding water to compensate for water lost through plant

transpiration and evaporation. Six weeks later, plants were randomly placed in a split plot design with 3 replications per treatment. Plants were treated with 0, 5, 10, 15, and 50 mg/L of Mn from $MnSO_4$ (Southern Agricultural Insecticides, Inc. Palmetto, FL). At 2, 4, or 6 weeks plants were harvested and leaf lamina, petioles and rhizomes were separated and sent to the lab for tissue analysis (3 reps per treatment). An analysis of the water used in the solution as well as the substrate was performed. For total nitrogen (N) and carbon (C), dry samples were combusted with oxygen (O) catalyst at 1150 ºC. Nitrogen, C, and sulfur (S) gases were measured by thermal conductivity using an Elementar Vario Macro CNS analyzer (Columbo and Giazzi, 1982; Kirsten, 1979). For dry ashing organic matter destruction, ~0.50 gram samples of dried plant material were ashed for 8 hours in a muffle furnace at 500 ºC. Samples were digested and analyzed by procedures outlined by Plank (1992), and Odom and Kone (1997).

Analysis of variance was performed on all response data using PROC GLIMMIX in SAS version 9.2 (SAS Institute, Cary, NC). The experimental design was a split plot with Mn concentration and tissue type in the main plot and harvest time in the subplot. Homogeneity of variance was tested using Levene's test at $\alpha = 0.05$. In case of heterogeneity, covariance parameters were calculated using the GROUP option on the RANDOM statement in PROC GLIMMIX, and a heterogeneous variance model was used. Differences among simple effect means for tissue type were determined using Tukey's means comparison test at $\alpha = 0.05$. Single degree of freedom, orthogonal contrasts were used to test linear and quadratic trends of Mn and other element concentrations over harvest time at $\alpha = 0.05$.

3.4. RESULTS

Nutrient analysis of the water, soil and final substrate (1:1 soil and pine bark) showed a high concentration of Ca and a low contribution for most of the trace elements (Al, Mn, Fe, B, Cd, Cr, Cu, Ni, Pb and Zn) from the water (< 0.1 mg/L) and a neutral pH (7.2). Soil analysis reported a much higher concentration of Al, Mn, and Na and after mixing with pine bark. The end result substrate showed a threefold increase in Ca concentration while the other elements remained very similar (Table 3.1).

Manganese (Mn): Mn concentrations in leaves from non-treated and those growing in 5 and 10 mg/L did not change (Table 3.2). However, as the concentration of Mn in solution increased, Mn levels in leaf tissues showed a quadratic response to time where the highest concentration occurred at 4 weeks. In rhizomes and petioles, all treatments showed a quadratic response and the highest concentrations were also observed at 4 weeks. The only exception was the concentrations in the petioles from non-treated plants, which did not show any effect. Mn levels increased linearly in all harvests (Table 3.2). In rhizomes, the trend was also linear in the first and last harvest, but quadratic at 4 weeks that showed a decrease in the Mn levels of those plants treated with 50 mg/L. In petioles, there was no response in the first harvest, but the trend was quadratic at 4 weeks and shifted to linear in the last harvest. Tukey's means comparison was used to determine which tissue had the highest concentration of Mn at each harvest (Table 3.2). At 2 weeks, no differences among tissues were found. At 4 weeks, Mn concentrations were higher in rhizomes and leaves and lowest in petioles. At 6 weeks, Mn concentration was higher in leaves followed by petioles and lowest in rhizomes.

Mn levels in solution did not affect the concentrations of some heavy metals (arsenic (As), chromium (Cr), copper (Cu), iron (Fe), nickel (Ni), and lead (Pb) in treated plants.

However, concentrations of the elements changed over time and varied depending of the tissue. Arsenic levels in leaves and petioles did not change over time and were always lower than the concentrations found in the rhizomes (Table 3.3). Cr levels remained high in rhizomes followed by petioles and lowest in leaves. In rhizomes and petioles, Cu concentrations showed a quadratic response to increasing time. Concentrations were highest in rhizomes, followed by leaves and lowest in the petioles. However, in the leaves there were no differences among the concentrations found at the different harvests. Iron accumulated primarily in rhizomes. Although the levels in leaves and petioles were lower than those in the rhizomes, the differences in those two tissues were small. Further evaluations (at 4 and 6 weeks) indicated that the concentrations remained higher in the rhizomes, but no differences were found in the leaves and petioles. Plants did not show visual signs of Fe toxicity. Pb concentration in the tissues showed a quadratic response over time and only at the end of the experiment did differences among tissues appear. Concentrations of Pb were higher in leaves followed by petioles and then, rhizomes. Concentrations of Ni in leaves and petioles did not change over time, and were lower than those in the rhizomes. Rhizome Ni concentrations showed a linear decrease over time.

Manganese treatments and time of exposure had an effect on the levels of aluminum (Al) and boron (B) in lotus tissues. Aluminum concentrations in leaves and petioles were not affected by Mn levels in solution, and the levels of Al accumulated in the leaves and petioles ranged from 96 to 180 mg/L, respectively (Table 3.4). Leaf concentrations were twice those previously reported by Nguyen (2001) but were similar in petioles (19 to 204 mg/L). Al concentrations reached levels of hyper-accumulation in rhizomes in the first 2 weeks of the study (> 9,500 mg/L) (Table 3.4). Analyzing simple effects over concentrations, leaf and petiole Al levels were not affected by the Mn levels in solution. However, in rhizomes, the trend was quadratic at 2

weeks, no changes were found at 4 weeks, and a linear increase was found at 6 weeks. With increasing Mn levels in solution, B levels in the tissue decreased over time (Table 3.4). Most of the B accumulated in the leaves.

Sodium (Na) concentrations in the leaves were not affected by the time of exposure to the treatments or by Mn levels in solution. Na concentrations ranged from 483 to 1,844 mg/L (Table 3.5). In rhizomes, Na concentrations responded quadratically with a decrease at 4 weeks and much higher levels accumulated at 6 weeks. In petioles, the control showed a linear accumulation of Na and the differences between the Na accumulated at 2 weeks and 6 weeks showed an increase in Na concentration of 41 %. Na concentration in treated responded quadratically. Na levels in the leaves and rhizomes over concentration were not affected. For petioles, no changes were found in the first 2 weeks, but quadratic trends were found in the subsequent harvest. Most of the Na accumulated in the petioles, lower levels in the rhizomes and the lowest levels in the leaves.

Zn concentrations were not affected by Mn in solution in leaves and rhizomes over time. Only in non treated rhizomes was a quadratic response detected. In petioles, similar results were found for the non-treated plants but the trend was opposite. The only treatment which exerted an effect was 10mg/L inducing a linear increase in Zn levels (Table 3.5). Simple effects over concentrations only showed changes for rhizomes and petioles at 4 weeks. The trend was quadratic. Zn accumulated preferentially in the rhizomes at higher Mn concentration. At the end of the experiment, concentrations in the leaves ranged from 33 to 41 mg/L and in rhizomes from 83 to 109 mg/L.

Tissue composition from ornamental lotus cultivars did not show differences among the As, B, Ca, Cu, Cd, P, Mn, and Pb accumulation (Table 3.6). Leaves and petioles showed similar

levels of Al (< 110 mg/L), but in much lower quantities than rhizomes which reached levels of hyper-accumulation. Cr levels in rhizomes were also very high ranging more than twice those from the leaves and petioles (1 to 2 mg/L). Fe levels in leaves and petioles showed no differences among them and concentrations reached levels averaging less that 130 mg/L. However, rhizomes showed very high levels (3,944 mg/L). Despite high levels, our plants did not show signs of Fe toxicity. Although, Mn concentrations in the tissues were higher than those reported by the literature (190 to 406 mg/L), there were no differences among tissues. Lotus cultivars showed Ni and Zn levels in rhizomes being higher (265 and 121 mg/L respectively) than those in leaves and petioles (<12 and < 66 mg/L respectively). Na levels were high in both petioles and rhizomes. However, concentrations in the petioles were not different from those in the leaves.

In the first and second harvests (2 and 4 weeks) of the plants exposed to different concentrations of Mn in solution, several young leaves died showing brown spots and a distinctive yellowing and later deteriorating to brown coloration. These leaves were collected and analyzed. High concentrations of Al, B, Cr, Cu, Fe, Mn, Na, Ni, and Zn were determined (Table 3.7). However, due to the lack of sufficient samples (1 to 3 leaves per treatment) no statistical analysis was conducted.

3.5. DISCUSSION

Remediation capacity of a plant is associated with the content of elements in the above ground biomass (petiole and leaf), and in the underground tissues (rhizomes), and the percentage of the element recovered per year from a determined volume of soil with respect to plant element concentration, and plant yield (Fischerová et al., 2006).

Aluminum: The variation in concentrations in treated plants at the different harvest does not allow an easy interpretation of the effects of Mn in solution on the concentrations of Al. Low r^2 suggest that the effects although significant were marginal. In a study of metal accumulation and Mn/Al interaction, it was noted that increasing Mn supply reduced concentrations of Al in shoots and leaves. These cations compete for negatively charged binding sites at the cell surface (Blair and Taylor, 1997). Al toxicity is frequently accompanied by high levels of Fe and Mn in the plant tissues (Mengel and Kirkby, 1987). Plants evaluated in this study did not show visual signs of Al, Mn or Fe toxicity. Concentrations of all 3 of these elements in the leaves were twice those previously reported by Nguyen (2001), but the concentrations in petioles were similar. Tolerance to excessive Mn levels has been reported previously associated with tolerance to excess Al (Foy et al., 1978). Our results correspond with the reports on hyper-accumulation of Al by some species. *Camellia sinensis* (tea) accumulated Al at 3,000 to 5,000 mg/L dry weight in its roots. In this plant, lack of Al toxic response can be brought about by organic acids and polyphenols that detoxify excess Al by chelation (Mengel and Kirkby, 1987). Lotus plants are known for their high content of phenolic compounds (Park et al., 2009). Other studies have documented the capacity of lotus to accumulate high quantities of Al. Nguyen (2001) reported that rhizomes accumulated from 2,054 to 7,324 mg/L.

Arsenic: There is very little information on toxic effects of arsenic on plant species (Carbonell et al., 1998). However, at very low concentrations As stimulates the formation of free radicals and reactive oxygen species either via direct electron transfer or as a metal-mediated inhibition of metabolic reactions (Mahmud et al., 2006). In *Spartina alterniflora*, concentrations as low as 2 mg/L of As can have a negative effect on biomass accumulation (Carbonell et al., 1998). Our study reported concentrations in tissues higher than those reported for other plants

[0.02 to 7.0 μg g^{-1} DW] (Prasad, 1999). Other phyto-remediation studies using lotus, have revealed that soils containing high levels of As (2,878 mg/L) led to accumulation of high concentrations of As in lotus rhizomes, leaves, and petioles [561, 315, 289 mg/L, respectively] (Aqeel-Ashraf et al., 2010).

Boron: In water-logged plants, researchers found that applications of B increased the stability of leaf membranes, chlorophyll content, soluble sugars, soluble proteins, amino acids content, and dry mass accumulation (Sayed, 1998). Boron can also be toxic at very low concentrations (0.5 to 2 nM) and can cause substantial reduction in leaf biomass (Cervilla et al., 2009). Plants treated with Mn showed higher levels of B than those considered toxic in other plants. This high concentration of B, might also explain the tolerance of lotus to high concentrations of Al. Aluminum inside the plant is usually in the form of aluminate $Al(OH)_3$ that is structurally similar to boric acid $B(OH)_3$. Based on the similarities of the molecules, it has been proposed that Al exerts its toxic effect by inducing boron deficiency (Blevins and Lukaszewski, 1998). High levels of B might prevent this from happening and the plant may use B reserves to prevent further damages from other heavy metals. In addition, $B(OH)_3$, when in solution, is a weak acid molecule and can be dissociated at physiological pH (e.g. 5.9), and its movement is greatly influenced by transpiration rates (Nable et al., 1997; Bacon et al., 1998). Lotus has an extremely high rate of transpiration that might have facilitated the movement of B to other tissues such us the premature dead leaves (Table 3.7).

Chromium: Trace metal concentrations in aquatic plants vary considerably according to the part of the plant and the chemical characteristics of the element. Cr usually lacks mobility from roots to the petioles and leaves. Cr concentrations in plants usually range from 0.2 to 1 μg g^{-1} DW (Prasad, 1999). Cr is considered a toxic heavy metal, and its deleterious effects on root

development, protein biosynthesis, petiole and leaf growth reduction on different plants has been well documented (Shanker, et al., 2005). In some species, soluble Cr toxicity (0.6 mg/L) induces a reduction of petiole lengths (Francko et al., 1993), and can reduce protein content in roots by 79 %, 75 % in leaves, and 57 % in rhizomes (Chandra and Kulshreshtha, 2004). Finding a plant with a high tolerance or hyper-accumulation capacity for chromium (Cr) is very important in some areas. In India, about 2,000 to 32,000 tonnes of Cr escape into the environment from the tanning industries alone and levels in the effluent usually exceed the recommended permissible limit (2 mg/L) by 100 to 250 % (Shanker et al., 2005). Cr has low mobility in plant tissues and the reason for that may be due to barriers or to lack of transport mechanisms (Baldantoni et al., 2004). This might explain why we measured higher concentrations in rhizomes than in the above ground tissues. Our data also indicated that leaves contained toxic levels of Cr. Others have determined that lotus can accumulate more than 3,000 mg/L (Gardea-Torresdey et al., 2005) of Cr that might explain why in some heavy Cr polluted areas lotus exhibited luxuriant growth (Vajpayee et al., 1999). The high production of rhizomes by lotus plants (Hicks, 2005) makes it a suitable candidate for phyto-remediation of Cr.

Copper: Concentration varies by species and is taken up by the plant in quantities ranging from 2 to 20 mg/L (Mengel and Kirkby, 1987). When evaluating different concentrations of Cu among ornamental cultivars of lotus (Table 3.6.), no differences were found among the tissues (leaves, petioles and rhizomes). However concentrations were higher than those reported in the literature. For plants exposed to different Mn concentrations, different tissues showed varying levels of Cu over time (Table 3.2). Cu accumulated in rhizomes 6 weeks after treatment, but there was not much change in leaves and petioles. These results are in agreement with other studies of Cu removal using wild species of lotus (*N. lutea*). Lung and

Light (1996) estimated that rhizomes showed their highest accumulation level at 2 weeks (37 mg/L) and 6 weeks (32 mg/L). Furthermore, Cu removal from the water column was estimated at a rate of 400 μg / m^2 beginning at week 14, reaching its peak at week 22 of the growing season. Four hundred μg / m^2 is an uptake value that seems to be low. However, a typical urban runoff contains 5 to 200 μg/L (Davis et al., 2001), and wastewater from metal finishing processes has exhibited levels of Cu from 25 to 40 mg/L (Monser and Adhoum, 2002). Compared with other aquatic plants used in phyto-remediation (Upadhyay et al., 2007), lotus is less efficient for Cu removal than water hyacinth (*Eichhornia* crassipes), water lettuce (*Pistia stratiotes*), duckweed (*Lemna minor*), and water velvet (*Azolla pinnata*). In India, *Hydrilla verticillata*, which is considered another heavy metal phyto-remediator, showed a bio-accumulation of 16.3 mg/L while lotus accumulated 1,617 mg/L of Cu (Kumar et al., 2008a).

Iron: Flood conditions increase Fe solubility; levels can increase from 0.1 mg/L to 100 mg/L inducing toxicity in some plants (Mengel and Kirkby, 1987). Our data suggest that lotus is a hyper-accumulator of Fe. Analysis of tissues from different cultivars showed that Fe concentrated mainly in the rhizomes where levels ranged from 2,165 to 5,723 mg/L (data not shown). On plants exposed to different Mn concentrations, concentrations of Fe were initially high, but levels decreased with time. Although, the data did not show an effect of Mn treatments on Fe accumulation, other studies have revealed that Mn is transported into cells via a mechanism similar to that for iron. Mn and Fe, as well as several other divalent transition metals, compete for uptake into a number of different cell systems (Roth and Garrick, 2003). Fe concentrations in leaves and petioles were much lower than in rhizomes (266 to 288 mg/L, respectively). Despite the high levels of Fe, our plants did not show signs of Fe toxicity. This data supports other findings that report that concentrations of Fe within the roots vary

substantially over time (Hansel et al., 2002). The trends observed in this study were also observed for As concentrations on plants treated with Mn (Table 3.3) suggesting a possible interaction of both elements in chemical and physical processes. In a study using *P. arundinacea* and *T. latifo*lia, it was found that As and Fe concentrations in root tissue were correlated [r = 0.7 and 1.0, respectively] (Hansel et al., 2002). Tolerance to high concentrations of Fe, Al, and Mn was thought to be related to the capacity of accumulating high concentrations of Calcium (Ca) and SiO_2 (Mengel and Kirkby, 1987) and older plants show higher tolerance to Fe. Normally, levels of Ca in higher plants range from 5 to 30 mg g^{-1} dry matter (Mengel and Kirkby, 1987). In lotus tissues (Table 3.6), the concentrations of Ca were higher than those reported.

Lead: Pb is considered a major environmental pollutant. Pb in its organic forms (Pb tetraethyl, Pb triethyl, Pb diethyl, etc.) is extremely mobile in the soil and readily taken up by plants (Fischerová et al., 2006). Organic Pb forms can induce a drastic reduction in growth at concentrations as low as 10 mg/L (Mengel and Kirkby, 1987) in some species. Phyto-toxicity and tolerance to Pb differs among species. Cattails (*Typha latifolia*) accumulated high amounts of Pb in their roots (Deng et al., 2004), and *Eichhornia crassipe*s concentrates Pb in the inner root tissues using the root epidermis as a barrier to transport Pb to aboveground tissues (Weis and Weis, 2004). Low concentrations of Pb were observed in the rhizomes (78 mg/L) of lotus. Aqeel-Ashraf et al. (2010) observed that lotus can accumulate 518 mg/L Pb in the rhizomes, 71 mg/L in petioles, and 110 mg/L in the leaves.

Manganese: In studies involving containerized lotus, high concentrations of Mn were measured in plants ranging from 1,580 mg/L (initial) to 2,014 mg/L 70 days after planting (Tian et al., 2009), and other researchers reported levels of hyper-accumulation of Mn in the leaves

(Hicks, 2005; Nguyen 2001). The high tolerance of Mn in lotus plants is related to its high biomass (Upadhyay et al., 2007). Lotus has the capacity of producing large amounts of biomass, developing a large leaf area index (Nohara and Tsuchiya, 1990), and its main rhizome can reach 11 m (Masuda et al., 2006). Plants have developed up to 215 leaves in a growing season (Nguyen, 2001) and these leaves have high concentrations of heavy metal chelating compounds (e.g. polyphenols). Hyper-tolerance to Mn concentrations is being attributed to these compounds in some other species. *Trapa natans* accumulate Mn in the floating lamina in the form of phenolic compounds (Baldisserotto et al., 2010). Analysis on changes in phenolic compound concentrations in lotus plants exposed to high Mn needs to be further evaluated.

Nickel: Relatively few plants are able to accumulate Ni exceeding concentrations of 15 mg/L (Brooks et al., 1977). Lotus cultivars showed levels in rhizomes higher than those in leaves and petioles. Lotus rhizomes do accumulate high levels of Ni as compared to underground tissues of 14 Iranian crops irrigated with urban wastewater (Harati et al., 2011). For phyto-remediation, plants must possess efficient mechanisms to detoxify the accumulated metals (Salt et al., 1998). Lotus and other Ni tolerant species might have the capacity to flush the metal from the cytoplasm into vacuoles (Krämer et al., 2000). In 'Camellia Red', high concentrations of Ni were found in the rhizomes (194 to 345 mg/L), leaves (13 to 32 mg/L), petioles (25 to 40 mg/L), and torus (141 mg/L) (data not shown). However, ultra-cellular studies in lotus are lacking and should be considered to evaluate where Ni accumulates in the tissue.

Sodium: Although Na is not considered essential to plant growth, its presence has a marked effect on the growth of some plants (Flowers et al., 1977) by affecting the electrical conductivity of the solution and consequently the nutrient availability of plant roots (Yeager, 2007). Some studies reported positive effects of Na on biomass accumulation by increasing the

relative amount of lipid, lignin, and cellulose present in organic dry matter. Miller (1938) reported that carbohydrate accumulation in K deficient onion plants could be enhanced if plant nutrition is supplemented with Na. In lotus, Na has been reported to range from 0.035 to 0.142% in leaves and 0.2 to 0.8 % in the petioles (Nguyen, 2001). In addition, Na affects the EC of the nutrient solution that has an effect on total dry mass accumulation. Hicks (2005) determined that total dry mass increased from 0.77 g plant $^{-1}$ (EC_0) to a peak of 12.7g (EC_{200}) before declining sharply to 3.0 g at higher EC (EC_{300}). Mn treatments led to higher accumulation of Na (Table 3.5) and concentrations in both the aboveground and underground tissues of the ornamental lotus were surprisingly high (Table 3.6). In this study, we found hyper-accumulation of Na in all tissues, but in much higher levels in the petioles followed by rhizomes with lower levels in the leaf. The data suggest that lotus might be suitable for growing in areas where saline water intrusion had occurred.

Zinc: Zn is an essential trace element for plants (Fischerová et al., 2006). It is an important component of vital enzymes; it is a structural stabilizer for proteins, membrane and DNA-binding proteins (Aravind et al., 2003). The mean concentration of Zn in normal plants (above ground tissues) is 66 mg/L and the toxic level is up to 230 mg/L (Deng et al., 2004). Water hyacinth (*Eichhornia crassipes*) accumulates Zn in the inner root tissues, especially cell walls and within the cells of the vascular bundles. In this study, Zn accumulated preferentially in the rhizomes, followed by the petioles and in lower concentration in the leaves. Consistent with observations from others (Cardwell et al., 2002) emergent aquatic macrophyte roots contained higher amounts of Zn than the corresponding stems and leaves. The mobility of this element has been reported to be very low, and in older leaves can become very immobile (Mengel and Kirkby, 1987). Other researchers (Kumar et al., 2008b) found similar

concentrations of Zn (128 mg/L) in lotus rhizomes as those found in this study. Recent studies (Aqeel-Ashraf et al., 2010) found that lotus rhizomes can accumulate as much as 810 mg/L of Zn. Zn concentrations from treated plants seem not to be affected by the increasing Mn treatment concentrations. Mn shows properties similar to other cations such as Mg^{2+}, Ca^{2+}, Zn and Fe. So these ion species affect uptake and translocation of Mn in the plant (Mengel and Kirkby, 1987).

Findings show highest levels of Al, Cr, Fe, Na, Ni and Zn in the rhizomes. Moreover, lotus rhizomes showed As concentrations that surpass the reported threshold toxicity level. The premature dying of some leaves and the unusual concentration of heavy metals (Table 3.7). High tolerance of lotus plants to high concentrations of heavy metals is related to its high biomass production (Upadhyay et al., 2007) in a short period of time (a growing season). These plants have the capacity to develop a large leaf area index (Nohara and Tsuchiya, 1990). Plants can produced up to 291 g dry weight m^{-2} during peak growth. In addition, its main rhizome reaches 11 meters (Masuda et al., 2006) and some cultivars can produce more than 40 tonnes of rhizomes per year, per hectare. This plant is a good candidate to be used as phyto-remediator of polluted waters from nurseries, intense aquaculture systems, waste water treatment plants and other industrial and agricultural facilities.

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Table 3.1: Nutrient composition of soil, substrate, water and fertilizer used in the study.

	Composition analysis (mg/L)									
Sample	Ca	K	Mg	P	Al	As	B	Cd	Cr	Cu
Water	8	1	3	0.3	0.1	0.1	0.1	0.1	0.1	0.1
Soil	90	17	34	2	37	13	1.1	0.1	0.1	0.3
Substrate mix	215	41	77	3	41	13	0.9	0.1	0.1	0.3

	Fe	Mn	Na	Ni	Pb	Zn	SS	pH	EC (mmhos/cm)
Water	0.1	0.1	3	0.1	0.1	0.1	65	7	0.1
Soil	45	12	15	0.1	0.3	0.3			
Substrate mix	41	14	17	0.1	0.2	0.8			

Chemical composition of the fertilizer (20-20-20 TotalGro™) solution use in the study.[z]

Macro nutrients	Percentage	mg/L in solution
Total nitrogen	20	150
Phosphate P_2O_5 44% P	20	66
Potassium K_2O 83% K	20	124.5
Micronutrients		
Boron	0.020	0.150
Copper	0.050	0.375
Iron chelated	0.100	0.750
Manganese chelated	0.050	0.375
Molybdenum	0.001	0.008
Zinc	0.050	0.375

[z]Compounds are derived from Ammonium phosphate, Potassium nitrate, Urea, Iron EDTA, Mn EDTA, Cu EDTA, Zn EDTA, Sodium borate, and Sodium molybdate.

Table 3.2: Effects of time, tissue type, and manganese (Mn) in solution (0, 5, 10, 15, and 50 mg/L) on the accumulation of Mn in lotus tissues.

	Leaves				Rhizomes				Petioles			
Trt	2 weeks	4 weeks	6 weeks	Sign.[wx]	2 weeks	4 weeks	6 weeks	Sign.[wx]	2 weeks	4 weeks	6 weeks	Sign.[wx]
0	632 ns[z]	1073 ab	792 ns	ns	328 ns	1635 a	347 ns	Q***	437.7ns	316 bc	231 ns	ns
5	688 ns	1118 b	1087 a	ns	405 ns	2170 a	188 c	Q***	499.1ns	2199 a	334 bc	Q***
10	882 ns	1507 ns	1459 a	ns	527 ns	2262 ns	282 c	Q***	804.1ns	1445 ns	474 bc	Q***
15	876 ns	1718 ns	1026 a	Q*	501 ns	2460 ns	332 c	Q***	607.9ns	2202 ns	433 bc	Q***
50	1632 ns	3446 a	2223 a	Q***	1154 ns	1632 bc	881 bc	Q***	731.9ns	1396 c	696 c	Q*
Sign.[wy]	L**	L***	L***		L**	Q**	L**		ns	Q***	L*	

[w]Orthogonal polynomial contrasts were used to determine the main effects on Mn accumulation in tissues. The tissue * Trt *harvest interaction was significant. Significant levels are described as: Non-significant (ns), linear (L), or quadratic (Q) at α= 0.05 (*), 0.01 (**); or 0.001 (***).

[x]Simple effects over time (rows):

Leaves: Trt 15.0 mg/L ($y= -59.24375 + 552.23958x - 133.31250x^2$, $r^2 = 0.33$); and Trt 50.0 mg/L ($y = -1518.6x^2 + 6369.4x - 3218.1$, $r^2 = 0.30$).

Rhizomes: Trt 0.0 mg/L ($y = -91.827x + 885.52$, $r^2 = 0.01$); Trt 5.0 mg/L ($y = -1873.2x^2 + 7384.1x - 5105.7$, $r^2 = 0.99$).

Trt 10.0 mg/L ($y = -1857.4x^2 + 7307.5x - 4923.5$, $r^2 = 0.98$); Trt 15.0 mg/L ($y = -2043.8x^2 + 8090.7x - 5545.8$, $r^2 = 0.95$).

Trt 50.0 mg/L ($y = -614.28x^2 + 2320.3x - 551.52$, $r^2 = 0.64$).

Petioles: Trt 5.0 mg/L ($y = -1782.5x^2 + 7047.3x - 4765.6$, $r^2 = 0.80$); Trt 10.0 mg/L ($y = -805.79x^2 + 3058.7x - 1449.3$, $r^2 = 0.85$).

Trt 15.0 mg/L ($y = -1681.8x2 + 6639.8x - 4350.2$, $r^2 = 0.41$); Trt 50.0 mg/L ($y = -681.91x2 + 2709.6x - 1295.2$, $r^2 = 0.46$).

[z]Simple effects over concentration (columns):

Leaves: 2 weeks ($y = 20.13x + 629.61$, $r^2 = 0.80$); 4 weeks ($y = 49.054x + 985.45$, $r^2 = 0.73$); and 6 weeks ($y = 26.112x + 897.62$, $r^2 = 0.38$).

Rhizomes: 2 weeks ($y = 16.494x + 319.03$, $r^2 = 0.72$); 4 weeks ($y = -1.5349x^2 + 75.297x + 1701.8$, $r^2 = 0.29$); and 6 weeks ($y = 13.189x + 187.49$, $r^2 = 0.69$).

Petioles: 4 weeks ($y = -2.5378x^2 + 140.3x + 713.41$, $r^2 = 0.20$); and 6 weeks ($y = 8.1882x + 303.34$, $r^2 = 0.42$).

[z]Least squares means. Mean separation was performed to determined at each harvest – column (2, 4, or 6 weeks), which tissue (leaf, rhizome, or petiole), at each treatment (row) accumulated the most Mn. Differences in means were determined using Tukey's test α = 0.05.

Table 3.3: Accumulation of As, Cr, Cu, Fe, Ni and Pb (mg/L) in lotus tissues over time (2, 4, or 6 weeks).

	Arsenic			Chromium			Copper		
Harvest	Leaves	Rhizomes	Petioles	Leaves	Rhizomes	Petioles	Leaves	Rhizomes	Petioles
2	1 b[y]	5 a	1 b	2 bc[2]	6 a	1 c	19 bc	37 a	15 c
4	1 ns	10 ns	5 ns	12 ns	8 ns	14 ns	21 ns	17 ns	22 ns
6	3 b	14 a	2 b	1 c	7 b	8 ab	21 b	32 a	16 c
[wx]Sign.	ns	L***	ns	Q**	ns	Q**	ns	Q***	Q**
	Iron			Nickel			Lead		
Harvest	Leaves	Rhizomes	Petioles	Leaves	Rhizomes	Petioles	Leaves	Rhizomes	Petioles
2	377 b	6405 a	191 c	21 bc	731 a	16 c	2 ns	5 ns	1 ns
4	145 ns	254 ns	131 ns	47 ns	40 ns	33 ns	6 ns	5 ns	10 ns
6	202 b	3478 a	200 b	16 c	163 a	35 bc	27 a	3 c	13 b
[wx]Sign.	Q***	Q***	Q*	ns	L***	ns	L***	ns	ns

[w] Orthogonal polynomial contrasts were used to determine the main effects on As, Cr, Cu, Fe, Ni, or Pb accumulation in tissues. Only main effects tissue and harvest were significant. Significant levels are described as: Non-significant (ns), linear (L), or quadratic (Q) at α= 0.05 (*), 0.01 (**); or 0.001 (***).

[x]Simple effects over time (columns):

Arsenic in leaves: ($y = 5.4023x - 0.5895$, $r^2 = 0.04$).
Chromium in leaves: ($y = -10.863x^2 + 42.938x - 29.861$, $r^2 = 0.47$) and in petioles: ($y = -9.9781x^2 + 43.82x - 33.27$, $r^2 = 0.08$).
Cooper in rhizomes: ($y = 17.598x^2 - 73.266x + 92.885$, $r^2 = 0.44$) and petioles: ($y = -10.044x^2 + 41.534x - 16.254$, $r^2 = 0.06$).
Iron in leaves: ($y = 134.34x^2 - 622.3x + 854.11$, $r^2 = 0.26$); in rhizomes: ($y = 4579.2x^2 - 19889x + 21714$, $r^2 = 0.58$), and petioles: ($y = 61.825x^2 - 245.36x + 374.6$, $r^2 = 0.07$).
Nickel in rhizomes: ($y = -238.15x + 817.1$, $r^2 = 0.34$).
Lead in leaves: ($y = 13.793x - 15.046$, $r^2 = 0.31$).

[y]Least squares means. Mean separation was performed to determine at each harvest – row (2, 4, or 6 weeks) which tissue (leaf, rhizome, or petiole) accumulated the most As, Cr, Cu, Fe, Ni, or Pb. Differences in means were determined using Tukey's test $\alpha = 0.05$.

Table 3.4: Effects of manganese (Mn) treatments, tissue type, and time of exposure on the accumulation of Aluminum (Al) and Boron (B) in lotus.

	Aluminum (mg/L)											
	Leaves				Rhizomes				Petioles			
Trt	2 weeks	4 weeks	6 weeks	Sign.[x]	2 weeks	4 weeks	6 weeks	Sign.[x]	2 weeks	4 weeks	6 weeks	Sign.[x]
0	191 b[z]	66 ns	135 b	ns	7094 a	101 ns	2084 a	L***	169 b	104 ns	184 b	ns
5	102 b	145 ns	77 ns	ns	9591 a	101 ns	917 ns	L***	106 b	71 ns	78 ns	ns
10	217 b	77 ns	120 b	ns	7308 a	113 ns	2099 a	L***	154 b	57 ns	111 b	ns
15	155 b	59 ns	67 b	ns	5349 a	107 ns	1334 a	L***	109 b	60 ns	96 b	ns
50	158 b	68 ns	106 b	ns	7835 a	88 ns	2825 a	L***	91 b	53 ns	204 b	ns
Sign.[wy]	ns	ns	ns		Q***	ns	L***		ns	ns	ns	
	Boron (mg/L)											
	Leaves				Rhizomes				Petioles			
Trt	2 weeks	4 weeks	6 weeks	Sign.[x]	2 weeks	4 weeks	6 weeks	Sign.[x]	2 weeks	4 weeks	6 weeks	Sign.[x]
0	107 a[z]	22 c	26 a	L***	47 bc	50 b	10 bc	L***	36 c	52 ab	8 c	L***
5	106 a	45 bc	26 a	L***	51 bc	82 a	7 b	L***	30 c	32 c	7 b	L***
10	99 a	37 bc	38 a	L***	42 bc	82 a	9 c	L***	27 c	25 c	17 bc	ns
15	98 a	26 c	21 ab	L***	44 bc	74 a	8 c	L***	29 c	32 bc	19 bc	ns
50	93 a	28 b	42 a	L***	46 bc	87 a	10 bc	L***	32 c	29 b	7 c	L***
Sign.[wy]	L***	ns	L***		ns	L**	ns		ns	Q*	Q***	

[w]Orthogonal polynomial contrasts were used to determine the main effects on Mn accumulation in tissues. The tissue * Trt interaction was significant. Significant levels are described as: Non-significant (ns), linear (L), or quadratic (Q) at $\alpha = 0.001$ (***).

[x]Simple effects over time (rows):

Al accumulation for different tissues: Rhizomes: Trt 0 ($y = -2009.2x + 7442$, $r^2 = 0.38$); Trt 5 ($y = -3692.8x + 11351$, $r^2 = 0.68$); Trt 10 ($y = -2030.6x + 7617$, $r^2 = 0.31$); Trt 15 ($y = -1603.3x + 5739.6$, $r^2 = 0.26$); and Trt 50 ($y = -1895.8x + 7780.8$, $r^2 = 0.30$).

B accumulation for different tissues (rows):

Leaves: Trt 0 ($y = -35.692x + 126.29$, $r^2 = 0.70$); Trt 5 ($y = -37.309x + 135.2$, $r^2 = 0.80$); Trt 10 ($y = -27.349x + 117.39$, $r^2 = 0.70$); Trt 15 ($y = -36.501x + 125.9$, $r^2 = 0.71$); and Trt 50 ($y = -20.416x + 98.114$, $r^2 = 0.44$).

Rhizomes: Trt 0 ($y = -20.464x + 74.855$, $r^2 = 0.59$); Trt 5 ($y = -28.797x + 99.733$, $r^2 = 0.53$); Trt 10 ($y = -23.687x + 86.883$, $r^2 = 0.42$); Trt 15 ($y = -23.961x + 86.207$, $r^2 = 0.51$); and Trt 50 ($y = -25.121x + 93.353$, $r^2 = 0.40$).

Petioles: Trt 0 ($y = -17.073x + 64.197$, $r^2 = 0.26$); Trt 5 ($y = -13.581x + 49.034$, $r^2 = 0.74$); Trt 10 ($y = -23.687x + 86.883$, $r^2 = 0.42$); and Trt 50 ($y = -13.54x + 49.008$, $r^2 = 0.78$).

[y]Simple effects over concentrations (columns):

Al accumulation for different tissues: Rhizomes: At 2 weeks ($y = 3.2859x^2 - 176.73x + 8390.4$, $r^2 = 0.07$); and at 6 weeks ($y = 25.496x + 1418$, $r^2 = 0.14$).

B accumulation for different tissues: Leaves: At 2 weeks ($y = -0.2723x + 104.97$, $r^2 = 0.05$); and at 6 weeks ($y = 0.0799x^2 - 4.3438x + 123.73$, $r^2 = 0.07$).

Rhizomes: At 4 weeks ($y = 0.4567x + 67.704$, $r^2 = 0.18$).

Petioles: At 4 weeks ($y = 0.0307x^2 - 1.8899x + 47.126$, $r^2 = 0.12$); and at 6 weeks ($y = -0.0243x^2 + 1.2565x + 5.1191$, $r^2 = 0.23$).

[z]Least squares means. Mean separation was performed to determined at each harvest – column (2, 4, or 6 weeks), which tissue (leaf, rhizome, or petiole), at each treatment (row) accumulated the most Al or B. Differences in means were determined using Tukey's test $\alpha = 0.05$.

Table 3.5: Effects of Mn treatments and time of exposure in the accumulation of Sodium (Na) and Zinc (Zn) in lotus tissues.

	Sodium (mg/L)										
	Leaves				Rhizomes				Petioles		
Trt	2 weeks	4 weeks	6 weeks	Sign.[wx]	2 weeks	4 weeks	6 weeks	Sign.[wx]	2 weeks	4 weeks	6 weeks	Sign.[wx]
0	772 c[z]	632 c	1844 c	ns	4292 ab	1143 bc	7643 ab	Q***	5200 a	5396 a	8789 a	L***
5	806 c	733 ns	1347 c	ns	4208 ab	2852 ns	6339 b	Q**	5324 a	526 ns	8257 a	Q***
10	851 c	525 ns	1469 c	ns	4779 ab	3009 ns	7022 ab	Q***	5500a	550 ns	5516 b	Q***
15	1201 c	592 ns	1162 c	ns	4945 ab	2787 ns	5789 ab	Q**	5080 a	2837 ns	4486 b	Q*
50	799 c	483 ns	1197 b	ns	4072 bc	2635 ns	6109 a	Q**	5293 a	606 ns	6821 a	Q***
Sign.[wy]	ns	ns	ns		ns	ns	ns		ns	Q*	Q***	
	Zinc (mg/L)											
	Leaves				Rhizomes				Petioles			
Trt	2 weeks	4 weeks	6 weeks	Sign.[wy]	2 weeks	4 weeks	6 weeks	Sign.[1y]	2 weeks	4 weeks	6 weeks	Sign.[wy]
0	51 b[z]	35 c	41 c	ns	96 a	50 bc	109 a	Q***	44 b	83 ab	50 bc	Q**
5	52 b	43 bc	33 c	ns	94 a	92 a	104 a	ns	45 b	36 c	52 bc	ns
10	39 c	44 bc	40 c	ns	102 a	119 a	101 a	ns	40 bc	34 c	74 bc	L**
15	39 c	42 b	38 c	ns	96 a	92 a	83 a	ns	47 bc	46 b	40 b	ns
50	59 ns	49 ns	39 c	ns	79 ns	63 ns	94 a	ns	50 ns	41 ns	61 b	ns
Sign.[wy]	ns	ns	ns		ns	Q***	ns		ns	Q*	ns	

[w]Orthogonal polynomial contrasts were used to determine the main effects on Mn accumulation in tissues. A three way interaction was significant. Significant levels are described as: Non-significant (ns), linear (L), or quadratic (Q) at α= 0.05 (*), 0.01 (**); or 0.001 (***).

[x]Simple effects over time (rows):
Na accumulation for different tissues: Rhizomes: Trt 0 ($y = 4439.4x^2 - 16468x + 16321$, r2 = 0.87); Trt 5 ($y = 2421.8x^2 - 8621.7x + 10408$, $r^2 = 0.64$); Trt 10 ($y = 2890.9x^2 - 10442x + 12330$, $r^2 = 0.52$); Trt 15 ($y = 2579.7x^2 - 9896.9x + 12262$, $r^2 = 0.38$); and Trt 50 ($y = 2455.4x^2 - 8803.6x + 10420$, $r^2 = 0.38$). Petioles: Trt 0 ($y = 1962.5x + 2649.1$, $r^2 = 0.59$); Trt 5 ($y = 6176.8x^2 - 23328x + 22475$, $r^2 = 0.86$); Trt 10 ($y = 4957.7x^2 - 19823x + 20365$, $r^2 = 0.63$); Trt 15 ($y = 1946x^2 - 8080.9x + 11215$, $r^2 = 0.38$); and Trt 50 ($y = 5451.6x^2 - 21043x + 20885$, $r^2 = 0.75$).
Effect Zn accumulation for rhizomes: Trt 0 ($y = 52.623x^2 - 203.28x + 245.47$, $r^2 = 0.60$). Petioles: Trt 0 ($y = -35.705x^2 + 145.93x - 66.302$, $r^2 = 0.51$); and Trt 15 ($y = 13.188x + 22.984$, $r^2 = 0.11$).

[y]Simple effects over concentration (columns):
Na accumulation in petioles: At 4 weeks ($y = 3.5429x^2 - 238.81x + 3784.5$, $r^2 = 0.21$); and at 6 weeks ($y = 7.4881x^2 - 425.03x + 9336.5$, $r^2 = 0.40$).
Zinc accumulation: Rhizomes: At 4 weeks ($y = -0.0952x^2 + 4.7927x + 60.171$, $r^2 = 0.21$). Petioles: At 4 weeks ($y = 0.0531x^2 - 3.2301x + 69.641$, $r^2 = 0.31$).

[z]Least squares means. Mean separation was performed to determined at each harvest – column (2, 4, or 6 weeks), which tissue (leaf, rhizome, or petiole), at each treatment (row) accumulated the most Na or Zn. Differences in means were determined using Tukey's test $\alpha = 0.05$.

Table 3.6: Elemental concentration of different tissues of ornamental lotus (base line).

	Percentage				mg/L			
Tissues [y]	Ca	K	Mg	P	Al	As	B	Cd
Leaf	1.6 ns[z]	2.2 ab	0.5a	0.5ns	83 b	2 ns	19 ns	0.4ns
Petiole	0.7 ns	2.8 a	0.5b	0.6	110 b	15 ns	6 ns	2 ns
Rhizome	0.7 ns	1.8 b	0.3ab	0.6	4589 a	14 ns	15 ns	1 ns

	mg/L							
	Cr	Cu	Fe	Mn	Na	Ni	Pb	Zn
Leaf	1 b	14 ns	127 b	406 ns	580 b	11 b	8 ns	38 b
Petiole	2 b	19 ns	93 b	190 ns	4461 a	12 b	10 ns	66 b
Rhizome	5 a	24 ns	3944 a	225 ns	3300 ab	265 a	22 ns	121 a

[y]Tissues were obtained from ornamental lotus cultivars: 'Ms. Slocum', 'Perry's Superstar', 'Rosea Plena' and 'Camellia Red'.
[z]Mean separation was performed using Tukey's test, to determined which tissue (leaf, rhizome, or petiole) have the highest concentration of the individual element (column). Different letters represent significant differences at $\alpha = 0.05$.

Table 3.7: Chemical composition of young lotus leaves from 'Camellia Red' that died prematurely showing Mn toxicity symptoms[y].

		Percentage				mg/L											
Harvest[z]	Trt	Ca	K	Mg	P	Al	As	B	Cd	Cr	Cu	Fe	Mn	Na	Ni	Pb	Zn
2 weeks	0	2	2	0.7	0.5	249	1	202	0.2	12	15	625	1142	2546	217	0.1	73
	5	2	1	0.9	0.6	254	2	182	0.1	6	11	741	1130	2178	50	0.3	154
	10	2	1	0.7	0.5	459	1	158	0.1	2	12	610	1261	832	62	0.1	51
	15	1	2	0.6	0.2	165	1	103	0.1	17	16	374	536	1585	144	4	77
	50	2	1	0.8	0.4	163	0.2	158	0.1	25	12	458	693	1299	79	0.1	91
4 weeks	0	2	1	0.8	0.4	104	0.1	71	2	3	12	204	2051	2753	7	6	109
	5	2	1	0.8	0.4	98	0.1	82	1.0	15	14	362	1856	2692	77	8	67
	10	2	1	0.8	0.4	105	0.1	69	1.0	6	14	201	2747	2711	22	2	97
	15	2	2	0.7	0.5	111	14	90	1.2	1	14	286	2024	2829	11	4	98
	50	2	2	0.8	0.5	101	0.1	78	3	5	18	235	2402	2842	17	2	62

[y]For some plants, Mn toxicity symptoms are generally characterized by brown spots in the leaves (Mengel and Kirkby, 1987).

[z]Damaged leaves (1 to 3) were collected only in harvest 1 and 2 (2 and 4 weeks), and harvest 3 (6 weeks) did not produced any.

CHAPTER 4

PHYTO-REMEDIATION OF NUTRIENT RUN-OFF FROM A RECIRCULATING AQUACULTURE SYSTEM USING LOTUS (*NELUMBO NUCIFERA* GAERTN.)

4.1. ABSTRACT

A limited variety of grasses and semi-aquatic plants have been used in constructed wetlands (CWs) to reduce water pollutants from sewage and industrial and agricultural runoff. Most of these plant species have low commercial/ornamental value, and the land used to build the CWs is lost to production. The potential of lotus to reduce nutrient runoff from intense aquaculture systems was evaluated because lotus produces a high biomass in a short season. The crop is appreciated as an ornamental plant and used as a vegetable and medicinal plant. In this study, planted CWs (V) and non-planted (NV) with 3 different Hydraulic Retention Times (HRT) of 24, 48 and 72 hours were used. NH_4^+ was significantly reduced in V CWs and increasing HRT induced a linear decrease in NH_4^+ levels. Independently of vegetation, nitrates and phosphorous levels were decreased with increasing HRT. Suspended solids decreased in both V and NV CWs with increasing HRT. Nutritional analyses from the lotus plant tissues (before and after 12 weeks) showed that HRT increased Ca concentrations in leaves. HRT reduced the levels of K and Mg concentrations and increased P levels. Al, Fe, Na and Ni in the rhizomes and petioles (Na) reached levels of hyper-accumulation with concentrations ranging from >1,000 to > 6,000 mg/L. Plants growing in CWs with a 24h HRT showed the highest biomass accumulation (fresh weight). Fish effluent provided an excellent source of nutrients for lotus while the plant substantially reduced nutrient run-off. In addition, lotus has the potential of hyper-accumulating some heavy metals.

4.2. INTRODUCTION

The Black Belt Region is the center of aquaculture business in Alabama and stretches from eastern, south-central Alabama into northwestern Mississippi. In the 1820s and 30s, the Black Belt was a rich cotton-growing area. By the 1950s, after years of soil erosion and the invasion of the boll weevil (*Anthonomus grandis*), the cotton industry collapsed. Failure to diversify agriculturally and economically along with urban exodus combined to mire the southern Black Belt in a seemingly irreversible decline. Environmental conditions now provide hope for new industries and crops. The Alabama Black Belt Region supports more than 10,000 ha of aquaculture. Despite current production, the state has the land and water resources to support an industry 10 times its current size (Crews and Chappell, 2007). However, to accomplish this expansion an adoption of more intensive culture techniques is required. Intensive aquaculture requires high fish stocking densities, large energy inputs, and thorough water quality monitoring and treatment (Lin et al., 2002). Recirculating systems are semi-closed systems in which water flowing through a series of tanks or raceways is captured, treated, and reused (Sindilariu et al., 2007). In intensive and semi-intensive aquaculture, large amounts of feed are used in the confined waters of ponds, lakes, and greenhouses. In the southern United States, catfish are fed during spring, summer, and early fall. Fish assimilate less than 30% of the nitrogen supplied through feeding. Large quantities of nitrogen, phosphorus, and other elements accumulate in ponds. In Alabama most catfish ponds are not drained and the pond assimilates most of the nutrients (Boyd et al., 2000). In other parts of the world, production ponds are commonly drained at harvest, making up a large constituent of water pollution in many developing countries (Lin et al., 2002). This culture technique is a large constituent of water pollution in many developing countries. Discharged water from aquaculture production can lead

to eutrophication of surrounding watersheds, ammonia toxicity, and increased biological oxygen demand [BOD] (Boyd and Queiroz, 2001; Neori et al., 1996). Discharged water from intensive systems has higher concentrations of nutrients and other pollutants than traditional culture techniques (Ferdoushi et al., 2008).

Today, the removal of excessive nutrients before the water is discharged to streams is regulated by the state through the implementation of the National Pollutant Discharge Elimination System (NPDES) permit (EPA, 2010). One of the most economical means of water treatment for aquaculture effluent is bioremediation and bio-filtration. Bioremediation is the process in which plants and/or bacteria assimilate nutrients into plant or organic biomass thereby increasing water quality of discharged effluents. These techniques have been suggested as reasonable and economic ways to improve the quality and reduce the volume of pond effluents (Boyd, 2003). Bio-filtration can be accomplished in combination with other methods including physical filtration, chemical filtration and biological filtration. At the Integrated Aquaculture Research Greenhouse at the E.W. Shell Fisheries and Aquaculture Research Station, the fish production system was based on biofloc technology (BTF) in which no external bio-filter or water treatment was used. BTF is based on running of the pond using minimal water exchange, with subsequent development of dense microbial population and management of the microbial population through the adjustment of the C/N ratio so that it controls inorganic nitrogen concentration in the water. The bacteria, forming bioflocs, assimilate TAN (Total Ammonia Nitrogen), produce microbial proteins and facilitate recycling of the unused feed protein (Avnimelech, 2010). Biological filtration is very important because the aquaculture wastewater contains high amounts of total nitrogen (N) as ammonia and ammonium ($NH_3 + NH_4^+$). These N forms are products of protein catabolism, and are excreted as NH_3 through fish gills and also is

produced by aerobic and anaerobic transformations (ammonification) of fish feces and uneaten food. Ammonium is also converted to nitrites (NO_2^-) and further to nitrate (NO_3^-) by aerobic bacteria (nitrification). Nitrite toxicity can occur and is due to diffusion across the fish gills and reaction with iron or copper in the hemoglobin creating methaemoglobin which is unable to bind and transport oxygen, killing the fish (Snow and Ghaly, 2008). Increasing water quality parameters to favorable conditions for increased nitrification allows for less water exchange and increased production efficiency. CWs integrated with plant production (aquaponics) can potentially clean and return water to the system for re-use and decrease the amount of discharged water entering surrounding watersheds. Plants produced can add extra income to the farmer's bottom line and also help maintain desirable water quality for fish production.

In Alabama, effluents from pond culture of channel catfish (*Ictalurus punctatus*) result mainly from storm overflow in winter and early spring. Treatment of storm overflow by sedimentation is not feasible because settling basins with enough volume to provide a hydraulic retention time of 8 h would often be larger than production areas (Boyd and Queiroz, 2001). Development of CWs planted with macrophytes might allow filtration of large volumes of water. To enhance the efficiency of CWs, new plants should be evaluated.

N. nucifera, has a high bio-mass production capacity [215 leaves/season/plant and a rhizome production of 40 t/ha] (Nguyen, 2001). Lotus rhizomes are very rich in starch (Lianjung et al., 2006) and their chemical composition has the potential to absorb some of the nutrients contained in fish effluents. Other qualities of lotus are its ornamental value with hundreds of cultivars (Wang and Zhang, 2004) and its medicinal value (Tian, 2008). Lotus has the potential to be used in CWs for improving water quality, providing natural habitats for wildlife, and

increasing aesthetics. The objective of this study was to determine the effects of growing lotus in CWs with different fish effluent retention times and to evaluate changes in water quality.

4.3. MATERIALS AND METHODS

Studies were performed at the Integrated Aquaculture Research Greenhouse Complex at the E.W. Shell Fisheries and Aquaculture Research Station, Auburn, AL (Latitude=34.18 N, Longitude = 86.85 W, Elevation = 242 m, USDA Zone 8b). The complex consists of 2 greenhouses. One greenhouse was the fish producing unit and the other greenhouse was the plant producing unit. In the fish producing greenhouse, there is an intense aquaculture system used for production of tilapia (*Oreochromis niloticus*). Fish were stocked at 80 fish/m^3 in two 125 m^3 polyethylene lined tanks (dimensions: 27.4 m x 3.8 m x 1.2 m). Feeding rates ranged between 22 to 34 kg of feed per tank per day increasing as fish biomass increased. Water (fish effluent) was exchanged at a rate between 1 to 6% daily.

Experiment I (March – December 2008)**:** Vessels (586 liter volume, with a length of 198 cm, width of 78.4 cm, and height of 63.3 cm) were filled with 0.35 m^3 of expanded clay (HydRocks®, Big River Industries, Alpharetta, GA) and fish effluent. A perforated PVC pipe was inserted beneath the substrate for drainage. One set of 3 vessels (CWs) were planted (V) with 3 lotus plants (*N. nucifera*) and one vessel without plants (NV). Medium size lotus plants from three different cultivars ('Rosea Plena'; 'Ms. Slocum', and 'Perry's Superstar') were selected for each vessel. Four vessels received fresh fish effluent (125 L) daily (24 hour Hydraulic Retention Time – HRT). The next set of vessels received fresh fish effluent every other day (48h HRT), and the next set of 4 vessels received fresh fish effluent every third day (72h HRT). Water samples were taken daily from the vessels at three locations (inlet of fish

effluent, center of vessel [30 cm below the water surface], and outlet or drainage pipe) during the first six weeks of the experiment. Greenhouse and water temperatures (day and night) were maintained at from 24 to 29 ^{o}C during the experiment. Cooling pads were used to reduce air temperature inside the greenhouses in the hottest days of the summer. At the end of the experiment, dormant plants were harvested and total rhizome biomass (FW) from each vessel was recorded.

Experiment II (May through August 2010)**:** A similar experiment was conducted in 2010. A similar set up from the first experiment was used for this evaluation, but instead of one vessel without plants per retention period, the number of vessels was increased to two, for a total of 15 vessels. Lotus plants (*N. nucifera* '04 – R – 31') were grown from propagules in 11.3 liter containers with substrate composed of sandy clay loam soil and pine bark (50:50). On a weekly basis container water levels were refilled to the same depth with a 150 mg/L N solution of soluble fertilizer 20 N - 8.8 P - 16.6 K (TotalGro™ 20-20-20, STD Industries, Winnsboro, La). Plants were forced to break dormancy in the greenhouse under night-interrupted lighting. For these procedures, from 2 AM to 4 AM, 60 watt incandescent bulbs spaced every meter and 60 cm above the plants, were turned on. Temperatures were maintained above 25 $^{\circ}$C as suggested by Wang and Zhang (2004). Twelve week old plants were placed in the vessels for the CW experiment (3 plants per vessel). Water samples used for analyses were taken every other week. Every 4 weeks, 30 g of chelated iron 10% (Sprint® 330. Becker Underwood, Ames, IA) was applied to the containers to prevent iron deficiency. At the end of the study leaf lamina, petioles and rhizomes (3 plants/ vessel) were collected for chemical analysis. Total fresh weight (FW) and dry biomass from leaves, petioles and rhizomes were recorded.

Plant tissue and effluent analysis: Nutrient characteristics of the wastewater and the effluent from CWs were analyzed at the Auburn University ALFA Soil and Plant Tissue Testing Laboratory, Auburn, Alabama. For total nitrogen and carbon analyses, samples were dried in a forced air oven at 60 °C. Mineral composition of the lotus plants were conducted before the start of the study and after 12 weeks of growth. Plant material or soil samples were prepared and combusted with oxygen catalyst at 1,150 °C. Nitrogen, carbon, and sulfur gases were measured by Thermal Conductivity using an Elementar Vario Macro CNS analyzer (Columbo and Giazzi, 1982; Kirsten, 1979). Ammonium and nitrates' analyses were based on a microscale determination of inorganic N in water and soil extracts (Sims et al., 1995). For dry ashing and organic matter destruction, dried plant material samples were ashed and analyzed by Inductively Coupled Plasma Emission Spectroscopy (Isaac and Johnson, 1985) on a Varian Vista - MPX Axial Spectrometer (Plank, 1992; Odom and Kone, 1997). Water nutrient analysis was determined by Inductively Coupled Plasma (ICP) Atomic Emission Spectrometry (Maxfield and Mindak, 1985) using a Varian Vista-MPX Axial Spectrometer. Electrical conductivity (EC) was based on University of Georgia's protocol for soil testing and plant analysis (Plank, 1992). Determination of EC was also accomplished using a Water YSI Model 31 Conductivity Bridge. Water pH was determined at 25 ^{o}C using a Fisher Accumet® Model 50 pH meter (Thermo Fisher Scientific Inc. IL, US).

Data analysis: Treatments were applied in a 3 x 4 factorial treatment design with a control in a completely randomized design. To estimate the statistical inferences for this generalized linear mixed model, PROC GLIMMIX was used in SAS® statistical software (SAS® version 9.2, SAS Institute, Cary, N.C.). In the case of heterogeneous variance, covariance parameters were calculated using the GROUP option on the RANDOM statement in

PROC GLIMMIX. The use of criterion for the GROUP option provided an improvement in the AIC (Akaike information criterion), and consequently, a more robust criterion for the model selection. Single degree of freedom orthogonal contrasts were used to test linear and quadratic trends over Hydraulic Retention Times – HRT (24, 48, and 72 hours) in V and NV CWs. Contrast analysis was used to determine if HRT (24, 48, and 72 h) have an effect in biomass accumulation (rhizomes) for the first experiment, and for FW and elemental composition of the different tissues (Experiment II).

4.4. RESULTS

Concentrations of various elements, such as arsenic (As), cadmium (Cd), molybdenum (Mo), and nickel (Ni), in the fish tank effluent were very low (<0.2 ± 0.1 mg/L) before and during the experiment. Aluminum (Al), boron (B), chromium (Cr), lead (Pb), manganese (Mn), and zinc (Zn) varied between different sampling days and treatments (data not shown). Iron (Fe) levels were not determined since periodically chelated iron was applied to prevent iron deficiency.

Retention time in the CWs (V and/or NV) had an effect ($P< 0.0004$) on calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), nitrates (NO_3), phosphorus (P), pH, and suspended solids [SS] (Table 4.1). Ca levels increased linearly as HRT increased in both V and NV CWs. In V CWs, potassium levels decreased linearly with increasing HRT. Mg levels increased linearly with increases in HRT but only in V CWs. When compared with the un-treated fish effluents Na levels in the effluent from CWs showed a higher level of Na, regarless of presence or absence of plants. Lotus plants did reduce the levels of NH_4^+ and NO_3, especially

at longer retention times. P concentrations decreased to higher levels in V than NV, while SS levels decreased with an increase in HRT, especially in vessels with plants (Table. 4.1).

Several macro and micro nutrients were affected by retention time and their concentration varied according to the type of tissue (Table 4.2). Ca levels increased linearly in leaves and quadratically in the petioles but they did not change in the rhizomes. The higher concentrations were always found in the leaves and there were no differences between the levels in the petioles and rhizomes. P: Phosphorous levels did not change in the leaf and the concentrations showed a quadratic trend in both petioles and rhizomes. The higher levels of P were found in the rhizomes, followed by stem and in lesser amounts in the leaves. Al, As, and Pb: Concentrations in leaves and petioles of these heavy metals were not affected by HRT. However, the levels in the rhizomes showed a quadratic reduction. Fe: Iron accumulations in leaf increased linearly. In rhizomes, levels decreased with an increase in HRT in a quadratic form. In addition, in all treatments (HRT), Fe concentrations in the rhizomes reached levels of hyper-accumulation. No changes in petiole concentrations were noticed. Mn and Nickel levels showed a quadratic reduction in rhizome and petioles. In the rhizomes from the control, Ni levels reached concentrations of hyper-accumulation (> 3,000 mg/L). Most Al, Fe, Ni and Pb accumulated in the rhizome and there were no differences among the other tissues.

Several elements were only affected by the HRT and the type of tissues (Table 4.3). K, Mg, and B: Levels showed a quadratic reduction with increases in retention time. K levels were higher in the petioles with no differences among the other tissues. Mg and B levels were higher in the leaf, followed by the rhizome, and in a lesser amount in the petioles. Cadmium and zinc levels suffered a linear reduction with increases in HRT. Cd levels were higher in both rhizome and stem. However, Cd levels in the leaves were not different from those in the stems. On the

other hand, Zn levels were higher in the rhizome and no differences were found among petioles and leaves.

HRT did not affect the accumulation of Cr and Na. However, tissue types have a significant effect on the levels accumulated. In the case of Cr, most of the element was concentrated in rhizome and petioles. Sodium levels hyper-accumulated in rhizome and petioles (>3,000 mg/L). In the case of copper, only HRT affected the concentrations in the different tissues. As time increased, Cu concentrations showed a linear reduction.

Biomass accumulation: In Experiment I, plants were harvested when they were dormant and only rhizome and runners fresh weight (FW) was recorded. As time increases, FW showed a linear decrease ($y=23.5556x - 0.25448$, $r^2 = 0.6$, $P=0.0007$). Average FW per HRT was 17.8 kg (24h), 9.8 kg (48h), and 5.5 kg (72h). In Experiment II, there were no differences among total FW produced at different HRT treatments averaging 6.1 kgs. Each CWs (with 3 plants each) produced an average of 0.9 kg of leaves; 2.5 kg of petioles; 2.8 kg of runners, 2.2 kg of rhizomes, and 36g of seed pods (torus).

4.5. DISCUSSION

To satisfy the demand for aquatic animal products, farmers are implementing a diversification of species and intensifying productions systems. Currently, aquaculture has been used to secure food supplies and generate income. However, this development also increases the amount of nutrient run-off that is produced by aquaculture farms. In the USA, several studies have recognized that effluent is an important point source of pollution, particularly for total nitrogen and chemical oxygen demand (Lin and Yi, 2003). To minimize the environmental impacts of modern intensive aquaculture, it is crucial to minimize the use of nutrients, retain the

nutrients in the system, and look for opportunities to re-utilize them. Aquatic plants have demonstrated considerable potential for nutrient removal from wastewater by various physical, biological and chemical mechanisms (Snow and Ghaly, 2008). Research has documented that aquatic plants reduce the concentrations of solids, NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} by settling suspended solids, adsorption and ion exchange, breakdown, uptake and transformation of contaminants by microorganisms associated with the plants, and by precipitation and chemical fixation reactions (Snow and Ghaly, 2008). They also play an important role in mineral transformation and cycling of nutrients (Horvat et al., 2008).

In aquaculture environments, fish growth results in the production of various nitrogenous waste products, primarily ammonia or NH_4^+ in solution (Tyson et al., 2007). Ammonia can be removed by absorption, nitrification, and denitrification of bacteria. These two forms compose the Total Ammonia Nitrogen (TAN). In most aquaculture environments, NH_4^+ predominates but fraction depends on pH, temperature and salinity. At a higher pH range, the environment is more conducive to nitrifying bacteria. Optimum pH for nitrifying bacteria is 7.5 to 9.0 (Tyson et al., 2007) in ionized ammonia form (NH_4^+) and exists as non-ionized ammonia (NH_3^+) (Nguyen, 2003). An anaerobic CWs' environment is favorable for nitrification and denitrification where NH_4^+ is transformed into NO_2^- and N_2 (Toscano et al., 2009; Nguyen, 2003). *Nitrosomas* uses NH_3 and O_2 to produced nitrite (NO_2^-) and water. *Nitrobacter* use NO_2^- to combine with O_2 to produce NO_3^-. Nitrates are used by aquatic organisms as a nutrient source (Nguyen, 2003). Many bacteria convert nitrates and nitrites to nitrogenous gases (NO, N_2O, N_2) through a process called denitrification, and the gas formed is released to the atmosphere (Mengel and Kirkby, 1987). NO_2^- and NO_3^- can be transformed by denitrification into N_2O and N_2 that goes into the atmosphere. This process might explain why nitrate concentrations were reduced despite the

presence or absence of lotus plants in our study. In a similar study using wetlands and growing giant reeds (*Arundo donax* L.), *Arundo* roots played a major role in NO_3^- removal. NO_3^- level reductions were further induced by increasing the HRT from 3 to 7 days (Abbassi et al., 2010).

Oxygen transported by vegetation is a key factor affecting the removal of nitrogen (organic nitrogen and NH_4^+) from CWs (Reddy et al., 1989). Lotus has high biomass production capacity. Selected cultivars are capable of producing up to 45 tons of rhizomes per ha. In addition, *Nelumbo nucifera* is able to improve its oxygen supply to the submerged and buried organs by a thermo osmotic gas transport. Investigations have shown that gas transport can increase up to 94% when there is a temperature difference of 3 °C and the gas flow system through fresh leaves may carry as much as 10 cm^3 air per minute to the buried rhizome (Mevi-Schutz and Grosse, 1988). On the other hand, ammonium uptake is influenced by the carbohydrate status of plants. High carbohydrate levels favor the uptake of NH_4^+ probably by enhancing NH_3^+ assimilation by the provision of carbon skeletons and energy (Mengel and Kirkby, 1987). Based on this data it will be interesting to do further evaluations to determine if the plant absorbs more ammonium as the plant matures and begins to accumulate starches in the roots. In this study, and despite significant reductions in NH_4^+, the levels remained higher than those recommended for the culture of aquatic organisms which should not exceed 0.02 mg/L (Snow and Ghaly, 2008). Longer retention times should have been evaluated to determine if the reduction of NH_4^+ can decline to acceptable levels for recycling. Planted CWs are very effective in reducing ammonium concentrations (Olguín et al., 2008; Tanner et al., 1995). In a comparative study of the purification of aquaculture wastewater using 3 different species and 2 HRT treatments, NH_4^+ removal was significantly affected by plant species, but was not influenced by hydraulic retention time. In their study using water hyacinth (*Eichhornia*

crassipes), water lettuce (*Pistia stratiotes*) and parrot's feather (*Myriophyllum aquaticum*), after 144 h HRT NH_4^+ was reduced by 32, 76 and 68 mg/L, respectively (Snow and Ghaly, 2008). Based on our data, there are other processes involved in nitrogen transformation because nitrate concentrations were significantly reduced with longer HRT resulting in higher reduction.

Fish effluent coming from the fish tank was characterized by a low pH (6.1). A low pH is not uncommon in fish tanks, especially where the buffering capacity has become exhausted. Over time, a drop in buffering capacity is common in filtered aquaculture systems. As the fish waste is broken down to nitrates, these nitrates (and other organic wastes) can form weak acids that gradually exhaust the buffers in the water. Once the buffers are completely exhausted (e.g. the carbonate hardness [KH] drops to zero) pH decreases. We noticed an increase in pH in the V CWs which was lower than those from NV CWS. The lower pH in the vegetated wetlands however, could be is explained by CO_2 production from decomposing plant litter and other wastewater components trapped in the roots as well as nitrification processes of ammonia (Kyambadde et al., 2005). The higher pH in unplanted CWs could be due to the absence of macrophytes favoring atmospheric aeration, algal growth, and photosynthesis. Another factor that could contribute to the changes in pH was the substrate of the CWs (calcinated clay). Calcined clays may contain traces of calcium oxide or calcium carbonate, and these may have increased the pH in the water (Table 4.1). White (2007) used calcined clays in her studies of CWs and nutrient assimilation and removal from nursery run-off using CWs. She found that pH stabilized between 6.9 NV CWs and 7.4 V CWs over the experiment (13 months).

Lotus has a high demand for K, especially when it reaches its peak of development (Dong-Bi et al., 2009). However, aquaponic systems that rely solely on fish waste to supply nutrients for plants are usually low in K (Tyson et al., 2007). K concentrations in the fish

effluents averaged 43 mg/L and we observed a reduction of approximately 5 % in the effluents from V CWs. The reductions observed in this study were much lower than reported by studies using other species (Trivedy and Gudekar, 1987) and in some cases longer HRT (Dipu et al., 2010). In one study, K reductions reached 89% using 168 h HRT (Abbassi et al., 2010). Using V CWs, the authors documented K reductions of 79 to 89% using 72 and 168 hours HRT, respectively.

Phosphate absorbed by plant cells rapidly becomes involved in metabolic processes and is incorporated into organic compounds. Organic phosphates form hexose phosphates and uridine diphosphate (Mengel and Kirkby, 1987) and become vital components of nucleic acids, proteins, lipid membranes, and carbohydrate structures (Hicks, 2005). Lin and Yi (2003) reported that annual nutrient losses from the pond's mud were 1 ton of P per hectare. Phosphorous removal can be accomplished using different mechanisms: plant uptake, chemical adsorption, and precipitation. Several plants can be used to remove excess P in intensive aquaculture systems. For instance, lettuce can remove P from an intensive rainbow trout aquaculture operation. The system removed from an inlet concentration of 0.7 mg/L to an outlet concentration of < 0.01 mg/L (Adler et al., 1996). Lotus uses large quantities of P during its growth and can be an effective phosphorous phyto-remediator than other macrophytes growing in CWs (Kanabkaew and Puetpaiboon, 2004). In co-cultures of lotus with tilapia or cultures in ponds, plants removed 70% of the P from old pond mud. This represented 43 kg P that was incorporated in the lotus biomass during a full growing season (Lin and Yi, 2003). Longer HRT resulted in enhanced P precipitation (Kanabkaew and Puetpaiboon, 2004). Our data shows that CWs with plants reduced the concentrations of P significantly and the longer HRT yielded greater reductions of the initial P concentration. Similar results were found by White (2007),

who determined that heavy rooted vegetation growing in CWs facilitated consistent P removal efficiency. In her study, original concentrations of P in the fish effluents (13 mg/L) were reduced by 20% in V CWs after 72h of retention vs. 5% in the NV. Similar results were obtained by evaluating CWs used to treat dairy farm wastewaters (Tanner et al., 1995). Vegetated CWs showed greater removal of P than NV CWs. P is mainly removed via uptake by plants and adsorption on porous media (Akratos and Tsihrintzis, 2007). According to White (2007), even small increments of P in effluents (0.01 to 0.05 mg/L) contribute to increased eutrophication rates. Unfortunately, with these HRT the levels of phosphorous in the treated effluents remain higher than those allowed by the US Environmental Protection Agency (USEPA). USEPA recommends that total P for surface waters entering lakes and reservoirs should not exceed 0.1 mg/L (Taylor et al., 2006).

High levels of suspended solids (SS) in fish effluents are a major problem. In the southeast United States, SS typically exceed water quality standards of 100 mg/L or less (Boyd, 2003). This is a great concern because it has been estimated that a ton of fish (e.g. catfish) can discharge 2.3 kg of total solids (Schwartz and Boyd, 1994). Feces, uneaten feed and bacterial biomass are some of the main sources of SS (Snow and Ghaly, 2008). If this organic matter accumulates in the system, dissolved oxygen decreases as it decays and produces CO_2 and NH_3. However, SS are decomposed by microorganisms, inorganic nutrients are mineralized and essential elements for plant growth are released in the water. Sand and gravel hydroponic substrates can remove solid waste from system water. Solids can also remain in the system to provide nutrients for the plant (Rakocy et al., 2006). In this study, the initial concentration of SS in the fish effluent ranged from 204 to 437 mg/L, a level exceeding water quality standards. Data showed that treated fish effluents had lower SS than the control and the levels were highly

influenced by vegetation and HRT. Our V CWs were able to reduce the SS levels approximately 20%. Other studies documented even higher SS removal using lotus (Kanabkaew and Puetpaiboon, 2004). Lotus and Hydrilla (*Hydrilla verticillata*) were evaluated for reducing pollutants from domestic wastewater. Lotus showed the highest removal efficiency (70%) followed by Hydrilla (54%) and higher than the NV control [22%] (Kanabkaew and Puetpaiboon, 2004).

Double cropping in ponds is a good way to increase productivity in the fish ponds and an effective way to remove accumulated nutrients. Fish feed can release effluents with 3 to 29 % N (Schwartz and Boyd, 1994). For each ton of fish produced, an average of 20 kg N (all forms) was discharged. During tilapia production, a fish yield of 23,000 (kg/ha/year) can produce 980 kg/ha/year of nitrogen run-off (Lin and Yi, 2003). Co-culture and rotation culture of lotus and fish have been practiced in China for many years (Yi et al., 2002). Studies demonstrated that in an old fish pond cultivated with lotus, the crop is capable of recovering more than 80% of N contained in mud (Yi et al., 2002). Based on the number of plants used in this experiment per treatment (9) and the number of plants planted in a lotus field (4,500), we estimated that '04-R-31' can produce: 1,425 kg of leaves, 4,015 kg of runners, 3,178 kg of rhizomes and 55 kg of seed pods in 12 weeks. All these tissues can be used for medicinal, ornamental, and edible purposes (Billing and Biles, 2007). However, there are cultivars that can produce more than 16 tons (32,000 kgs) of rhizomes/ha and 1.5 tons (3,000 kgs) of seeds per hectare (Hicks, 2005; Nguyen, 2001).

In this study, we determined that the accumulation of heavy metals and other elements in quantities surpassed the threshold of toxicity for some species. For instance, arsenic levels in the rhizomes of the control plants reached 7 mg/L. There is very little information on toxic effects of

arsenic on plant species (Carbonell et al., 1998). However, at very low concentrations As stimulates the formation of free radicals and reactive oxygen species either via direct electron transfer or as a metal-mediated inhibition of metabolic reactions (Mahmud et al., 2006). In *Spartina alterniflora*, concentrations as low as 2 mg/L of As can have a negative effect on biomass accumulation (Carbonell et al., 1998). Our study reported initial concentrations in tissues higher than those reported for other plants (0.02 to 7.0 $\mu g\ g^{-1}$ DW) (Prasad, 1999). Other phyto-remediation studies using lotus revealed that soils containing high levels of As (2,878 mg/L) led to accumulation of high concentrations of As in lotus roots, leaves, and petioles (561, 315, 289 mg/L), respectively (Aqeel-Ashraf et al., 2010).

Levels of hyper-accumulation of heavy metals such as Al, Fe, Ni, and Na were noticed in the lotus tissue. However, plants evaluated in this study did not show any signs of Al, Mn or Fe toxicity, and the concentrations in the leaves were twice those previously reported by Nguyen (2001). Although Na is not considered essential to plant growth, its presence has a marked effect on the growth of some plants (Flowers et al., 1977) by affecting the electrical conductivity of the solution and consequently the nutrient availability to plant roots (Yeager, 2007). Some studies reported positive effects of Na on biomass accumulation by increasing the relative amount of lipid, lignin, and cellulose present in organic dry matter. Miller (1938) reported that carbohydrate accumulation in K deficient onion plants could be enhanced if plant nutrition was supplemented with Na. In lotus, Na has been reported to range from 0.03 to 0.14 % in leaves and 0.2 to 0.8 % in the petioles (Nguyen, 2001). Initial analysis of the tissue before this study detected hyper-accumulation of Na in rhizomes and petioles (> 3,000 mg/L or 0.3%). Lotus plants used in the study did maintain high levels of Na without phenotypic effects (Table 4.2). Data suggest that lotus might be suitable for growing in areas where saline water intrusion has occurred.

Aquaculture intensifies (feed inputs, organic matter, nutrients, and suspended solids added to the water bodies increase) directly impacting oxygen depletion, eutrophication and turbidity in the receiving waters. To minimize environmental impacts, it is important to use nutrients efficiently, retain nutrients applied in the system, and look for ways to recycle and reuse excess nutrients. Plants enhance the environment for remediation of nutrient run-off by assimilating nutrients, and converting them into organic forms which are returned to the CWs as litter, leachates and exudates (Tanner et al., 1998). Good performance of wetlands for wastewater treatment depends in part on the growth potential and ability of macrophytes to develop sufficient root systems for microbial attachment and microbial transformation (Kyambadde et al., 2005). Evaluation of different lotus cultivars and native species may help nursery owners and small fish producers incorporate CWs into viable production areas thus improving water quality. Lotus potential to hyper-accumulate heavy metals opens the venue of exploring its use for industrial waste water phyto-remediation. This study determined that fish waste water provides an excellent source of nutrients for lotus plant growth yielding healthy, robust plants in CWs. Our results also demonstrate that ornamental lotus growing in CWs can substantially reduce the nutrient run-off from intense aquaculture systems. Nitrogen concentrations were affected by the HRT and presence of plants enhanced the uptake. The effective reduction of nitrate, phosphorous and potassium will help maintain water quality in recirculating aquaculture systems. The potential of extending the HRT to further reduce the concentration of nutrients from the effluent should be explored. It is possible that increasing the CW footprint would further mitigate various nutrients and compounds in the effluents.

4.6. LITERATURE CITED

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Table 4.1: Comparisons of compositional analysis of fish effluents in simulated constructed wetlands (CWs) with lotus ('Camellia Red') plants (V) or without plants (NV).

HRT (hours)	Vegetation	Chemical composition (mg/L)[y] Ca	K	Mg	Na	NH_4^+	NO_3^-	P	pH	SS
	Control[y]	30	43	16	103	7	25	13	6	426
24	No plants	32	42	17	106	6	15	13	7	413
48	No plants	31	41	16	104	6	11	12	7	406
72	No plants	31	43	17	116	5	8	12	8	412
24	Plants	32	41	16	104	6	13	13	7	399
48	Plants	33	39	16	104	4	10	12	7	393
72	Plants	34	40	17	116	3	9	11	7	394

[z]Effect of retention time (24,48 or 72 hours) and vegetation on chemical composition of fish effluent.

Control vs. Treated[y] Sign.[x]	*	*	ns	***	**	***	***	***	***
No plants vs. Plants Sign.[z]	**	***	ns	ns	***	ns	***	***	***
HRT No plants Sign.[z]	L***	ns	ns	L***	ns	L***	L***	L***	ns
HRT Plants Sign.[z]	L***	L***	L**	L***	L***	L***	L***	L***	L***

[y]Control was the untreated fish effluents coming from the intense aquaculture system, and treated means the fish effluent after being retained in the CWs at different hydraulic retention time (24, 48, or 72 hours). For this study if there was an interaction between the sample day and treatments, model reduces to main effect model. Significant levels are described as: Non-significant (ns), linear (L) at α= 0.05 (*), 0.01 (**), or 0.001 (***).

[z]Orthogonal contrasts were used to determine if chemical composition changes after exposing effluents to treatments (retention time and vegetation). Significant levels were express as: Non-significant (ns) or Linear (L), α = 0.05 (*), 0.01 (**), or 0.001 (***).

Ca: CWs without plants ($y = 4.7207x + 24.71$, $r^2 = 0.0276$), and CWs with plants ($y = 0.2784x + 28.104$, $r^2 = 0.0004$).
K: CWs with plants ($y = -2.1615x + 49.338$, $r^2 = 0.0053$).
Mg: CWs with plants ($y = 0.0565x + 13.673$, $r^2 = 8E-05$).
Na: CWs without plants ($y = -9.0899x + 70.393$, $r^2 = 0.0857$), and CWs with plants ($y = 1.9311x + 37.204$, $r^2 = 0.002$).
NH_4: CWs with plants ($y = -0.6702x + 8.1261$, $r^2 = 0.0455$).
NO_3: CWs without plants ($y = -5.4883x + 24.294$, $r^2 = 0.3923$), and CWs with plants ($y = -2.4999x + 26.683$, $r^2 = 0.2296$).
P: CWs without plants ($y = 0.178x + 11.829$, $r^2 = 0.0034$), and CWs with plants ($y = -0.3286x + 12.891$, $r^2 = 0.0298$).
pH: CWs without plants ($y = 0.8234x + 6.0796$, $r^2 = 0.338$), and CWs with plants ($y = 0.1645x + 6.2819$, $r^2 = 0.3284$).
SS: CWs with plants ($y = -9.0132x + 381.3$, $r^2 = 0.0061$).

Table 4.2: Accumulation of macronutrients (%) and micronutrients in lotus tissue affected by hydraulic retention times (0, 24, 48, or 72 hours).

	Calcium			Phosphorus			Aluminum			Nickel		
HRT	Leaf	Rhizome	Petiole	Leaf	Rhizome	Petiole	Leaf	Rhizome	Petiole	Leaf	Rhizome	Petiole
0	1.24 a[z]	0.66 b	0.53 b	0.05 ns	0.31	0.35	108.4 b	6277.8 a	138.1 b	21.8 b	3122.1 a	55.2 b
24	1.98 a	0.71 b	0.71 b	0.28 c	1.28 a	0.76 b	331.9 b	1172.1 a	126.9 b	33.6 ns	19.5	12.1
48	2.16 a	0.58 b	0.66 b	0.29 c	1.16 a	0.67 b	240.8 b	982.2 a	112.0 b	21.6 ns	126.0	12.4
72	2.47 a	0.58 b	0.69 b	0.29 c	1.20 a	0.75 b	263.4 b	1064.7 a	89.5	15.0 ns	228.2	17.6
Sign.[yz]	L***	ns	Q*	ns	Q***	Q***	Q***	ns	Q***	ns	Q*	Q***
	Arsenic			Iron			Manganese			Lead		
HRT	Leaf	Rhizome	Petiole	Leaf	Rhizome	Petiole	Leaf	Rhizome	Petiole	Leaf	Rhizome	Petiole
0	0.10 b	7.03 a	0.10 b	159.1 b	5399.5 a	169.9 b	693.3 ns	330.1	278.9	0.32 b	4.80 a	0.09 b
24	0.22 ns	0.21	0.33	440.6 b	2131.9 a	407.1 b	526.9 a	144.8 b	159.1 b	0.10 ns	0.48	0.10
48	0.11 ns	1.00	0.25	335.2 b	1763.8 a	320.5 b	715.9 a	162.3 b	202.0 b	0.62 ns	0.39	0.10
72	0.15 ns	0.53	0.26	418.5 b	2210.1 a	348.0 b	982.5 a	172.0 b	220.7 b	0.10 ns	0.86	0.32
Sign.[yz]	ns	Q**	ns	L**	Q***	ns	ns	Q***	Q*	ns	Q**	ns

[y]Orthogonal polynomial contrasts were used to determine if chemical composition changes after exposing effluents to treatments (retention time and type of tissue). The tissue * Trt (HRT) interaction was significant. Significant levels are described as: Non-significant (ns), linear (L), or quadratic (Q) at α= 0.05 (*), 0.01 (**), or 0.001 (***).

[z]Mean separation was performed using Tukey's to determined at each treatment - row (2, 4, or 6 weeks), which tissue (leaf, rhizome, or petiole) accumulated the most macro or micro nutrient. Different letters represent significant differences at means were different at $\alpha = 0.05$.

Table 4.3: Accumulation of macronutrients (%) and micronutrients in lotus tissue affected by hydraulic retention times (0, 24, 48, or 72 hours).

	Potassium[w]				Magnesium[w]				Boron[w]			
HRT		Tissue		HRT		Tissue		HRT		Tissue		
0	3.04	Leaf	2.44 b[z]	0	0.66	Leaf	0.59 a	0	49.8	Leaf	45.4 a	
24	2.34	Rhizome	2.19 b	24	0.48	Rhizome	0.54 b	24	28.4	Rhizome	31.9 b	
48	2.32	Stem	2.93 a	48	0.47	Stem	0.42 c	48	28.5	Stem	24.4 c	
72	2.38			72	0.46			72	28.9			
Sign.[xy]	Q**				Q***				Q**			

	Cadmium[w]				Zinc[w]			Chromium[x]		Sodium[x]			Copper[y]
HRT		Tissue		HRT		Tissue		Tissue		Tissue		HRT	
0	0.18	Leaf	0.10 b	0	69.9	Leaf	37.2 b	Leaf	1.1 b	Leaf	954.6 b	0	23.7
24	0.13	Rhizome	0.16 a	24	60.1	Rhizome	87.5 a	Rhizome	21.0 a	Rhizome	3097.7 a	24	13.5
48	0.10	Stem	0.13 ab	48	44.7	Stem	37.6 b	Stem	8.1 a	Stem	3454.5 a	48	14.5
72	0.10			72	41.7							72	12.2
Sign.[xy]	L***				L**								L*

[uv]Orthogonal polynomial contrasts were used to determine if chemical composition changes after exposing effluents to treatments (retention time and type of tissue).

[vw]The tissue * Trt (HRT) interaction was significant.

[wx]Only main effects Tissue and Trt were significant.

[xy]Only main effects Tissue was significant.

[yz]Only main effects Trt was significant.

Significant levels are described as: Non-significant (ns), linear (L), or quadratic (Q) at α= 0.05 (*), 0.01 (**), or 0.001 (***).

[z]Mean separation with Tukey's was used to determine which tissue accumulated the most macro or micro nutrient. Different letters represent differences at α= 0.05.

CHAPTER 5

EFFECT OF MANGANESE ON ANTIOXIDANT CAPACITY OF LOTUS (*NELUMBO NUCIFERA* GAERNT.)

5.1. ABSTRACT

Manganese (Mn) toxicity has important implications environmentally and there are some reports of high Mn accumulation in lotus. However, little information is available reporting the physiological effects of Mn in lotus. Mature lotus plants (cv. 'Camellia Red') were grown in solutions (0.0, 5.0, 10.0, 15.0 and 50.0 mg/L of Mn) for 6 weeks and different biomarkers were used to determine the effects of Mn on the antioxidant capacity (AA) of the plant. Over time, treatments induced a linear increase in Mn levels in the tissue, accumulating mainly in leaves. Treated plants showed increases in Chlorophyll *a* (Chl *a*) content over time while Chl *b* content decreased. Total phenols (TPH) concentrations and lipid peroxidation (MDA) were not affected by treatments or time of exposure to Mn in solution. No effect on radical scavenging activity (DPPH) was measured over time. Scavenging activity measured with ABTS, decreased in a quadratic fashion with increasing Mn levels in solution, but increased in leaves exposed to the highest concentration (50 mg/L Mn). The opposite pattern was determined using ferric reducing ability of plasma (FRAP) assays.

Lotus antioxidant defensive mechanisms allow the plant to prevent oxidative damage and lipid peroxidation when exposed to polluted Mn environments. In the presence of high concentrations of Mn, lotus absorbs high amounts of Mn and stores the Mn in its tissues. Lotus is a promising candidate for phyto-remediation of Mn polluted environments.

5.2. INTRODUCTION

Heavy metal deposits in soils, as a result of mining, smelting, pesticide use, and fertilizers have resulted in serious contamination of terrestrial and aquatic environments (Lei et al., 2007). Remediation of sites contaminated with heavy metals is very difficult and expensive. However, the use of plants for environmental restoration is an emerging cleanup technology.

Plants can absorb and accumulate inorganic contaminants, primarily metals, from polluted soil and water environments. In this approach, mature plants capable of accumulating high levels of metals can be harvested and a fraction of the metal contamination removed (Lasat, 2002). However, higher concentrations of heavy metals can induce abiotic stress (Guo et al., 2004). Most heavy metals are strong oxidants and may disturb cellular metabolism by increasing the production of reactive oxygen species (ROS) (Gwóźdź et al., 1997). ROS include superoxide radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide ($H_2.O_2$) that can potentially cause oxidative damage to lipids, and disrupt cellular metabolism (Guo et al., 2004). $H_2.O_2$ has weak toxicity, however, in the presence of transition metals (e.g. Mn) produces the hydroxyl radical (OH^-) that is considered the most reactive oxygen species. Therefore, scavenging of $H_2.O_2$ is essential to avoid oxidative damage of plant tissues (Sakihama et al., 2002).

Plants under heavy metal stress invest energy and other resources in protective enzymatic and non-enzymatic antioxidant defenses (Matés and Sánchez-Jiménez, 1999; Posmyk et al., 2008). Oxidative stress occurs because of an imbalance between the production of ROS and plant detoxification (Kachout et al., 2009). Plant phenolic compounds such as flavonoids and water soluble vitamin C (ascorbate) are potent antioxidants. $H_2.O_2$ is detoxified to water by the ascorbate-glutathione cycle. In the scavenging system ascorbate acts as the electron donor for

ascorbate peroxidase to remove $H_2.O_2$, and ascorbate is regenerated by monodehydro ascorbate reductase (Sakihama et al., 2002).

Manganese (Mn) when present at abnormally high concentrations in the soil/water becomes cytotoxic and can cause injury to plants (Mashhadi et al., 2007). The most well documented role of Mn is water splitting and O_2 evolution system in photosynthesis (Mengel and Kirkby, 1987; Lidon et al., 2004). Mn also takes part in respiration, protein synthesis, acyl lipids and carbohydrates synthesis (Mashhadi et al., 2007). Absorption of Mn by plant organs depends on the ability of plants to transfer Mn across the soil-root interface and on the total and available amount of Mn in the soil (Mashhadi et al., 2007). Mn uptake by root tissue is characterized as a biphasic process and initially takes place rapidly and in a reversible and non-metabolic way. It appears to be absorbed by negatively charged cell wall constituents of the root cell apoplastic space (Millaleo et al., 2010). Mn is adsorbed at a much lower speed in the symplast via plant metabolism and is less readily exchanged. Mn is xylem transported from roots to the above-ground parts by the transpiration stream (Millaleo et al., 2010). In the phloem, and depending on species and age of the plant, Mn redistribution is very limited (Page et al., 2006). Mn generally tends to accumulate predominantly in the plant shoots, not in the roots (Page and Feller, 2005). Excess Mn is stored in vacuoles, cell walls and in chloroplast thylakoids.

Manganese toxicity has important implications in regard to global change and on ecosystems prone to Mn stress (Mashhadi et al., 2007). The deleterious effects of Mn toxicity are often observed in the shoots as stunted growth, chlorosis, crinkled leaves, and brown lesions (Gonzales et al., 1998). Mn toxicity leads to oxidative stress and stimulates the production of ROS (Mashhadi et al., 2007) as well as the production of free radicals (Gonzales et al., 1998). Free radicals' lifespan and diffusion into the cellular space are closely controlled by the cell's

antioxidant system (Gonzales et al., 1998). Under normal conditions, production and scavenging of these ROS is regulated in vivo by the antioxidant system. However, under Mn toxicity normal metabolic pathways are disrupted, and induce macromolecular damage (Mashhadi et al., 2007) such as lipid peroxidation (Gonzales et al., 1998), increase abscisic acid (ABA), and malondialdehyde (Lei et al., 2007). Excess Mn can damage the photosynthetic apparatus (Millaleo et al., 2010) and can significantly decrease chlorophyll contents (Lei et al., 2007). In addition, excessive Mn concentrations in plant tissues can alter absorption, translocation and utilization of other elements (Ca, Mg, Fe and P) also causing oxidative stress (Millaleo et al., 2010).

Normal concentrations of Mn in plant dry matter are observed within a 20 to 500 µg/g and occasionally exceed 1,000 µg/g (Xue et al., 2004). There are reports of concentrations of 200 mg/L affecting corn biomass accumulation and as high as 5,300 mg/L for sunflowers (Mengel and Kirkby, 1987). In containerized lotus (*Nelumbo nucifera*), concentrations of Mn were measured ranging from 1,580 mg/L (initial) to 2,014 mg/L, 70 days after planting (Tian et al., 2009). Australian researchers (Hicks, 2005; Nguyen, 2001) also reported high concentrations of Mn in healthy lotus leaves (8,360 mg/L) and petioles (2,511 mg/L). According to Upadhyay et al. (2007), plants with relatively high biomass may have a greater metal uptake capacity, due to lower metal concentration in their tissues. Some lotus cultivars (e.g. 'Big Sleeping Dragon', 'Hondong Big Lotus') have the capacity of producing 24 to 30 tons/ha of rhizomes (Nguyen and Hicks, 2004; Qingdong, 2004). Seed producing cultivars ('Number 36 Outer Space Seed Lotus', 'Chuzhou White Seed Lotus') can produce from 1.3 to 3.7 tons /ha of seeds (Qingdong, 2004). Lotus massive biomass production and growth habit are attributes that can be used for phyto-remediation purposes (Hicks, 2005).

Under various abiotic and biotic stress conditions, higher contents of non-enzymatic constituents are important for plants to adapt to stress, and ultimately tolerate and alleviate oxidative stress (Kachout et al., 2009). Several assays have been used to estimate antioxidant capacities in fruit and vegetable *in vivo* and *in vitro* including 2,2-diphenyl-1-picrylhydrazyl - DPPH (Cho et al., 2003,; total phenol content –TPC (Hu and Skibsted, 2002), ferric reducing ability of plasma –FRAP (Thaipong et al., 2006), and lipid peroxidation – MDA determination (Lei et al., 2007). When evaluating antioxidant capacity, synergism among the various antioxidants *in vivo* is dependent on the concentration, structure, and interaction among antioxidants (Me, et al., 2007). For example, radical species used in DPPH assay are typically soluble in organic solvents (lipophilic antioxidants) and do not detect hydrophilic antioxidants. Yet, the Folin assay works detects hydrophilic antioxidants (Park et al., 2009b). That is a reason why it is important to use different methods based on different mechanisms for measurement of antioxidant activity. Furthermore, some of those techniques can be used to estimate oxidative stress induced by heavy metal toxicity. In this study, we used different assays to characterize the response and levels of antioxidants from plants exposed to different concentrations of Mn, to compare the efficiency of DPPH, FRAP, MDA with other assays, and to correlate them with total phenolics (TPH) in lotus leaves.

5.3. MATERIALS AND METHODS

In May 2010, 64 plants of lotus cultivar 'Camellia Red' plants were grown in plastic containers (11.3 liters) in a mix of top soil (sandy clay-loam) and pine bark (1:1 by volume). Every week, container water levels were refilled to the same height with a 150 mg/L solution of soluble fertilizer complete formula 20-20-20 TotalGro™ (STD Industries, Winnsboro, LA) with micronutrients. The volume of the solution was maintained constant by adding water to

compensate for plant transpiration and evaporation. Six weeks later, plants were randomly placed in a split plot design with 3 replications per treatment. Plants were treated with 0, 5, 10, 15 and 50 mg/L of soluble $MnSO_4$ 29.0% (Southern Agricultural Insecticides, Inc. Palmetto, FL). Every 2, 4, and 6 weeks plants were harvested and leaf lamina, petioles and rhizomes were separated and sent to the lab for tissue analysis (3 replications per treatment). An analysis of the water used in the solution as well as the substrate was performed. Samples were digested and analyzed by procedures outlined by Plank (1992) and Odom and Kone (1997). For dry ashing - organic matter destruction, ~0.50 gram samples of dried plant material were ashed for 8 hours in a muffle furnace at 500 °C. For total nitrogen (N) and carbon (C), dry samples were combusted with Oxygen (O) catalyst at 1,150 °C. Nitrogen, C, and sulfur (S) gases were measured by thermal conductivity using an Elementar Vario Macro CNS analyzer (Columbo and Giazzi, 1982 and Kirsten, 1979).

Chlorophyll content: Chlorophyll *a*, *b* and *total* (Chl *a*, Chl *b* and Chl *total*) were determined according to the method by Hipkins et al. (1986). Approximately 100 mg of leaf tissue were pulverized with a mortar and pestle in liquid N, homogenized by the addition of 6.0 mL of 100 % HPLC grade methanol. The homogenized sample was transferred to a glass Corex centrifuge tube and incubated in the dark for 2 hours. Samples were centrifuged at 10,000g for 10 minutes (Beckman J2-21, San Antonio, TX). The supernatant was filtered with Miracloth (Calbiochem, La Jolla, CA). Final volume was raised to 10 mL with methanol and measured using a microplate reader (Synergy HT, Bio – Tec Instruments Inc. Winooski, VT).

Chl *a*, Chl *b* and Chl *total* values were calculated according to the following equation:

Chlorophyll a ($\mu g/mL$) = $16.5 \times A_{665} - 8.3 \times A_{650}$

Chlorophyll b ($\mu g/mL$) = $33.8 \times A_{650} - 12.5 \times A_{655}$

Total Chlorophyll (µg/mL) = 25.8 x A_{650} + 4.0 X A_{655}

Note: A stands for absorbance. One sample per rep (3n) was used for the extraction and 4 subsamples were analyzed in the spectrophotometer. Chlorophyll concentration was converted to micrograms of chlorophylls per gram of fresh according to the following equation:

(µg chlorophyll/mL methanol) x 6 mL methanol / (g FW tissue)

Lipid peroxidation: Lipid peroxidation was determined by quantifying the amount of malondialdehyde (MDA) production as described by Hodges et al. (1999). Leaf samples (100mg) was pulverized using liquid N, in a mortar and pestle, followed by addition of 5.0 mL of 80% (v/v) HPLC grade ethanol and vortexed for 1 minute. The combined extract was centrifuged at 10,000*g* at 4 °C for 10 minutes, and the clarified supernatant was filtered with Miracloth. Volume was raised to 10.0 mL using 80% (v/v) HPLC grade ethanol and vortexed for 1 minute. The resultant supernatant was used for MDA determination. To 1.0 mL of the combined clarified supernatant, 1.0 ml of a solution of 20% (Trichloroacetic acid) TCA and 0.01% butylated hydroxyl toluene (BHT) was added. This solution represented the -TBA solution. To another series of sample tubes, a solution of 20% TCA (v/v), 0.01% BHT (w/v) and 0.065% (w/v) thiobarbituric acid (TBA) was added. This solution represented the +TBA solution. Samples were carefully vortexed and heated at 95 ^{o}C for 25 minutes in water bath. Samples were cooled to room temperature and centrifuged at 10,000g at 4 ^{o}C for 10 minutes, 200 µL of each sample (4 n) were transferred to a microplate, and the absorbance was read at 440, 532 and 600 nm. MDA content was calculated according to the following equation:

MDA content (nmol/ mL) =

$$A = Abs\ 532_{[+TBA\ solution]} - (Abs\ 532_{[-TBA\ solution]} - Abs\ 600_{[-TBA\ solution]})$$

$$B = (Abs\ 440_{[+TBA\ solution]}) - [(Abs\ 600_{[+TBA\ solution]}) \times 0.0571]$$

MDA content nmol/ mL= (A – B / 157,000) x 10^6

Total phenolic content (TPH): TPH was determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965) with slight modifications. Frozen leaf tissue (100 mg) was powdered using liquid N, mortar and pestle, followed by the addition 3.0 ml of extraction solvent (1.2 M HCl in 80% HPLC grade methanol / Milli–Q water). Extracts were maintained at 4 °C overnight using a platform shaker (Barnstead Lab-Line Max-Q 2508, Thermo Fisher Scientific Inc. Pittsburgh, PA). The following morning the homogenate was transferred to a 50 ml Oak Ridge Centrifuge Tube (Nalge Nunc International Corporation, Rochester, NY). Samples were incubated in a water bath (Fisher Scientific model ISOTEMP 210, Dubuque, Iowa) at 60 °C for 2 hours followed by a 10 minute sonication (Branson, model 5510 Branson Ultrasonic Corporation, Danbury, CT). Sonicated samples were clarified by centrifugation (Beckman Centrifuge, model J2-21, San Antonio, TX) at 10,000g for 10 min at 4 °C, filtered with Miracloth, and diluted to a final volume of 5 mL1.2 M HCl in 80% HPLC grade methanol / Milli–Q water. Samples were placed in 1.5 mL Eppendorf® microtubes and stored in a -80 °C ultra low freezer until later analysis. For determination, 200 µL of diluted samples (1:8) or standards were mixed with 1.4 mL of Milli-Q water. Generation of a standard curve was achieved by constructing five different concentrations of gallic acid (20, 40, 60, 80 and 100 mg/L). A blank was prepared using Milli-Q water instead of sample. Subsequently, 200 µL of Folin-Ciocalteu's Reagent (FCR-1:5 dilution with Milli-Q water) were mixed with the sample, standard or blank. The reaction mixture was vortexed, incubated for 30 minutes at 40 °C, and absorbance was read at 765 nm against a blank (0 µL Gallic acid standard) using a microplate reader (Synergy HT, BIO-TEK Instruments, Inc., Winooski, Vermont). Results were expressed as mg gallic acid equivalent per 100g fresh weight (mg GAE/100g FW).

Reduced ascorbic acid (vit C): Vit C was extracted based on the solvent system (Wall, 2006). Frozen leaf tissue (100mg) was macerated with liquid nitrogen in a mortar and pestle, followed by the addition of 6.0 mL of cold *m*-phosphoric acid-acetic acid solution. The homogenate was transferred to Oak Ridge Centrifuge Tubes (Nalge Nunc International Corporation, Rochester, NY), followed by a 10 minute sonication (Branson, model 5510 Branson Ultrasonic Corporation, Danbury, Conn.). Sonicated samples were clarified by centrifugation (Beckman Centrifuge, model J2-21, San Antonio, TX) at 13,000g for 15 min at 4 ^{o}C, filtered with Miracloth and diluted to a final volume of 10.0 mL. Vitamin C was determined according to a procedure reported by Gossett et al. (1994), with modifications which permit adaptations for micro-plate determinations (Hodges et al., 1996). A standard curve was generated by constructing six different concentrations of L-ascorbic acid (0, 20, 40, 60, 80, and 100 µM). The dilution factor was 4:1. Extracts and standards were immediately transferred to a multichannel pipette reservoir and 200µl was pipetted into 96 well flat bottom plates (Costar cat # 3370, Corning Inc., Corning, NY). The absorbance was measured at 525 nm in a micro plate reader (Synergy HT, BIO-TEK Instruments, Inc., Winooski, VT) maintained at 25 ^{o}C for the duration of sample determination. For the microplate blank, the mix was used as described in the micro-Eppendorf method for Vit C analysis. Results were expressed as mg/100g FW. Although several dilutions were conducted, our concentration readings were out of range. We presumed that the heavy concentrations of Mn interfered with the assay.

Antioxidant radical scavenging activity (DPPH): DPPH was measured according to the method outlined by Brand-Williams et al. (1995). 100µM of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) obtained from Wako Chemicals USA Inc. (Richmond, VA, USA) was prepared by combining 7.9 mg / 200 ml of 80% HPLC grade methanol. The radical solution was stirred at

room temperature for 20 minutes. Generation of a standard curve was achieved by constructing five different concentrations of either two standards consisting of L-ascorbic acid or vitamin C at concentrations of 0, 25, 50, 75, 100 and 125 mg/L.

Frozen leaf tissue (100mg) was macerated with liquid nitrogen, followed by the addition of 10.0 ml of 80% HPLC grade methanol, covered with aluminum foil, and shaken on a platform overnight (approximately 12 to 14 hours) at room temperature. The homogenate was sonicated for 15 minutes and centrifuged for 15 minutes at 10,000g. Supernatant was filtered with Miracloth, and final volume made to 10.0 mL. Antioxidant activity was determined by adding 10.0 µL of leaf extract solution (4 replicates) into test tubes with the addition of 4.0 ml HPLC water and 1.0 ml of 250 µM DPPH solutions. Tubes were vortexed and allowed to stand at room temperature for 30 minutes in the dark, then vortexed again. From the reaction mixture two hundred (200 µL) were pipetted into 96 well flat bottom plates (Costar cat # 3370, Corning Inc., Corning, NY) and the decrease in absorbance was measured at 517 nm in a microplate reader (Synergy HT, BIO-TEK Instruments, Inc., Winooski, VT).

Antioxidant activity was calculated as percent of inhibition relative to the control using the following equation:

(%) = (Abs blank – Abs sample / Abs blank) x 100,

Where: Abs blank is the absorbance of the control reaction (control consisted 10 µL of methanol instead of sample extract), and Abs sample was the absorbance of the test compound. Results are expressed in vitamin C equivalent antioxidant capacity, (VCEAC mg/100gfw).

Ferric Reducing Activity of Plasma (FRAP): FRAP assay was performed according to Benzie and Strain (1996). Potential antioxidants will reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}); the latter forms a blue complex (Fe^{2+}/TPTZ), which increases the absorption at 593 nm.

The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 ml glacial acetic acid), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer (pH 3.6), 2.5 ml of 10 mM TPTZ solution in 40 mM HCL and 2.5 ml $FeCl_3 \cdot 6H_2O$.

Leaf extracts (15 µl) were allowed to react with 285 µl of the FRAP solution for 30 min in the dark. Generation of standard curves was achieved by constructing five different concentrations of either three standards consisting of Trolox, a synthetic vitamin E analogue (±) (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) at concentrations of 0, 150, 300, 450, 600, and 750 µM , L-ascorbic acid (vit C) at concentrations of 0, 20, 40, 60, 80, and 100 mg/L, and Gallic Acid at concentrations of 0, 20, 40, 60, 80, and 100 mg/L. Samples were diluted to approximately a 1:8 dilution (1.0 mL of sample + 7 mL of Ultrapure Milli-Q water). Then, 10 µL of diluted sample or standard was added to 290 µL of freshly prepared FRAP reagent directly into a microplate. Samples were incubated at room temperature in the dark for 30 minutes. After incubation, the colored product (ferrous tripridyltriazine complex) was immediately transferred to a multichannel pipette reservoir and 200 µl was pipetted into 96 well flat bottom plates (Costar cat # 3370, Corning Inc., Corning, NY) and absorbance was measured at 593 nm, 620 nm, and 700 nm in a micro plate reader maintained at 37 ^{o}C for the duration of sample determination (Synergy HT, BIO-TEK Instruments, Inc., Winooski, VT). In the microplate blank either water (Vit C and Gallic acid), or 80% methanol (FRAP) was used. Results were either expressed as vitamin C equivalent antioxidant capacity, (VCEAC mg/100g FW), Trolox equivalent antioxidant capacity (µM TEAC/100g FW), or gallic acid equivalents (GAE mg/100g FW).

Reagents: DPPH (1, 1– diphenyl-2-picrylhydrazyl) was obtained from Wako Chemicals USA Inc. (Richmond, VA, USA). Trolox and Gallic acid were purchased from Sigma / Aldrich. All other chemical reagents, solvents or standards were either purchased from Sigma / Aldrich Chemical Co. (St. Louis, MO) or Fischer Scientific (Fischer Scientific, Raleigh, NC) and were either high–performance liquid chromatography (HPLC) or analytical grade quality. Ultrapure Milli–Q water was used throughout this study and had electrical conductivity of 18.2 MΩ cm^2 (Millipore Corp., Bedford, MA).

Statistical analysis: For the statistical analysis, Mn concentrations in the tissue were evaluated by analysis of variance performed on response data using PROC GLIMMIX in SAS version 9.2 (SAS Institute, Cary, NC). The experimental design was a split plot with Mn concentration and tissue type in the main plot and harvest time in the subplot. Homogeneity of variance among treatments was tested using Levene's test at $\alpha = 0.05$. Where heterogeneous variance was found, covariance parameters were calculated using the GROUP option on the RANDOM statement in PROC GLIMMIX, and a heterogeneous variance model was fitted. Differences among simple effect means for tissue type were determined using Tukey's means comparison test at $\alpha = 0.05$. Single degree of freedom, orthogonal contrasts were used to test linear and quadratic trends of effects of Mn concentration and time at $\alpha = 0.05$. For the antioxidant activity and oxidative stress, analysis of variance was performed on the response data using PROC MIXED in SAS. The experimental design was a completely randomized design with harvests and Mn concentrations in a factorial treatment arrangement, and the four samples taken per plant were sub-samples. The homogeneous variance assumption was tested using the COVTEST option on the MODEL statement, and heterogeneous variance, where significant, was corrected using the GROUP option on the REPEATED statement. Single degree of freedom

orthogonal contrasts were used to test linear and quadratic trends over Mn concentration, and paired comparison contrasts were used to compare harvests within a concentration. Where only main effect (Mn concentration in solution) was significant, data was combined and analyzed using polynomial regression analysis. PROC CORR was also performed to evaluate the antioxidant activity responses and the Pearson Correlation Coefficients are expressed in terms of *r* values.

5.4. RESULTS

Manganese in solution and accumulation in lotus tissue: Accumulation in leaves were only affected by high levels of Mn (15 and 50 mg/L) in solution indicating a quadratic response with an increase at 4 weeks and a reduction at 6 weeks (Table 5.1a). In rhizomes and petioles, in non-treated and treated plants the response was quadratic with a three time increment at 4 weeks. In rhizomes, the response to Mn concentrations was linear at 2 and 6 weeks but quadratic at 4 weeks. Similar to rhizomes, the response the petioles' Mn levels showed a quadratic and linear trend at 4 and 6 weeks. However, Mn levels were not affected in the first harvest (2 weeks). This indicates that Mn was most abundant in leaves followed by rhizomes with the smallest amount in petioles.

The deleterious effect of Mn toxicity is often observed in leaves as well as shoots. Typical symptoms are stunted growth, chlorosis, crinkled leaves, and brown lesions (Gonzales et al., 1998). At the first 2 harvests (2 and 4 weeks), young leaves with brown lesions were noted that eventually senesced. Comparing data with concentrations reported in the literature (Mengel and Kirkby, 1987; Prasad, 1999) damaged leaves showed high concentrations of Al (> 100

mg/L), Fe (> 200 mg/L), Zn (> 60 mg/L), and very high concentrations of B (>70 mg/L), and hyper-accumulation of Mn (> 2500 mg/L), and Na (>2800 mg/L) (Table 5.1b).

Manganese effects in leaf chlorophyll content: In aquatic plants exposed to heavy metals, changes in chlorophyll levels are considered a suitable marker for oxidative stress (Lyubenova and Schroder, 2011). The analysis of simple effects over concentration provided the following results for chlorophyll *a*, and *b* (Table 5.2). There was an interaction between harvest time and Mn concentrations in solution. Chl *a*: At the first harvest (2 weeks) there was a linear increase in Chl *a* content ($r^2 = 0.9$, $P<0.001$). In subsequent harvests the response was quadratic. Chl *b*: At all harvests the concentrations responded in a quadratic fashion. The first harvest showed the highest concentrations ranging from 884 to 1,319 mg/g FW. Analyzing simple effects over time: Chl *a* increased linearly but the levels of Chl *b* decreased. Levels of Chl *total* were not affected. An analysis of variance of accumulations of Chl *a* at 4 weeks, were highest followed by 6 weeks of treatment to Mn. Analyzing all the data from the different harvests, Mn in solution induced a linear increase in Chl *a* ($y = 1.5742x + 493.39$, $R^2 = 0.1756$). Opposite to the trend observed for Chl *b*, simple effects over concentration induced a linear decrease. Chl *total*: Contents were not affected by the Mn concentration in solution or the time of exposure and the concentrations ranged from 1,186 ± 456 mg/g FW.

Manganese effects in lipid peroxidation and total phenols concentration: Lipid peroxidation determined by the accumulation of MDA was not affected by the Mn in solution nor the time of exposure. Values ranged from 44.2 ± 30.4 nmol/mL. Total phenols concentrations did not change over time and were not affected by the Mn in solution (Table 5.3).

Manganese effects in vit C concentration: Although, several dilutions were performed the concentrations were out of range. We hypothesized that Mn interfere with the assay.

Manganese effects in radical scavenging activity: These assays were performed ABTS assay with vitamin E equivalent (Trolox) as a standard, and antioxidant radical scavenging activity of 1-1-diphenyl-2-picrylhydrazyl (DPPH). Simple effects over concentration and/or time did not affect the antioxidant activity of lotus leaves' extract measured with ABTS assay. Concentrations ranged from 2888 ± 1476 μM TEAC/100g FW. DPPH showed a linear decrease with increase concentrations of Mn in solution but were not affected by time of exposure. The ability to scavenge the DPPH radical is related to the inhibition of lipid peroxidation (Sohn et al., 2003) and the concentration of total phenols. We correlated the levels of MDA at each treatment and harvest with the scavenging activity of DPPH and the Pearson's correlation coefficient was 0.24 (Table 5.4). The ability to scavenge the DPPH radical is related to the concentration of total phenols in the leaf tissue. We correlated the levels of TPH recorded in each treatment with the DPPH activity and we found the Pearson's correlation coefficient found was $r = 0.85$ (Table 5.4).

Manganese effects in antioxidant capacity of the leaf: AA was determined using FRAP assay and 3 standards. Using L- ascorbic acid or gallic acid standards, AA showed a linear decreased as the concentration of Mn in solution increased (Table 5.3). Using Trolox as standard was not affected by the treatments or time of exposure (Table 5.3). Concentrations ranged from 2259 ± 1780.2 μM TEAC/100g FW.

5.5. DISCUSSION

Manganese toxicity is not restricted to a narrow critical concentration range. There are reports of concentrations of 200 mg/L affecting corn biomass accumulation and others as high as 5300 mg/L for sunflowers (Mengel and Kirkby, 1987). In aquatic macrophytes such as soft rush (*Juncus effuses*) concentrations of Mn in the leaves varied from 123 to 1500 mg/L, and in shoots and roots concentrations varied from 571 to 596 mg/L (Ghaly et al., 2008). Concentrations of Mn

in tissues increased progressively as the concentration of Mn increased in solution. Mn accumulates primarily in the leaves, less in the stems and least in rhizomes. A similar pattern of Mn accumulation and distribution has been reported in *Phytolacca acinosa* Roxb. However, over time concentrations in stems and roots first increased and then decreased (Xue et al., 2004). Although, Mn concentrations used in this study were high, the only apparent symptoms of toxicity were noted at the beginning of the experiment (young leaves with brown spots and premature dying). We hypothesize that lotus assimilated the excessive Mn with rapid growing tissues (new leaves) and continued growing. High concentrations of Mn in lotus tissues suggest tolerance and potential hyper-accumulation of this metal. In studies involving containerized lotus, high concentrations of Mn were measured in plants ranging from 1,580 mg/L (initial) to 2,014 mg/L 70 days after planting (Tian et al., 2009). Australian researchers (Hicks, 2005; Nguyen 2001) also reported high concentrations of Mn in healthy lotus leaves (8,360 mg/L) and petioles (2,511 mg/L). High tolerance of Mn by lotus plants is related to its high biomass (Upadhyay et al., 2007). Lotus has the capacity of producing large amounts of biomass, developing a large leaf area index (Nohara and Tsuchiya, 1990), and its main rhizome can reach 11 meters (Masuda et al., 2006).

It is well documented that higher metal concentrations cause the generation of ROS and if the accumulation of them is not controlled, oxidative chain reactions can occur. Proteins, nucleic acids, and lipids can withstand oxidation to a certain extent; however, plants usually respond to this stress by increasing activities of the antioxidant system (Lyubenova and Schroder, 2011). Manganese toxicity induces oxidative stress and stimulates the production of ROS (Mashhadi et al., 2007; Gonzales et al., 1998). Metal ions block the electron flow in photosystem II (PSII) leading to the formation of excited chlorophyll, which in turn causes the production of free

oxyradicals (Singh, et al., 2006). Mn in excess can damage the photosynthetic apparatus (Millaleo et al., 2010) inducing alterations in the photosynthetic pigment pattern (Lei et al., 2007). The changes in concentrations differ according with the species. In poplar (*Populus cathayana*) cuttings exposed to increasing concentrations of Mn, chlorophyll content decreased with increasing Mn concentrations (Lei et al., 2007). On the contrary, 4 week-old water chestnut (*Trapa natans*) leaf, Chl *total* increased 11% with respect to controls (Baldisserotto et al., 2007). In our study, Chl *total* concentrations were not affected by the Mn in solution nor the time of exposure. Manganese treatments induce an increase in Chl *a* concentration while Chl *b* showed a decrease with time.

Repeated metal exposure usually affects plant cell membranes and their destabilization is frequently attributed to the production/accumulation of free radicals (Singh et al., 2006) inducing an increase of MDA production (Rai et al., 2004). MDA is the final product of peroxidation of membrane lipids and accumulates when plants are subjected to various abiotic stresses. Therefore, MDA is considered a reliable biomarker of oxidative stresses (Guo et al., 2004). Mn treatments increased the accumulation of malondialdehyde (MDA) content (Lei et al., 2007) in poplar (*Populus cathayana*) and wheat (Gonzales et al., 1998). In our study, MDA concentrations (44 ± 30 mg/100 g FW) did not change with time on increases in Mn concentration in solution. The data suggest that Mn concentrations were not high enough to induce membrane damage in the plants.

Scavenging of free radicals is one of the major anti-oxidant mechanisms to inhibit the chain reaction of lipid peroxidation (Omale, 2010). Antioxidants interrupt lipid oxidation in the propagation phase (chain-braking mechanism) by protecting the oxidation substrates against the first formed radicals in the initiation phase (Hu and Skibsted, 2002). Lipid peroxidation is

accelerated when free radicals are formed as a result of losing a hydrogen atom from the double bond in the structure of unsaturated fatty acids. Free radicals are widely accepted as factors that contribute to oxidative modification of DNA, proteins and other molecules. Antioxidants protect plants against the oxidative damage by radical-scavenging activities (Cho et al., 2003). They can seize the free radical chain of oxidation and form stable free radicals, which would not initiate or propagate further oxidation (Lee et al., 2005; Wang et al., 2003). Michalak (2006) reported that during heavy metal stress phenolic compounds can act as metal chelators and scavenge molecular species of active oxygen. Several reports support the concept that lotus leaf extracts scavenge reactive free radicals, such as ROS and DPPH radicals (Cho et al., 2003; Hu and Skibsted, 2002). An evaluation of lotus blossoms and leaves extractions (Korean liquor known as Yunyupju) show that lotus liquor has scavenging activities of DPPH as good as those shown by α-tocopherol (Lee et al., 2005). There have been many reports of induced accumulation of phenolic compounds and peroxidase activity in plants treated with high concentrations of metals (Michalak, 2006). Overall, in this study TPH content decreased as the plants matured. Similar observations had been reported in other crops. In golden delicious apple fruits, phenolic levels decreased 5-fold on a dry weight basis during the seasonal development of leaves (Mayr et al., 1995). In our study, high Mn concentrations might have stimulated the defensive mechanisms. According to Pinto et al., 2003 enhanced levels of cellular antioxidants allowed leaves to acclimate to increased steady state concentrations of ROS. In contrast, an abrupt generation of high levels of ROS over a short period of time of acute stress will exceed the total antioxidant capacity. We also recorded a close correlation between the contents of total phenolics and radical scavenging and antioxidant activities as shown by DPPH ($r=0.85$), FRAP ascorbic acid ($r=0.82$), FRAP Trolox ($r=0.80$) and FRAP gallic acid ($r=0.79$). When comparing the

antioxidant capacity of lotus extracts against other antioxidants, similar results were obtained by Park et al. (2009b). Lotus extracts show a higher antioxidant capacity than Trolox and ascorbic acid (Hu and Skibsted, 2002).

Concentrations of TPH in the leaf were very high (1169 ± 888 mg/100g FW). Other plants showed relatively high antioxidant activity and placed among those with the highest levels of antioxidants (Park et al., 2009a). For example, TPH in Korean lotus can be compared with those in green peppers which can reach a DPPH activity of 2.1 μM Trolox equivalents/g DW (Park, et al., 2009b). Furthermore, lotus root knot extract has a much higher content of phenolic compounds (72.6 g/100g DW) than green tea, and (77.0 g/100g DW) and Ginkgo extracts [19.2 g /100g DW] (Hu and Skibsted, 2002). Hyper-tolerance to Mn concentrations is attributed to the production of phenolic compounds. Different aquatic species tolerate high concentrations of Mn by chelating it with phenolic compounds. *Trapa natans* accumulates Mn in the floating lamina in the form of phenolic compounds (Baldisserotto et al., 2010). In methanol extracts of waterlilies (*Nymphaea spp*.), direct chelation or binding of Cr, Pb and Hg to polyphenols was observed (Michalak, 2006). Lotus plants have high contents of TPH which might help them to tolerate concentrations that reach the toxicity threshold.

The evaluation of lotus leaves for antioxidative activity should not be dependent on a single method, but should include measurement of reactions characteristic of both initiation and propagation phases. We used 3 different standards for the ferric reducing antioxidant power (FRAP), an equivalent of vitamin E (Trolox) and an assay to determine antioxidant radical scavenging activity (DPPH). The high correlation coefficients between FRAP ascorbic acid by FRAP Trolox, and FRAP ascorbic acid by FRAP Gallic acid were similar to those results obtained by Park et al. (2009b). In studies of ferrous ion chelating activity it was found that lotus

plumule (seed embryos) possessed noticeable chelating activity of the ferrous ion (IC_{50} was estimated as 4.8 ± 0.4 mg/L). Authors mentioned that although the concentration was higher than what can be achieved under physiological conditions, it may be significant because it reduces the concentration of metal in lipid peroxidation and DNA oxidation (Wang et al., 2003). Hydrophilic antioxidants are very abundant secondary metabolites. Most techniques used to determine antioxidant activity showed high correlation with TPH (Thaipong et al., 2006). For instance, the correlation between total antioxidant capacity (DPPH) and total phenolics had correlation values of 0.93 to 0.96 for peaches, plums and nectarines (Gil et al., 2002). We observed a correlation between the antioxidant activities of FRAP ascorbic acid by FRAP Trolox (r=0.92), and the activities of FRAP ascorbic acid by FRAP Gallic acid (r=0.94). Similar results were obtained by Park et al. (2009b).

Excessive Mn concentrations in plant tissues can alter absorption, translocation and utilization of other elements (Ca, Mg, Fe and P) causing oxidative stress (Millaleo et al., 2010). Manganese toxicity is not restricted to a narrow critical concentration range. There are reports of concentrations of 200 mg/L affecting corn biomass accumulation and others as high as 5,300 mg/L for sunflowers (Mengel and Kirkby, 1987). Lotus antioxidant defensive mechanisms allowed the plant to prevent oxidative damage and lipid peroxidation for more than 6 weeks of high exposure to exogenous concentrations of Mn. Our studies show that lotus leaves have high concentrations of phenolic compounds and the leaves have the capacity of absorbing extremely high concentrations of Mn. Supportive of our work, Tian et al. (2009) reported high concentrations of Mn ranging from 1,580 mg/L (initial) to 2,014 mg/L 70 days after planting. Australian researchers (Hicks, 2005; Nguyen 2001) also reported high concentrations of Mn in healthy lotus leaves (8,360 mg/L) and petioles (2,511 mg/L). The high tolerance of Mn by lotus

plants is related to its high biomass (Upadhyay et al., 2007). Lotus has the capacity of producing large amounts of biomass, developing a large leaf area index (Nohara and Tsuchiya, 1990). Plants can develop up to 215 leaves in a growing season (Nguyen, 2001) and have produced up to 291 g dry weight m^{-2} during peak of growth.

5.6. LITERATURE CITED

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Table 5.1a: Effects of time, tissue type, and manganese (Mn) in solution (0, 5, 10, 15, or 50 mg/L) on the accumulation of Mn in lotus tissues.

	Leaves				Rhizomes				Petioles			
Trt	2 weeks	4 weeks	6 weeks	Sign.[wy]	2 weeks	4 weeks	6 weeks	Sign.[wx]	2 weeks	4 weeks	6 weeks	Sign.[wx]
0	632 ns[x]	1073 ab	792 ns	ns	328 ns	1635 a	347 ns	Q***	437.7ns	316 bc	231 ns	ns
5	688 ns	1118 b	1087 a	ns	405 ns	2170 a	188 c	Q***	499.1ns	2199 a	334 bc	Q***
10	882 ns	1507 ns	1459 a	ns	527 ns	2262 ns	282 c	Q***	804.1ns	1445 ns	474 bc	Q***
15	876 ns	1718 ns	1026 a	Q*	501 ns	2460 ns	332 c	Q***	607.9ns	2202 ns	433 bc	Q***
50	1632 ns	3446 a	2223 a	Q***	1154 ns	1632 bc	881 bc	Q***	731.9ns	1396 c	696 c	Q*
Sign.[wz]	L**	L***	L***		L**	Q**	L**		ns	Q***	L*	

[w]Ortoghonal polynomial contrasts were used to determine the main effects in Mn accumulation in the different tissues. There was a significant 3 way interaction. Significant levels are described as: Non-significant (ns), linear (L), or quadratic (Q) at $\alpha = 0.05$ (*), 0.01 (**), or 0.001 (***).

[x]Significant differences between individual means from tissues were determined using the Tukey's multiple comparisons test at each tissue (leaf, rhizome, or petiole), at each treatment (row). Letter codes indicate significant differences ($\alpha = 0.05$).

[y]Simple effects over time (rows):

Leaves: Trt 15.0 mg/L ($y = -59.24375 + 552.23958x - 133.31250x^2$, $r^2 = 0.33$); and Trt 50.0 mg/L ($y = -1518.6x^2 + 6369.4x - 3218.1$, $r^2 = 0.30$).

Rhizomes: Trt 0.0 mg/L ($y = -91.827x + 885.52$, $r^2 = 0.01$); Trt 5.0 mg/L ($y = -1873.2x^2 + 7384.1x - 5105.7$, $r^2 = 0.99$).

Trt 10.0 mg/L ($y = -1857.4x^2 + 7307.5x - 4923.5$, $r^2 = 0.98$); Trt 15.0 mg/L ($y = -2043.8x^2 + 8090.7x - 5545.8$, $r^2 = 0.95$).

Trt 50.0 mg/L ($y = -614.28x^2 + 2320.3x - 551.52$, $r^2 = 0.64$).

Petioles: Trt 5.0 mg/L ($y = -1782.5x^2 + 7047.3x - 4765.6$, $r^2 = 0.80$); Trt 10.0 mg/L ($y = -805.79x^2 + 3058.7x - 1449.3$, $r^2 = 0.85$).

Trt 15.0 mg/L ($y = -1681.8x2 + 6639.8x - 4350.2$, $r^2 = 0.41$); Trt 50.0 mg/L ($y = -681.91x2 + 2709.6x - 1295.2$, $r^2 = 0.46$).

[z]Simple effects over concentration (columns):

Leaves: 2 weeks ($y = 20.13x + 629.61$, $r^2 = 0.80$); 4 weeks ($y = 49.054x + 985.45$, $r^2 = 0.73$); and 6 weeks ($y = 26.112x + 897.62$, $r^2 = 0.38$).

Rhizomes: 2 weeks ($y = 16.494x + 319.03$, $r^2 = 0.72$); 4 weeks ($y = -1.5349x^2 + 75.297x + 1701.8$, $r^2 = 0.29$); and 6 weeks ($y = 13.189x + 187.49$, $r^2 = 0.69$).

Petioles: 4 weeks ($y = -2.5378x^2 + 140.3x + 713.41$, $r^2 = 0.20$); and 6 weeks ($y = 8.1882x + 303.34$, $r^2 = 0.42$).

Table 5.1b: Chemical composition of young lotus leaves from 'Camellia Red' which died prematurely showing Mn toxicity symptoms[z].

	Percentage				mg/L											
Harvest[y]	Trt	Ca	K	Mg	P	Al	As	B	Cd	Cr	Cu	Fe	Mn	Na	Ni	Pb	Zn
	0	2	2	0.7	0.5	249	1.1	202	0.2	12	15	625	1142	2546	217	0.1	73
	5	2	1	0.9	0.6	254	1.9	182	0.1	6	11	741	1130	2178	50	0.3	154
2 weeks	10	2	1	0.7	0.5	459	1.0	158	0.1	2	12	610	1261	832	62	0.1	51
	15	1	2	0.6	0.2	165	1.3	103	0.1	17	16	374	536	1585	144	4	77
	50	2	1	0.8	0.4	163	0.2	158	0.1	25	12	458	693	1299	79	0.1	91
	0	2	1	0.8	0.4	104	0.1	71	1.7	3	12	204	2051	2753	7	6	109
	5	2	1	0.8	0.4	98	0.1	82	1.0	15	14	362	1856	2692	77	8	67
4 weeks	10	2	1	0.8	0.4	105	0.1	69	1.0	6	14	201	2747	2711	22	2	97
	15	2	2	0.7	0.5	111	13.8	90	1.2	1	14	286	2024	2829	11	4	98
	50	2	2	0.8	0.5	101	0.1	78	2.8	5	18	235	2402	2842	17	2	62

[y]Damaged leaves (1-3) were collected only in harvest 1 and 2 (2 and 4 weeks); harvest 3 (6 weeks) did not produced any.

[z]For some plants Mn toxicity symptoms are generally characterized by brown spots in the leaves (Mengel and Kirkby, 1987).

Table 5.2: Effect of Mn (mg/L) and time in photosynthetic pigments in lotus leaves.

		Mn in solution (mg/L)					
Harvests	Parameter	0	5	10	15	50	Sign.[wz]
1		433	433	433	434	527	L***
2	Chl *a*	535	588	581	590	606	Q**
3	(mg/g FW)	552	462	491	520	591	Q**
	Sign.[wx]	L***	L**	L***	L***	L***	
1		1319	1319	1319	1319	884	Q***
2	Chl *b*	375	647	576	506	548	Q***
3	(mg/g FW)	797	311	337	362	636	Q***
	Sign.[wx]	L***	L***	L***	L***	L***	

[x]Orthogonal polynomial contrasts were used to determine the main effects on the concentrations of Chl a and Chl b. There was a significant interaction between harvest*Mn concentration. Significance levels are described as: Non-significant (ns), linear (L), or quadratic (Q) at α= 0.01 (**); or 0.001 (***).

[y]Simple effects over concentrations (rows):

Chl *a*: Harvest 1 ($y = 2.0487x + 419.35$, $r^2 = 0.89$); harvest 2 ($y = -0.062x^2 + 4.2352x + 548.65$, $r^2 = 0.50$); harvest 3 ($y = 0.0937x^2 - 3.2713x + 522.37$, $r^2 = 0.47$).

Chl *b*: Harvest 1 ($y = -0.2414x^2 + 3.4735x + 1313.9$, $r^2 = 0.97$); harvest 2 ($y = -0.1836x^2 + 10.782x + 463.25$, $r^2 = 0.16$); harvest 3 ($y = 0.7699x^2 - 39.194x + 676.83$, $r^2 = 0.56$).

[z]Simple effects over time (columns):

Trt 0: Chl *a* ($y = 59.596x + 387.46$, $r^2 = 0.64$), Chl *b* ($y = -261.11x + 1352.4$, $r^2 = 0.28$), Trt 5: Chl *a* ($y = 14.885x + 467.26$, $r^2 = 0.03$), Chl *b* ($y = -503.87x + 1760.8$, $r^2 = 0.95$),

Trt 10:Chl *a* ($y = 28.889x + 443.63$, $r^2 = 0.14$), Chl *b* ($y = -491.03x + 1732.1$, $r^2 = 0.88$), Trt 15: Chl *a* ($y = 42.894x + 428.15$, $r^2 = 0.30$), Chl *b* ($y = -478.18x + 1685.3$, $r^2 = 0.85$),

Trt 10: Chl *a* ($y = 31.949x + 511.08$, $r^2 = 0.49$), Chl *b* ($y = -123.98x + 937.08$, $r^2 = 0.44$).

Table 5.3: Effect of Mn in solution and time in total phenol content, lipid peroxidation and antioxidant capacity (AA) of lotus (cv. 'Camellia Red') leaves.

	Mn in solution (mg/L)					
Assay	0	5	10	15	50	Sign.[y]
Total phenols (mg GAE/100g FW)	1264.4	1191.2	1305.4	1146.2	946.2	ns
DPPH (VCEAC mg/100g)[z]	3205.0	2775.9	2917.3	3246.1	266.6	L**
Lipid peroxidation (nmol/mL)	47.8	46.9	47.2	36.7	42.3	ns
FRAP standard[z]						
(VCEAC mg/100g)	1928.6	1639.7	1703.8	1779.1	1312.9	L**
(μM TEAC/100g FW)	2455.7	2201.2	2391.2	2451.1	1809.0	ns
(GAE mg/100g FW)	724.4	633.0	686.4	698.7	534.0	L*

[y]Orthogonal polynomials contrast were used to determine if main effect had an effect over time or over concentration. Only main effect treatment (Mn concentrations) has an effect. Significant levels are described as: Non-significant (ns), linear (L), or quadratic (Q) at α =0.05 (*), or 0.01 (**).

DPPH ($y = - 16.146x + 3138.1$, $r^2 = 0.04$)

FRAP L-ascorbic acid ($y = - 10.305x + 1836.4$, $r^2 = 0.03$).
FRAP gallic acid ($y = - 3.2961x + 707.29$, $r^2 = 0.04$).
[z]AA was measured using scavenging radical assays (DPPH), and ferric reducing ability of plasma (FRAP) with three different standards (vit C, Trolox, and gallic acid).

Table 5.4: Pearson's correlation coefficients of antioxidant capacity and oxidative stress on lotus leaf treated with manganese.

	Lipid peroxidation	Antioxidant radical scavenging activity		Ferric Reducing Antioxidant Power (FRAP)		
Assays[z]	MDA	TROLOX	DPPH	FRAP $_{\text{L-Ascorbic acid}}$	FRAP $_{\text{Trolox}}$	FRAP $_{\text{Gallic acid}}$
TPH	0.34	0.09	0.85	0.82	0.80	0.79
TROLOX			0.24	0.23	0.17	0.30
DPPH	0.23			0.91	0.86	0.90
FRAP $_{\text{L-ascorbic acid}}$					0.92	0.94
FRAP $_{\text{Trolox}}$						0.90

[z]Assays: TPC- Total Phenolic; Trolox equivalent antioxidant capacity, FRAP using 3 different standards (L-ascorbic acid, Trolox, and Gallic acid).

APPENDIX 1

EVALUATION AND INTRODUCTION OF LOTUS (*NELUMBO NUCIFERA* GAERTN.) AS AN ALTERNATIVE CROP FOR PRODUCTION IN ALABAMA: A PRELIMINARY APPROACH

A.1.1 ABSTRACT

Lotus' wide diversity of edible, ornamental and medicinal uses suggest there may be opportunities for growing lotus and supplying local niche demands, as well as providing export opportunities. An active cultivar collection has been developed with the idea of gathering a broad spectrum of diverse cultivars and seed from wild stands from India, China, Japan, Australia, Thailand, United States, Canada, and Russia. Sources of high quality propagation material are limited and very often the cultivar names are mislabeled. AU researchers developed a cultivar registration system as a tool to help nursery producers, researchers and hobbyists with a description of some of the most popular cultivars. We evaluated practical production cultivation techniques including container fertilization, flower removal for increased rhizome production and alternative substrates. Consumer preferences were surveyed, which determined that consumers prefer medium or large size plants with pink, red, yellow or white flowers, in double or multipetal forms. Over 150 cultivars were evaluated at Auburn University and its Experiment Stations and 34 cultivars were selected as the most promising for commercial production. Cooperating nurseries worked with Extension personnel to force lotus cultivars into bloom in 45 to 60 days in high-tunnel production systems with extended photoperiods to target peak spring season sales and measured economic benefits. This venture continues to evolve with promising opportunities for Alabama farmers.

A.1.2. INTRODUCTION

The Alabama Cooperative Extension System, ACES, focuses on helping people and communities improve their quality of life and economic well-being. ACES selects and studies economically depressed communities and regions and identifies or develops opportunities to assist people in reversing and overcoming their depressed conditions as well as providing educational opportunities and support information based on scientific research (Whatley, 2011). In Alabama, there is an area known as the Black Belt Region. Black Belt refers to the dark prairie soil of south central Alabama (Gibson, 1941). Although the area was very prosperous in the antebellum United States (Tullos, 2004), the displacement of the plantation aristocracy, soil erosion, the cotton boll weevil (*Anthonomus grandis* Boheman) invasion, and the failure to construct a vigorous, diversified economy, all combined to mire the Black Belt Region in a seemingly irreversible decline (Ransom and Sutch, 1972).

Channel catfish (*Ictalurus punctatus*) farming has been expanding in Alabama from few farms in the early 1960s to more than 10, 000 ha in 2000 (Boy et al., 2000). Commercial aquaculture businesses are present in Bibb, Dallas, Greene, Hale, Marengo, Perry, and Tuscaloosa Counties, with much of the production concentrated in the Blackland Prairie region (Boyd et al., 2000; Pine, 2008). Soils, terrain, climate and adequate water resources have allowed the establishment and expansion of aquaculture farms which provide a much needed source of employment, opportunity and income (Cline, 2011). The area has the land/water resources to support an expansion of the industry 10 times its current size (Crews and Chappell, 2007). However, like much of agriculture, Alabama's aquacultural production has been under stress due to domestic and international competition (Holliman et al., 2008). Alabama's catfish industry must comply with the United States Environmental Protection Agency (USEPA)

effluent regulations (Boyd et al., 2000). To cope with environmental issues, enhance production and achieve sustainability, aquaculture must increase yield per unit of water, lower cost of production and continue to develop the variety of products from the farm (Holliman, et al., 2008). Aquaculture should be integrated with other production systems like horticultural ornamental plants and vegetable production (Sleeper, 2009).

Aquatic vegetables are potential plants capable of growing in fish water and producing edible, medicinal and ornamental economic crops. In China and other Asian countries, lotus (*Nelumbo nucifera* Gaertn.), water bamboo (*Zizania latifolia*), water caltrop (*Trapa natans*), Chinese celery (*Oenanthe japonica*), water chestnut (*Eleocharis dulcis*), swamp potato (*Sagittaria sagittifolia* ssp. *leucopetala*), Prickly Waterlily (*Euryale ferox*), watercress (*Nasturtium officinale*), cattail (*Typha latifolia*), taro (*Colacasia esculenta*) and Benghal dayflower (*Commelina benghalensis*) are very popular aquatic vegetables (Qingdong, 2005). Among these crops in China, lotus covers the largest area (200,000 ha) and has been cultivated the longest for more than 2,000 years for its rhizomes and seeds (Guo, 2009; Nguyen, 2001). Lotus is usually classified into three types: rhizome lotus, seed lotus and flower lotus (Guo, 2009) and can be used as part of a poly-culture with fish, shrimp, crab, or grown with other vegetables (Yi et al., 2002). In addition, lotus has its use as an ornamental plant in water gardens with hundreds of cultivars being developed over the last century (Wang and Zhang, 2004). It is also popular as a cut flower and extensively used for its medicinal qualities (La-ongsri et al., 2009). Lotus normally begins blooming in late May or June and continues until late August (Wang and Zhang, 2004). Research indicates that *Nelumbo sp*. is responsive to photoperiod and can be forced to bloom out of season when subjected to critical water temperatures and long days (Masuda et al., 2006). American gardeners do not use lotus as much as gardeners in other

countries. However, environmental conditions in the Southeast, and the availability of hundreds of lotus cultivars make lotus a suitable candidate for an alternative crop in the Southeast (Tian et al., 2009b; Wang and Zhang, 2004; Yamaguchi, 1990). Forcing potted lotus for spring sales offers the opportunity to provide consumers with a highly desirable alternative ornamental plant (Creamer, 2008).

International production and marketing of lotus is being evaluated by ACES to determine the efficacy of introducing lotus as a new product that could stimulate Alabama's farm economy (Tian et al., 2006, Ward, 2009; Creamer, 2011). Areas evaluated included the study of international production and marketing of lotus and adaptation of best management practices [synergistic and sustainable production; development of new products and uses; post-harvest handling; economics and marketing; and alternative substrates that use traditional and waste material] (Orozco-Obando et al., 2009b; Tian, 2008; Yeager, 2007; Nyakatwa et al., 2001). The project also attempted to provide basic education about the new crop and to assess producers and consumer's attitudes and concerns towards lotus as an ornamental and edible crop. In addition, researchers identified leaders in the industry to partner, trial, demonstrate, adjust, document and share the suggested technology transfer for others to benefit (Orozco-Obando et al., 2010a).

A.1.3. MATERIALS AND METHODS

Substrate evaluation: Sandy loam soil was used as a control since it was the standard substrate in other studies with containerized lotus (Tian et al., 2009a; Tian et al., 2009b). Alternative substrates, pine bark and chicken litter were evaluated with the objective of finding a light weight, easy to mix, and soft loose texture that provided good conditions for root

development. Pine bark offered the best alternative at 50:50 (vol:vol) with sandy loam soil for growth and harvest of lotus.

Cultivar performance: In 2004, the Auburn University Lotus Project Team began assembling a collection of species (*N. nucifera* and *N. lutea*) with cultivars from sources in China, Japan, Thailand, United States, and other places for seed and rhizome production. Seeds from India, Japan, Australia, Canada, Thailand, Cambodia and other countries were collected, scarified, germinated in test tubes and transplanted to 3.7 L and grown at Auburn University to evaluate the diversity and provenance adaptability.

Performance was evaluated at the Department of Horticulture's Paterson Greenhouse Complex at Auburn University's main campus facility in Auburn, Alabama (Latitude=34.18 N, Longitude = 86.85 W, Elevation = 242 m, USDA Zone 8b). In 2004 through 2006, an evaluation of 21 cultivars began. Medium size (petiole length 60 to 160 cm) lotus cultivars were grown in 14 L containers, and the large size cultivars were grown in 83 L containers with 50:50 (vol:vol) pine bark : sandy loan soil. Pro·Sol® soluble fertilizer 20-20-20 was applied (Frit Ltd. Ozark, AL) at 4 and 8 g for medium and large containers, respectably, at 3-week intervals. At the same time, three seed producing selections ('04 - R - 40', '04 - R - 31', '04 - R - 7') and two rhizome production selections ('E#2' and 'E#3') were added to the evaluation and grown from June to late September in 83 L containers. Parameters evaluated (2004) were: survival rate (%), number of mature leaves (emergent), and plant height. Plant height was determined by measuring the length of the emergent leaf petiole from soil level to the lamina. Flower number, flower color, production of seeds; biomass (FW) was determined. For 2006, FW, propagule number, number and FW of rhizomes were also documented.

Another evaluation plot was implemented at the North Alabama Horticultural Research Center in Cullman, Alabama (Latitude = 32.60 N, Longitude = 85.50 W, Elevation = 197 m) in 2005 and 2006. One hundred and sixteen cultivars (3 replications per cultivar) were grown in 19 L containers with ½ of the volume filled with soil and 4 g of soluble fertilizer at 3-week intervals was applied. In 2005 number of emergent leaves (mature leaves) and number of flowers were counted. In 2006, floating leaves (first leaves emerging from the rhizomes) were counted, as well as emergent leaves, number of flowers and petiole length. In the peak of the flowering season (July 15), and based on number of flowers, number of mature leaves and overall aspect of the plant a quality rating was given. For quality rating a Hedonic scale was used: 1: plant did not survive; 2: few floating leaves, no buds or standing leaves; 3: some standing leaves and no buds, 4: standing leaves with 1 to 2 buds; 5: standing leaves and 3 buds. Capacity of cultivars to produce viable seeds was recorded. Flower production was documented every 2 weeks from May to September. Plants size categories were 3: small, medium and large. Plant size was based on the description provided by the source/nursery: small (S), medium (M) and large (L).

Environmental conditions of the sites (Auburn and Cullman) were monitored on the daily basis using AU Mesonet (AWIS, 2011) and the summary of temperature; relative humidity, precipitation, vegetative wetting and chilling hours accumulated from April to August are reported. Data from the evaluations was analyzed using SAS version 9.1 (SAS Institute, Cary, NC). For Auburn 2004, Pearson Correlation Coefficients were estimated using PROC CORR to determine the possible relationship between survival rate and number and weight of rhizomes produced by the cultivars. For Cullman 2005, analysis of variance between cultivars (leaf and flower number) were evaluated using PROC GLM with Duncan's Multiple Range test (α =0.05). For 2006, 3 replications per cultivar were grown in the site, however, at the end of the season

only the average data was reported. Since analyzing average would remove all the variance from the treatments, no comparisons were made. PROC FREQ was used to determine flower color and size.

Market assessment of lotus potential as a new crop and cost of production: In 2007, a survey instrument was developed at Auburn assessing the trait preferences and likelihood of a consumer to purchase ornamental lotus. This survey was given to Master Gardeners during Plant Expo 2007 at the Birmingham Convention Center. Participants completed a two section questionnaire regarding their familiarity with ornamental lotus and their interest in acquiring it. Participants were presented with choices of photos of 15 lotus flowers to indicate preference of type (single or multi petal flower, size of the plant (large, medium, small) flower color, etc. Partial demographic data was collected. Respondents were asked to rate 16 different cultivars based on the flower form and color (Fig. 1). Descriptive statistics were analyzed using SPSS© Version 17 (2009. SPSS Inc. Chicago, Illinois). To estimate the cost of production, an Agriculture economist worked with a collaborating nursery (Ten Mile Creek Nursery, Harford, AL.) to establish a budget for the new industry to provide sound economic data for pricing and marketing the crop (Creamer, 2008; Ward, 2009).

Forcing lotus: In February 2008, dormant rhizomes with 3 growing points each from six different cultivars ('Camellia Red', 'Embolene', '1266', '1267', '04-R-7', and 'E # 3') were planted in 28 L containers with 8 cm layer of top soil. All the containers were installed in a greenhouse at the Auburn University Horticulture Research Station in Mobile, Alabama. Before planting, the substrate was saturated with water and individual roots were planted at a 15 degree angle into the container substrate. Plants were fertilized with 2.5g per pot of Peters Professional 20-10-20 soluble fertilizer (Scotts-Sierra Horticultural Products Co, Marysville, OH). Water

level was maintained 5 to 10 cm above soil level during the growing cycle. Greenhouse fans were used to circulate air and reduce pockets of high humidity to help reduce disease pressure. The greenhouse was heated with a forced air gas heater set at 18 °C. Interrupted night lighting was introduced from 10 PM-2 AM provided by 60 Watt incandescent bulbs (1.3 m between bulbs) and suspended 75 cm above the benches (60 cm above the water surface) and 4 cm above mature plants. Every 2 weeks the diameter of the emergent leaves and lengths of their petioles was measured. Flower production (flowering time and number of flowers) and the length of stems were recorded. At the end of the growing cycle, plants were rated for quality and marketability. Data from all the different parameters were analyzed using PROC GLM Tukey's test (SAS Institute, Inc. 2010) to determine if there were differences among treatments at p=0.05 (Table 3).

Promotion of a specialty crop: It is important to be able to produce a high quality crop. If people do not understand the value and benefit of the new crop, the success of the introduction will be at risk. Knowledgeable people, sufficient time, money, and educational materials are essential for a successful introduction. The Lotus Team visited and studied the components of success by other lotus growing and marketing countries. Signing of formal Memoranda of Understanding (MOU) with other research oriented academic institutions has allowed us to share and develop information ((e.g. Wuhan Aquatic Vegetable Research Institute, Wuhan-China; National University of Costa Rica, Heredia, Costa Rica). We have applied and received funding for travel, research and Extension activities. We have created an agricultural advisory committee composed of community and industry members. Training and getting commitment from Extension regional and county agents helps to disseminate educational efforts and opportunities to interested parties. College students and Master Gardeners have been involved in the project

(e.g. Russell County Master Gardeners, 2011). By promoting the cultivation and display of lotus in regional and in-state public gardens (e.g. Atlanta Botanical Garden, Bellingrath Gardens and Birmingham Botanical Gardens) and surrounding areas, we demonstrated to Alabamians the value of lotus to their gardens. We partnered with Ten Mile Creek Nursery located in Hartford, Alabama to begin commercial production of out-of-season containerized lotus using high tunnel greenhouses and marketing the plants during Mother's Day (May 10). Media outlets were identified to publish popular articles to outline the value of lotus and relay research/demonstration results (Bancroft, 2009; Brinda and Kirk, 2008; Creamer, 2008, Creamer, 2011; DeGarmeaux, 2010; Orozco-Obando et al., 2008; Orozco-Obando et al., 2009a ; Orozco-Obando et al., 2009b; Orozco-Obando et al., 2009c; Orozco-Obando et al., 2010a, Orozco-Obando et al., 2010b; Tian et al., 2011; Tilt et al., 2008; Tilt et al., 2009; and Ward, 2009). Information gathered and developed were organized and published in an on-line, dynamic web site (AU, 2011).

A.1.4. RESULTS

Cultivar selection and cultivation techniques depend on the environmental setting where the plants are going to be produced (Tian et al., 2009b). Most of the cultivars were grown in sandy loam soil. However, the 1:1 mix of sandy loam soil and pine bark showed a great potential for further evaluations (Table 1). This substrate was light, easy to use, and easy to mix and provided a desirable texture for proper root development and plant growth (Slocum and Robinson, 1996). Chicken litter by itself or mixed with soil or pine bark showed a high pH (7.3 to 9.0, respectively). In addition, electrical conductivity (EC) was too high (15 to 19 mmhos/cm)

and the levels of ammonium were very high (300 to 1078 mg/L) as well as the nitrate levels (192 to 500 mg/L) when chicken litter was introduced to the substrate.

For proper development, lotus requires at least 6 months with temperatures greater than 15 °C (Tian, 2008; Follett et al., 2003; Zhen-Ming et al., 2006). During the growing season of lotus in Auburn, average temperatures during 2004 and 2005 ranged from 17 to 27 °C with a minimum of average of 11 °C in 2004. However, during the months of April and May, temperatures dropped to 3.3 °C and 7.2 °C, respectably. In 2005, average temperatures ranged from 17 to 27 °C. Lowest temperatures were recorded in April (3 °C) and May (9 °C). In 2007, temperatures were much warmer, ranging from 20 °C to 28 °C, with a minimum average temperature in the month of April: 14 °C. Lowest temperature of April reached 5 °C and 9 °C for the month of May (Table 2). In Cullman, average temperatures during 2005 ranged from 15 to 26 °C with a minimum of average of 9 °C in 2004. In the same year, the lowest temperature for the month of April was -0.6 °C, 4 °C (May), and 15 °C (June). In 2006, average temperatures ranged from 19 to 28 °C with a minimum temperature average of 13 °C (April). The lowest temperature for the month of April was 4 °C, 9 °C (May), and 12 °C (June) (Table 2).

Cultivar performance: For those cultivars planted in 2004, a survival assessment was conducted in 2005. Sixteen cultivars had a survival rate ranging from 70 to 100%. Cultivars with a lower survival rate were: 'Alexander the Great' (30%), 'Baby Red Baby' (40%), 'Mrs. P.D. Slocum' (43%), 'Big Red' (50%), and 'Big Ben' did not survive. Number of propagules (dormant rhizomes with 2-3 internodes) varied from 10 to 53 with an average weight of 0.8g. Selected cultivars for seed production ('04 – R – 31', '04 – R – 40', '04 – R – 40') and cultivars for rhizome production ('E#2', and 'E#3') produced from 9 to 23 propagules with an average

weight of 0.9 grams. Pearson Correlation coefficient between survival rate (%) and rhizome number was r=0.6 and for survival rate and weight of the rhizomes was r=0.8 (Table 3).

At the Cullman evaluation site, for the first evaluation (2005) the performance of most of the cultivars was very similar (Table 4a, 4b, 4c, and 4d). Comparing the number of leaves produced by all cultivars (116), only 'Space lotus', 'Jicui' and 'New Red' showed higher leaf number when compared with other cultivars (14.5 to 18.5 standing leaves). Cultivars with the lowest production of leaves were: 'Large Green House', 'Leader', 'Lanceolate Red', and 'Sparks'. For flower number, 'Pink Exquisite' and 'Space lotus' were on top of the flowering frequency list (>3.5 flowers) but this flower number was not statistically significant among the other cultivars. In 2006, seventeen (15%) of the lotus plants were classified as small size reaching petiole lengths of 24 to 78 cm and averaging 53 cm. Fifty two plants (45%) fell within the category of medium size with petioles lengths ranging from 21 to 76 cm (average 56 cm). The large group was represented by 6% of the lotus plants with petiole lengths ranging from 38 to 92 cm (average, 57 cm). For evaluation of seed production capabilities, 4% of the cultivars within this category were fertile. Color distribution was as follows: 26 % pink, 17% white, 1 % was red, and no data was recorded on yellow flower cultivars. Production of seeds was determined in 13% of the cultivars. Flowering began 5 weeks after planting, and by fall 2006, (early September) most of the late bloomers had finished flower production (data not shown). Most cultivars had more than 2 flowers and the quality rating was acceptable (>3). Differences possibly existed among cultivars; however, we were limited to the use of averages of the three replications for this demonstration project. Therefore recorded variation among the treatment replications was lost.

The Extension programming effort to share concurrent research/demonstration information was accomplished by developing an internet version of the International Cultivar Registration Authority (ICRA) registry to offer official documentation and registering of *Nelumbo* cultivars. The internet version of the registry was enhanced by offering specific indentifying photos of each registration entry (example: http://www.ag.auburn.edu/hort/landscape/LOTUS_Camellia_Red.html) and registering began by the submission of a description, photos and measurements of the bud and flower. Pictures of the flower were submitted for each of its first four days as well as details of the view of pistils; seed pod (young receptacle, mature receptacle). Also received were leaf photos (floating and emergent leaves) and pictures of the entire mature plant and dormant rhizomes. In 2007, the International Waterlily and Water Gardening Society (IWGS) recognized Auburn University lotus research project as the International *Nelumbo* registry.

Forcing lotus: Air temperatures in the greenhouse ranged between 11 to 21 ^{o}C (8 AM) and 18 to 42 ^{o}C (1 PM). Water temperatures ranged between 8 to 20 ^{o}C (8 AM) and 9 to 20 ^{o}C (1 PM) (Fig. 2).Two to three weeks after planting, vegetative buds produced coin leaves, followed by floating leaves and emergent leaves. Some of the genetically larger cultivars began to produce flowers at the end of the 6th week. For this study, six cultivars were selected and two of them did not sprout ('E # 3' and '04-R-7'). The number of emergent leaves and their size has an effect on the consumer's perception of product quality and helps to determine marketability of the final product. 'Camellia Red', '1266' and '1267' did not exhibit differences on number of leaves per neither plant (4) nor diameter (17 cm^2). 'Embolene' production of leaves and its ultimate size was less than the other cultivars. Some of the genetically larger cultivar selections began to produce flowers at the end of the 6th week. Flower production, in this study did not show much

variation among cultivars. However, plant quality and marketability as defined and considered in subjective ratings by color, fullness and uniformity of the final plant was better for 'Camellia Red' and '1267' (Table 5).

Economics: An Agriculture economist helped to establish a budget for the new industry to provide sound economic data for production, pricing and marketing the crop (Creamer, 2008; Ward, 2009). The bottom line figures generated by the budget process showed the production cost to be $56,000 for 2009 with the product selling for $75,000, a 25.3% profit was generated for the company. First years sales were greater than 5,000 potted plants followed by a similar amount in the second season and an increase to 10,000 lotus container plants in the third season.

Market Assessment: Consumer preference survey results showed that the majority of the participants (82% female) were familiar with ornamental lotus but 85% of survey participants did not own a lotus plant (Table 6). A high percentage (66%) of the participants who did not have a lotus plant was willing to acquire one. Respondents were asked to categorize their preference for traits exhibited by assorted cultivars. The majority preferred cultivars with pink flowers (50%), followed by red (27%), yellow (13%) and white (10%). In addition, most preferred double flowers (57%) and medium size plants (57%) followed by large plants (24%). The four most popular cultivars selected from a group of 16 were 'Holy Fire', 'Senior Red', 'Garton's 98', and 'Apricot Yellow'. Plants that people rated less preferable were: 'Gold Jade', 'Senior Red', and 'Dense Dew'. It was observed that 'Garton's 98' was frequently rated with lowest preference (Table 7). The form of flower preferred was double or multi-petaled compared to single flowered cultivars.

Based on the consumer preferences we offered a preliminary selection of cultivars. Pink flower lotus selections included: 'Dense Dew', 'Friendship Pink', 'Pink in Green layers', 'Guifei

Zhuijiu', 'Garton's # 6', and 'Holy fired'. Red color flower lotus with potential are: 'Camellia Red', '04 – R – 31', '04 – R – 07', '04 – R – 40', 'Big Red', Garton's # 5', 'Garton's' seed 98' and 'Garton's #6'. 'E#2' is a good candidate for a yellow-flowered lotus. Desirable white flower cultivar candidates for introduction include: 'Karizma', 'Birthday Peach', and 'Garton's # 4'. Cullman research station results for pink flower lotus with the best performance were: '136','Beautiful Bowl', 'Brocade Flag', 'Dense Dew', 'Friendship Pink', 'Golden Sun', 'Illustrious Youth', 'Juicy Peach', 'Leadership', 'Red Dragonfly', and 'Splendor Red'. Within the red color flower lotus category, the best performer was 'aH3'. No yellow cultivars were identified. However, there were several outstanding whites: 'Apricot Yellow', 'Decorated Lantern', 'Jewel Flower', 'Little Brocade-edge', and 'Wenjun Fuhong'.

A.1.5. DISCUSSION

Lotus requires little care in nature and grows well in Alabama's typical climate of mild winters and the very hot and humid summers. Soils in some areas are very poor for traditional agricultural crops but work well for lotus. The heavy clay texture aids in the construction of ponds and creates an ideal root environment with the incorporation of organic matter for commercial lotus production. Knowledge of ornamental lotus production in nursery containers or field conditions is very limited in the United States. To develop this plant as an alternative ornamental crop, it was important to study and understand the plant's basic biology, growth habits, and the relationship of those factors to best management cultural practices for propagation and production. Seed propagation is used most in breeding programs to develop new cultivars because lotus seeds are highly heterozygous and offer needed diversity for cultivar selection. Asexual propagation is considered the best and simplest method to ensure uniformity, a

consistent yield, and a harvestable crop in one season. Enlarged rhizomes (propagules) from the previous year are frequently used for commercial cultivation (Masuda et al., 2006). Rhizomes with at least two segments sealed at either end by an intact node and growing point are planted before breaking dormancy (Nguyen, 2001).

Lotus cultivars are available in a range of sizes from the small teacup size suitable for a pot to large plants that would be appropriate for a large body of water (Billing and Biles, 2007). Container size restrictions can inhibit growth and not allow complete development of the plants potential growth. Growth and performance of lotus can be affected by genotype, media, water depth, light, planting time, and propagation methods (Billing and Biles, 2007; Tian, 2008). At Cullman, the range of stem size (Table 4a, 4b, 4c, and 4d) suggested that some cultivars did not reach their maximum size reported by the literature (Billing and Biles, 2007). Soil level used at the research station occupied 50 % of the container volume. This represents a higher volume/depth of soil over the rhizomes which in ornamental lotus can result in a reduction on the number of flowers (Tian et al., 2009b). Fertilizer and frequency might also affect the growth of the plants. Tian et al., 2005 reported that cultivars respond differently to the amount of fertilizer applied. For instance, 'Garton's #1' produced fewer leaves and was less efficient with macronutrients uptake when fertilized with 8 grs of fertilizer at 3 weeks interval. Plants growing in smaller containers (26.5 L) with an initial application 4 g of soluble fertilizer (20-10-20) provided adequate nutrition for a healthy, saleable plant to develop (Tian, 2008). According to Billing and Biles (2007), petiole length in small, medium and large lotus should reach 33, 60 to 120 and 120 to 240 cm, respectably. At Cullman, the minimum petiole length from the small sized category plants almost reached the medium sized category designation. Literature designated medium plants matched the description but cultivars defined in the literature as large

lotus plants only reached a size of medium designated plants. These discrepancies suggest that container size selection, substrate and environmental conditions should be further explored. However, the use of means per cultivar instead of the actual values from each replication reduces statistical variation. If there were differences, the statistical design and analysis of the demonstration was not designed to detect them.

As mentioned before, lotus requires at least 6 months with temperatures greater than 15 °C. Temperatures above 15 °C break rhizome dormancy and shoots begin to grow (Tian, 2008). Higher temperatures (> 22 to 28 °C) stimulate growth of more rhizome branches, nodes and leaves, as well as, flower development (Follett et al., 2003; Zhen-Ming et al., 2006). In Auburn, in the months of April and May (2004 to 2006) and in the Cullman research station (2005 and 2006) in April, 2004 the lowest minimum temperatures were below those that stimulate vegetative growth in lotus. In the month of May the minimum temperatures were marginal and the average temperatures were warm enough to induce the breaking of the rhizomes. However, for some cultivars, these conditions can halt growth and in some cases kill the plant. In previous experiments, 'Embolene' was more sensitive to low temperatures. In a preliminary study growing lotus cultivar 'Embolene', a sudden drop in temperature below the target temperature of 15 °C halted the growth of the plants. Although plants survived without noticeable injury, they never recovered the normal growth pattern (data not shown). In another study with the same cultivar, Tian et al., (2009b) reported a positive response of 'Embolene' to higher temperatures.

The use of greenhouse/high tunnel infrastructures that protects plant from sudden drops in temperatures is conducive to uninterrupted development of a good crop in these areas (Follet et al., 2003). Lotus can be forced into flower out-of-season with the right cultivars and providing plants with appropriate growing conditions (Wang and Zhang, 2004). Japanese growers use

greenhouses to force early commercial lotus vegetable production in greenhouses (Nguyen, 2001) and in other countries tunnel houses and under-glass production techniques are used to ensure year-round lotus crop supply (Guo, 2009; Follett et al., 2003). *Nelumbo* is classified as a long-day plant, where long days or interrupted night length affects growth and flowering (Masuda et al., 2006). Long-day-length accelerates rhizome elongation and upright leaf production, and short-day-length promotes rhizome enlargement and inhibits upright leaf production (Masuda et al., 2006). Long day cycles could be provided to stimulate the plant's growth out of season. Chinese producers use 200 watt lamps to break dormancy (Zhen-Ming et al., 2006). However, 60 to 100 watt minimum of 108 lux or 10 foot-candles of light can be sufficient to interrupt photoperiod in some crops. Lotus rhizomes have no dormancy chilling requirement and generate new stems, leaves and flowers if the temperature is suitable (Wang and Zhang (2004). Forcing lotus for March or April bloom would be advantageous to have lotus in bloom and available at retail outlets during the height of retail garden sales in early spring (Bancroft, 2009; Creamer, 2008; Tian et al., 2009b). Our evaluation in Mobile, Alabama determined that lotus plants can be harvested in February and the rhizomes can be grown in greenhouses where temperatures were set to be higher than 18 ^{o}C . This allowed the production of flowering plants after six weeks. Currently, a commercial nursery (Ten Mile Creek Nursery, Harford, AL) is forcing lotus cultivars into bloom in 45 to 60 days in a high-tunnel production system with extended photoperiod for peak season sales and measured economic benefits (Orozco-Obando et al., 2010b).

When developing a crop for commercial production, it is important to begin with plants that are true-to-name to avoid confusion, protect the consumer, and give proper credit and possible financial royalties to the creative works of the plant's originator. The International

Society of Horticultural Science's (ISHS) Commission of Nomenclature and Cultivar Registration (ICRA) is the organizational group trying to assure accuracy in plant naming. ISHS verifies and publishes any new cultivar registration on an official record and date so the new name can be verified. The International Waterlily and Water Gardening Society (IWGS) is the designated International Cultivar Regulator authority (ICRA) and in 2007; the Auburn Lotus Research Project was chosen to help with the development of the lotus cultivar registrar. Bringing technology to a previous paper-based program provided an Extension educational tool that is available to everyone for verification of the work and education of potential plant developers and gardeners. We used reliable data from respected authors in other countries to mine information and names of cultivars. The registrar does not assure superior plants and is not responsible for policing uniqueness, only that each cultivar has a unique name within the species. An on-going active germplasm collection is being conducted with over 160 cultivars, to gather a broad spectrum collection of diverse cultivars and seed from wild stands from India, China, Japan, Australia, Thailand, USA, Canada, and Russia for breeding and to determine diversity within the species (Tilt et al., 2008). The web-based database also provides researchers and *Nelumbo* enthusiasts easy international instant access to lotus cultivars for review and an opportunity to offer collaborative oversight.

Potential Market: The potential for growing lotus crops for domestic markets needs to be explored. Currently, ornamental roots are offered by specialized growers on the internet with prices ranging from $2 to $35 per propagule. Fully developed plants are sometimes found in stores and nurseries for $21 to $160 depending on size, container value and the specific value of the cultivar. A few U.S. growers offer an extensive assortment of sizes, colors and shapes of miniature teacup lotus. This size lotus is appropriate for smaller landscapes, patio container

gardens and ponds and could be a good niche market which could appeal instantly to avid collectors and present a unique gardening introduction for the general public. Another business opportunity that has proven highly successful around the world is edible lotus (Ward, 2009). The Lotus Team in conjunction with Piedmont Aquaculture LCC, (Guntersville, AL) began the exploration and development of field production of edible lotus to take advantage of their established east coast Asian market distribution channel. The Black Belt Region and their successful aquaculture production industry and infrastructure provide the right conditions and opportunity to double-crop lotus (Creamer, 2008). Work continues in this area to determine production best management practices, nutritional data, post-harvest storage and handling techniques for optimum quality, comparison of imports vs. locally grown products and marketing and economic data that will offer answers for positioning growers for competition of local and international markets. Initial lotus marketing evaluations suggest great opportunities for a new specialty crop and use as a possible model crop for future research on more proven aquaculture commercial successes enjoyed by the international agricultural business community.

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Table A.1: Summary reports from AU Mesonet station from the Horticultural Research station at Cullman, AL. Parameters air temperatures, precipitation, vegetative wetting, and chilling hours for the months of April to August 2006 and 2007.

	[z]Auburn 2004					[z]Auburn 2005					[z]Auburn 2006				
Parameters	April	May	June	July	August	April	May	June	July	August	April	May	June	July	August
Air temperatures (°C):															
Avg Maximum	23.6	28.9	30.2	32.4	30.6	22.9	26.7	29.9	31.5	31.3	26.4	27.4	32.3	33.8	32.9
Avg Minimum	10.9	17.8	21.0	22.1	20.8	10.5	14.9	20.9	22.7	22.6	13.9	16.0	19.8	22.0	22.8
Average	17.3	23.3	25.6	27.2	25.7	16.7	20.8	25.4	27.1	26.9	20.1	21.7	26.1	27.9	27.8
Highest	30.0	32.8	33.9	36.1	34.4	27.2	31.7	33.9	34.4	35.6	30.0	34.4	37.2	37.2	36.7
Lowest	3.3	7.2	17.2	19.4	14.4	2.8	9.4	17.8	19.4	20.6	5.0	9.4	15.6	18.9	20.6
Relative humidity (%)															
Avg Maximum	97.1	99.7	95.8	99.2	99.7	94.2	93.2	97.7	99.3	99.3	94.2	93.2	97.7	99.3	99.3
Avg Minimum	46.3	48.8	41.9	48.3	61.4	45.4	36.3	43.2	57.0	44.8	45.4	36.3	43.2	57.0	44.8
Average	71.7	74.2	68.9	73.7	80.5	69.8	64.7	70.5	78.1	72.1	69.8	64.7	70.5	78.1	72.1
Highest	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Lowest	13.0	27.0	30.0	32.0	39.0	18.0	18.0	25.0	38.0	21.0	18.0	18.0	25.0	38.0	21.0
Precipitation (mm)															
Total	65.2	86.2	76.9	79.2	121.2	109.5	52.2	135.1	142.1	170.1	44.3	58.3	146.8	102.5	83.9
Greatest daily	32.6	21.0	16.3	41.9	67.6	30.3	11.7	72.2	53.6	32.6	21.0	44.3	83.9	21.0	16.3
Rain days	7.0	7.0	6.0	10.0	13.0	9.0	2.0	8.0	12.0	6.0	9.0	2.0	8.0	12.0	6.0
Vegetative wetting (hours/day)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chill hours/month	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

	[y]Cullman 2005					[y]Cullman 2006				
	April	May	June	July	August	April	May	June	July	August
Air temperatures (°C):										
Avg Maximum	21.9	24.9	28.7	30.9	30.8	26.4	26.4	32.2	34.4	34.6
Avg Minimum	9.1	12.2	18.7	21.4	20.4	12.9	14.7	18.3	21.4	22.2
Average	15.5	18.6	23.7	26.2	25.6	19.6	20.6	25.3	27.9	28.4
Highest	26.7	31.7	32.2	35.0	31.7	32.2	33.9	37.8	38.3	37.8
Lowest	-0.6	4.4	15.0	18.9	19.4	3.9	7.8	12.2	16.1	18.9
Relative humidity (%)										
Avg Maximum	94.4	97.5	95.0	94.2	96.1	92.4	71.7	90.6	95.1	83.9
Avg Minimum	38.4	49.1	36.2	37.0	41.4	36.2	66.0	30.7	47.1	78.3
Average	66.4	73.3	65.6	65.6	68.7	64.3	68.9	60.7	71.1	81.1
Highest	100.0	100.0	99.0	99.0	99.0	100.0	74.0	98.0	99.0	85.0
Lowest	18.0	30.0	25.0	16.0	24.0	17.0	62.0	17.0	31.0	77.0
Precipitation (mm)										
Total	131.6	40.5	174.6	182.9	0.0	115.4	139.2	30.4	58.2	48.1
Greatest daily	48.1	17.7	45.5	58.2	0.0	38.2	35.4	17.7	20.2	20.2
Rain days	9.0	14.0	6.0	11.0	11.0	9.0	3.0	7.0	11.0	6.0
Vegetative wetting (hours/day)	3.2	6.3	3.0	2.6	3.3	3.1	3.2	2.4	3.5	2.2
Chill hours/month	18.0	0.0	0.0	0.0	0.0	115.0	1.0	0.0	0.0	0.0

xObservations for 24 hours ending 7 AM Central Standard time. Source: AWIS Weather Services, Inc. (2011).
yDaily Weather Observations for: Cullman_CR10, AL. StationID= CULA1. Latitude= 34.18 N Longitude= 86.85 W Elevation= 800 Feet.
zDaily Weather Observations for: Auburn_CR10, AL. StationID= AAFA1. Latitude= 32.60 N Longitude= 85.50 W Elevation= 652 Feet.

Table A.2: Physico-chemical analysis of experimental substrates and soil used for container lotus production.

			Chemical composition (mg/L)									
Soil type	Ratio	Ca	K	Mg	P	Al	B	Cu	Fe	Mn	Na	Zn
Sandy loam	100%	27.9	9.3	21.7	1.3	1.7	<0.1	<0.1	1.7	<0.1	8.2	<0.1
Loam/Pine bark	1:1	7.9	24.4	6.6	1.7	4.8	<0.1	<0.1	3.7	<0.1	11.0	<0.1
Loam/Chicken litter	1:1	93.4	6771.0	22.4	278.0	7.0	9.8	68.7	50.5	2.3	2232.0	17.7
Chicken litter/Pine bark	1:1	154.0	5621.0	174.0	664.0	1.1	5.8	49.5	26.0	2.9	1938.0	9.9
Chicken litter	100%	70.4	8002.0	15.1	343.0	1.7	10.5	79.5	42.8	1.7	2506.0	18.2

	C (%)	N (%)	OM (%)	pH	SS (mg/L)	EC (mmhos/cm)	NH_4 (mg/L)	NO_3 (mg/L)
Sandy loam	2.5	0.3	4.3	7.2	203.0	0.29	82.0	25.0
Loam/Pine bark	8.1	0.3	13.9	6.2	112.0	0.16	52.0	6.8
Loam/Chicken litter	11.0	1.1	19.0	8.9	11900.0	17.0	302.0	192.0
Chicken litter/Pine bark	24.7	1.8	42.4	7.3	10500.0	15.0	1078.0	504.0
Chicken litter	26.7	2.3	46.0	9.0	13300.0	19.0	666.0	409.0

	Physical composition				
	Sand (%)	Silt (%)	Clay (%)	Textural class	H_2O availability
Sandy loam	75.0	20.0	5.0	Loamy Sand	0.06

Table A.3: Cultivar performance of lotus evaluated in Auburn during (2004 - 2006).

[u]Cultivar	Petiole (cm)	[w]Plant size	Emergent leaf (number)	Flowers (number)	Color	[x]Seed production	[y]Quality	Propagule number	Fresh weight [(g)]	[z]Survival (%)
04 - R - 31	.[v]	Large	.	15	Red	.	5	23	1620	.
04 - R - 40	.	Large	.	20	Red	.	4	9	180	.
04 - R - 7	.	Large	.	21	Red	.	4	13	540	.
Alexander The Great	.	.	.	.	.	.	.	.	.	30
Baby Red Baby	.	.	.	.	.	.	4	.	.	40
Big Ben	.	.	.	.	.	.	4	.	.	.
Big Red	100	Medium	22	2	Red	x	4	.	.	50
E#2	.	Large	.	0	Yellowish	.	5	16	1210	
E#3	.	Large	.	0	.	.	5	11	880	.
Embolene	97	Medium	81	17	Pink	x	5	53	860	90
Flora	.	.	.	.	Pink	x	3	.	.	80
Garton's 1	.	Medium	6	0	.	.	1	10	340	70
Garton's 2	.	.	.	.	.	.	.	.	.	100
Garton's 3	.	.	.	.	.	.	.	.	.	100
Garton's 4	.	.	.	.	White	.	4	.	.	83
Garton's 5	.	.	.	.	Red	x	5	38	1350	100
Garton's 6	.	.	.	.	Pink	x	4	.	.	100
Garton's 7	96	Medium	65	3	Red/Pink	x	5	29	910	100
Gartons' 98	78	Medium	20	3	Red	x	4	.	.	93
Karizma	.	.	.	.	White	x	5	28	560	91
Mrs. Perry D. Slocum	.	.	.	.	.	.	.	.	.	43
Purple Gold	.	.	.	.	White/Pink	.	.	.	.	75
Sacred Alba	.	.	.	.	.	.	.	.	.	60
White # 17	.	.	.	.	.	.	.	.	.	100
Yangzhou Bowl	.	.	.	0	.	.	1	.	.	100

[u]Cultivars were growing in medium size container (28 L) and Large containers (83 L) with half container level of soil and fertilized with 8 grs for medium size container or 16 grs for large size container at 3 week intervals.

[v]A period represent data absent.

[w]Plant size: Large plants are characterized for reaching a size of 2 meters while medium size cultivars reach from 60 to 120 cms (Billing and Biles, 2007).

[x]Seed production: An x represents the production of seeds.

[y]Quality rating quality rating a Hedonic scale was used: 1: plant did not survive; 2: few floating leaves, no buds or standing leaves; 3: some standing leaves and no buds, 4: standing leaves with 1 to 2 buds; 5: standing leaves and 3 buds.

[z]Survival based on 2004 evaluation.

Table A.4a: Lotus cultivar performance evaluated in Cullman (2005 – 2006).

		Type and number of leaves							
		Floating	Emergent	Emergent	Petiole length	Flower number			[z]Quality
Cultivar	[w]Size	[y]2006	[x]2005	[y]2006	[y]2006	[x]2005	[y]2006	Color	2006[z]
136	Medium	13	15 abc	9	75	.	8	Pink	4
a16(red)	Medium	5	.	10	40	.	4	Pink	3
aH3	Small	5	.	11	40	.	4	Red	4
American Three-colour	.	7	10 d	9	53	.	6	Pink/White	3
American Yellow	Medium	7	.	12	76	.	1	.	3
Apricot Blossom	Medium	17	.	6	35	.	.	.	3
Apricot Yellow	.	7	.	12	76	.	2	White	4
Beautiful Bowl	Medium	5	.	26	29	.	1	Pink/White	3
Birthday Peach	Medium	9	.	11	58	.	4	White	4
Brocade Flag	Medium	6	.	9	56	.	1	Pink/White	4
Camellia Red	Medium	7	10 d	9	28	.	1	Red	3
Celebration	Large	8	.	10	51	.	2	Pink	3
Charming Lips	Medium	9	.	7	61	.	.	.	3
Chongshui Hua	.	5	.	14	61	.	2	Pink	3
Chrysanthemum	.	15	.	7	70	.	.	.	4
Colorful Brocade	Small	3	.	9	78	.	.	Pink	2
Crab Claws Red	Medium	5	.	3	38	.	2	White/Pink	2
Crown	.	8	11 c	7	63	2	1	White	3
Crystal Beauty	Medium	9	.	10	71	.	.	White	3
Dancing Phoenix	.	15	.	12	71	.	.	.	3
Decorated Lantern	Small	10	.	10	68	.	2	White	4
Dense Dew	Small	6	.	10	71	.	1	Pink	4
Diaochan Lanji	Medium	11	.	6	71	.	.	Pink\White	4
Dongu	.	12	.	8	27	.	.	.	3
Double-petal Bayi	Medium	8	11 c	8	45	2	.	.	4
Duplicate Pink	.	17	.	7	61	.	1	White	4
East Red	Medium	6	.	13	76	.	.	.	4
Elegance	Large	7	.	12	92	.	.	.	4
Elite Red	.	16	10 d	4	43	.	.	.	3
Flowing Cloud	Medium	9	13 c	12	73	.	0	.	3
Fragrant Snow	Medium	3	.	12	71	.	.	.	3

[w]Size of plants is based on the length of the petiole were large plants reach more than 2m. In medium size cultivar petiole lengths range from 60 to 120 cms; and small reached 33 cm (Billing and Biles, 2007). Missing values represented by a period.

[x]Emergent leaves and flower number from 2005 evaluation was compared using Tukeys' ($\alpha = 0.05$). No differences were found when comparing the number of flowers.

[y]Data from 2006 is the average of 3 repetitions.

[z]Quality rating quality rating a Hedonic scale was used: 1: plant did not survive; 2: few floating leaves, no buds or standing leaves; 3: some standing leaves and no buds, 4: standing leaves with 1 to 2 buds; 5: standing leaves and 3 buds.

Table A.4b: Lotus cultivar performance evaluated in Cullman (2005 – 2006).

		Type and number of leaves							
		Floating	Emergent	Emergent	Petiole length	Flower number			[z]Quality
Cultivar	[w]Size	[y]2006	[x]2005	[y]2006	[y]2006	[x]2005	[y]2006	Color	[y]2006
Friendship Pink	Medium	11	.	13	75.9	.	1	Pink	4
Gold & Resplendence	.	14	.	9	63.3	.	3	Pink/White	3
Golden Peony	Medium	9	13 c	12	72.1	3	.	.	3
Golden Sun	Medium	9	.	12	72.1	.	1	Pink	4
Green Clouds	Medium	14	.	7	72.1	.	.	Pink	3
Green in Pink Layers	Medium	8	.	15	73.4	.	1	Pink	3
Green-adorned Red	Medium	7	.	6	69.6	.	0	.	3
Guifei Chuyu	Medium	10	.	8	67.0	.	1	Pink/White	3
Guifei Zhuijiu	Medium	9	7 d	9	71.6	.	2	Pink	3
Holy Fire	Medium	8	8 d	10	68.3	.	2	Pink/White	3
Hope	Medium	.	18 a	5	72.1	.	2	White	3
Hubei Edible Lotus	.	10	.	10	70.3	.	1	Pink	3
Icy Heart	.	18	.	5	20.2	.	.	.	2
Illustrious Youth	Large	9	13 c	10	68.3	.	4	Pink/White	4
Jade-tower with drunkard	Small	9	.	6	73.4	.	3	White	4
Jasmine Lotus	Medium	11	.	9	70.8	.	4	White	4
Jewel Flower	Medium	10	.	7	69.1	.	1	White	4
Jicui	.	12	15 abc	6	49.3	3	0	.	3
Juicy Peach	Medium	9	10 d	11	73.4	.	6	Pink/White	4
Lanceolate Pink	.	10	.	6	32.9	.	1	Pink/White	3
Lanceolate Red	.	13	.	3	37.2	.	.	.	3
Laotu Fenzhuang	.	12	.	4	19.5	.	.	.	3
Large Green	Large	8	.	8	43.0	.	.	Pink	3
Large Green House	Medium	8	6 e	10	45.5	.	0	.	3
Large Purple Jade	.	12	.	8	70.8	.	.	.	3
Large Versicolor	.	17	10 d	4	62.5	.	.	.	3
Leader	Small	9	.	9	40.5	.	.	White	3
Leadership	Medium	10	.	9	53.1	.	1	Pink	4
Little Antelope	Medium	10	13 c	6	50.6	2	1	Pink/White	3
Little Brocade-edge	Small	10	.	11	53.1	.	1	White	4
Little Duplicate Pink	Medium	8	.	7	48.1	.	1	White	4

[w]Size of plants is based on the length of the petiole were large plants reach more than 2m. In medium size cultivar petiole lengths range from 60 to 120 cms; and small reached 33 cm (Billing and Biles, 2007). Missing values represented by a period.

[x]Emergent leaves and flower number from 2005 evaluation was compared using Tukeys' ($\alpha = 0.05$). No differences were found when comparing the number of flowers.

[y]Data from 2006 is the average of 3 repetitions.

[z]Quality rating quality rating a Hedonic scale was used: 1: plant did not survive; 2: few floating leaves, no buds or standing leaves; 3: some standing leaves and no buds, 4: standing leaves with 1 to 2 buds; 5: standing leaves and 3 buds.

Table A.4c: Lotus cultivar performance evaluated in Cullman (2005 – 2006).

		Type and number of leaves							
		Floating	Emergent	Emergent	Petiole length	Flower number			[z]Quality
Cultivar	[w]Size	[y]2006	[x]2005	[y]2006	[y]2006	[x]2005	[y]2006	Color	[y]2006
Little Fairy	.	16	13 c	13	66.5	2	2	Pink	3
Little Peony	.	20	.	10	65.8	.	.	.	4
Little Princess	Small	9	11 c	7	45.5	.	0	.	3
Little Red Missing	.	12	.	3	19.0	.	.	.	3
Long-petal Peach-red	Medium	9	17 ab	6	35.4	2	.	.	3
Loyalty Son	Large	9	11 c	9	38.0	3	.	.	3
Missing	.	8	9 d	8	38.0	.	.	.	3
Mr. Huang	Medium	13	.	7	50.6	.	.	.	3
New Brocade-edged	Small	12	7 d	7	53.1	.	.	.	3
New Leaders	Small	9	.	7	45.5	.	.	.	3
New Red	.	17	14 c	3	36.2	3	.	.	3
Olympic Lotus	Medium	13	7 d	9	48.1	2	.	.	3
Orange Loquat	Medium	9	9 d	11	38.0	.	.	.	3
Outstanding Talent	.	9	10 d	7	48.1	.	.	.	3
Pine-cone pink	Small	13	11 c	9	50.6	.	.	.	3
Pink Beauty	.	13	.	4	53.1	.	.	.	3
Pink Bowl	Small	10	.	7	50.6	.	.	.	3
Pink Butterfly	.	15	.	16	73.4	.	.	White	3
Pink Exquisite	Medium	12	12 c	9	38.0	3	.	.	3
Prospect	Medium	22	10 d	8	55.7	.	.	.	3
Pure and Clean	.	18	.	4	22.8	.	.	.	3
Pure Girl	Small	12	.	4	24.5	.	.	.	3
Purple Gold	Small	10	.	10	53.1	.	.	.	3
Qintai Singer	.	10	.	13	55.7	.	.	.	3
Red Bowl	Medium	11	.	10	50.6	.	.	.	3
Red Cherry	Medium	10	.	6	50.6	.	.	.	3
Red Dragonfly	Medium	10	.	6	48.1	.	1	Pink	3
Red Lantern	Small	13	10 d	5	50.6	.	2	Pink/White	3
Red Peony	Medium	11	9 d	6	36.7	.	.	White/Pink	3
Red River	.	12	11 c	8	53.1	.	2	White/Pink	3
Red Rolling Silk Ball	Medium	11	.	8	52.4	.	1	White/Pink	3.5

[w]Size of plants is based on the length of the petiole were large plants reach more than 2m. In medium size cultivar petiole lengths range from 60 to 120 cms; and small reached 33 cm (Billing and Biles, 2007). Missing values represented by a period.

[x]Emergent leaves and flower number from 2005 evaluation was compared using Tukeys' ($\alpha = 0.05$). No differences were found when comparing the number of flowers.

[y]Data from 2006 is the average of 3 repetitions.

[z]Quality rating quality rating a Hedonic scale was used: 1: plant did not survive; 2: few floating leaves, no buds or standing leaves; 3: some standing leaves and no buds, 4: standing leaves with 1 to 2 buds; 5: standing leaves and 3 buds.

Table A.4d: Lotus cultivar performance evaluated in Cullman (2005 – 2006).

	Type and number of leaves							
		Floating	Emergent	Emergent	Petiole length	Flower number		[z]Quality
Cultivar	[w]Size	[y]2006	[x]2005	[y]2006	[y]2006	[x]2005	[y]2006	Color	[y]2006
Red Rose	.	10	.	7	58.2	.	.	.	3
Red-edged White	Medium	13	.	6	48.1	.	.	.	3
Rosy Peach Flower	.	20	.	6	72.6	.	1	Pink/White	3
Rosy Red Duplicate	.	10	.	8	29.1	.	.	.	3
Senior Red	Medium	12	.	5	55.7	.	.	.	3
Snow Beauty	.	14	.	6	20.2	.	.	.	2
Space Lotus 36	Large	12	7 d	7	43.8	.	.	.	3
Sparks	Medium	13	.	6	58.2	.	.	.	3
Spilling Yellow	.	13	.	9	58.2	.	1	White	3
Splendors Red	Small	14	.	7	55.7	.	.	Pink	3
Sweet Acacia	.	8	.	4	50.6	.	.	.	3
The Wonderful	Medium	12	11 c	6	62.5	.	.	.	3
Welcoming Red	Small	11	.	5	45.5	.	.	White	3
Wenjun Fuhong	.	12	.	10	65.8	.	1	White/Pink	4
White Chrysanthemum lotus	Medium	12	13 c	10	55.7	.	.	.	3
White Crane	.	14	.	11	61.5	.	.	.	3
White Crystal	.	18	.	8	45.5	.	.	.	3
White Pear Flower	.	15	.	8	53.1	.	.	.	3
Winter Chrysanthemum	Large	15	9 d	9	60.7	3	.	.	3
Yellow Antelope	Medium	13	.	7	38.0	.	2	Pink	3
Yellow Peony	Medium	16	10 d	7	65.8	.	.	.	3
Zhaojun Guying	Medium	15	.	4	21.5	.	.	.	3
Zhongshan Bailian	.	8	.	5	60.7	.	.	.	3

[w]Size of plants is based on the length of the petiole were large plants reach more than 2m. In medium size cultivar petiole lengths range from 60 to 120 cms; and small reached 33 cm (Billing and Biles, 2007). Missing values represented by a period.

[x]Emergent leaves and flower number from 2005 evaluation was compared using Tukeys' ($\alpha = 0.05$). No differences were found when comparing the number of flowers.

[y]Data from 2006 is the average of 3 repetitions.

[z]Quality rating quality rating a Hedonic scale was used: 1: plant did not survive; 2: few floating leaves, no buds or standing leaves; 3: some standing leaves and no buds, 4: standing leaves with 1 to 2 buds; 5: standing leaves and 3 buds.

Table A.5: Parameters measured to determining growth characteristic of different lotus cultivars forced in greenhouses.

	Number of leaves		Leaf diameter (cm)					
[z]Cultivar	Floating	Emergent	Floating	Emergent	Petiole length (cm)	Flower number	Bud number	[x]Quality
Camellia Red	.	6 a	.	18 a	34 a	54 a	2 ab	4 b
Embolene	14 ns	1 b	.	9 b	21 b	11 c	1 b	2 c
1266	.	4 a	.	17 a	28 ab	37 ab	3 a	4 a
1267	10 ns	5 a	.	17 a	28 ab	30 bc	1 a	3 c

[x]Quality rating was based on: 1=dead, 2=few floating leaves, no buds or standing leaves. 3=some standing leaves, no buds. 4= Standing leaves, 1-2 buds.

[y]Marketability rating: 0= not salable. 1 = salable.

[z]Analysis of variance among cultivars (columns) was performed using PROC GLM (Waller-Duncan). Different letters represent significant differences at α=0.05.

Table A.6: Demographics and type of plant that potential customer wants.

Variable	Descriptive statistics	
Age	Frequency	Percentage
25 or younger	3.0	2.9
26-35	7.0	6.9
36-45	15.0	14.7
46-55	35.0	34.3
56-65	26.0	25.5
66-75	13.0	12.7
76 or higher	2.0	2.0
Missing values	1.0	1.0
Gender		
Male	17.0	16.7
Female	82.0	80.4
Missing values	3.0	2.9
Interest on purchasing		
Definitely Yes	39.0	38.2
Probably Yes	28.0	27.5
Maybe	26.0	25.5
Probably Not	9.0	8.8
Lotus characteristics		
Flower color		
Pink	51.0	50
White	10.0	9.8
Yellow	13.0	12.7
Red	27.0	26.5
Flower form		
Single Flower	32.0	31.4
Double flower-multi-petal	69.0	67.6
Plant size		
Small (30-60 cm)	16.0	15.7
Medium (1.2 - 1.5 m)	58.0	56.9
Large (1.8 - 2.5 m)	24.0	23.5

Table A.7: Rating of different forms and colors of ornamental lotus cultivar.

Cultivar		Quality Rating[x]	Quality Rating Frequency[z]				
Type	Common name	Observations	1	2	3	4	5
1	Xiamen Bowl	22	6	5	4	4	3
2	Jasmine Lotus	28	5	6	4	4	9
3	Olympic	43	8	8	12	9	6
4	Large Green House	20	4	6	2	5	3
5	Apricot Yellow	40	12	10	6	4	8
6	Golden Peony	19	3	3	3	3	7
7	Gold and Jade	26	3	3	4	3	13
8	Prospect	31	5	9	4	5	8
9	Welcoming Red	26	5	5	5	4	7
10	Taikong # 36	33	5	9	10	5	4
11	Long Petal Peach	33	5	13	6	3	6
12	Dense Dew	34	4	5	6	12	7
13	Friendship	26	9	5	5	3	4
14	Garton's 98	46	13	7	5	12	9
15	Senior Red	55	18	13	8	6	10
16	Holy Fire	42	18	5	4	8	7

[x]Quality rating: 1[z]=One of the Best. 2=Above Average. 3=Average. 4=Below Average. 5= One of the Worst.

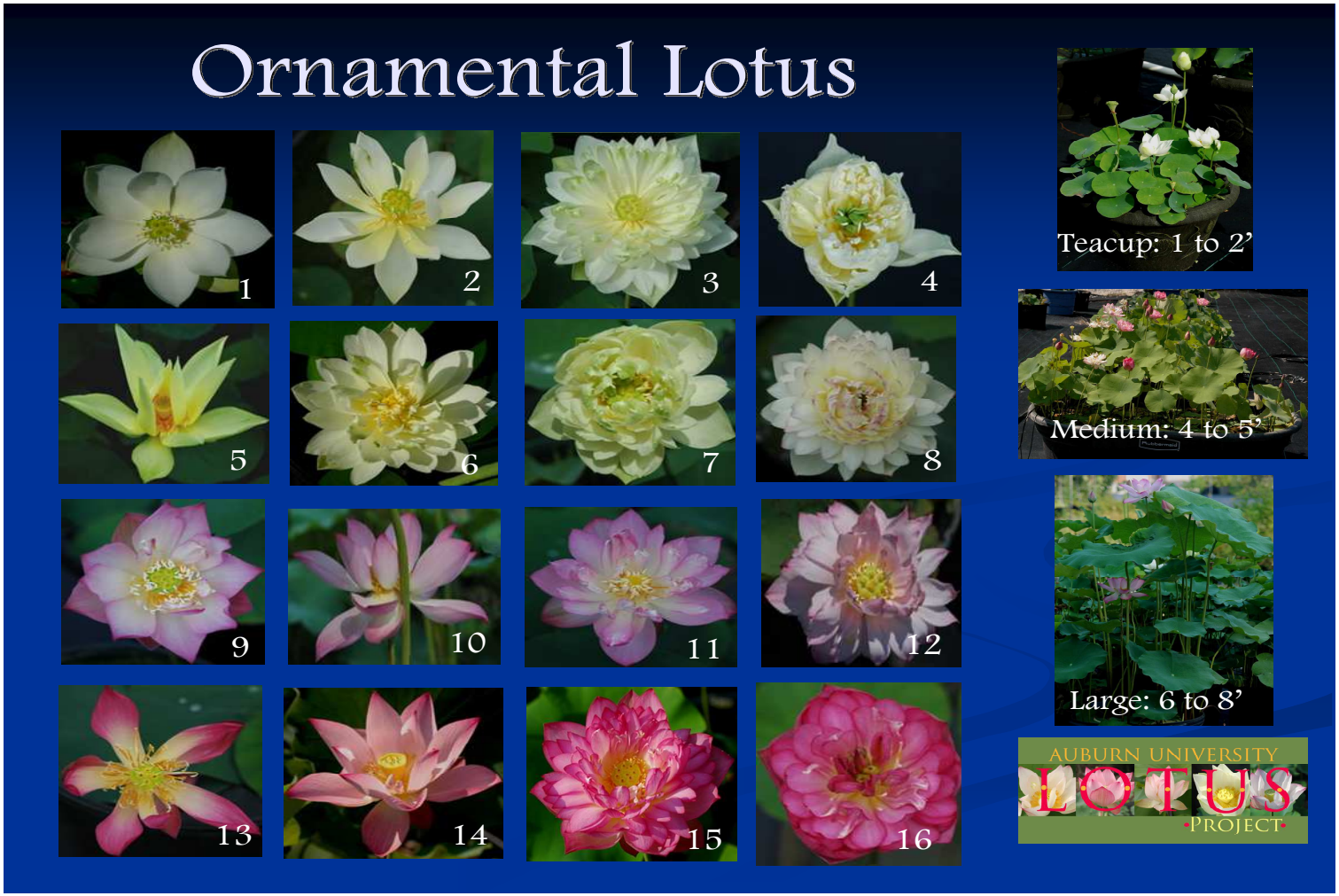

Fig.A.1. Poster used to assess customer flower color, plant size and flower form preference. Pictures by Tian (2008).

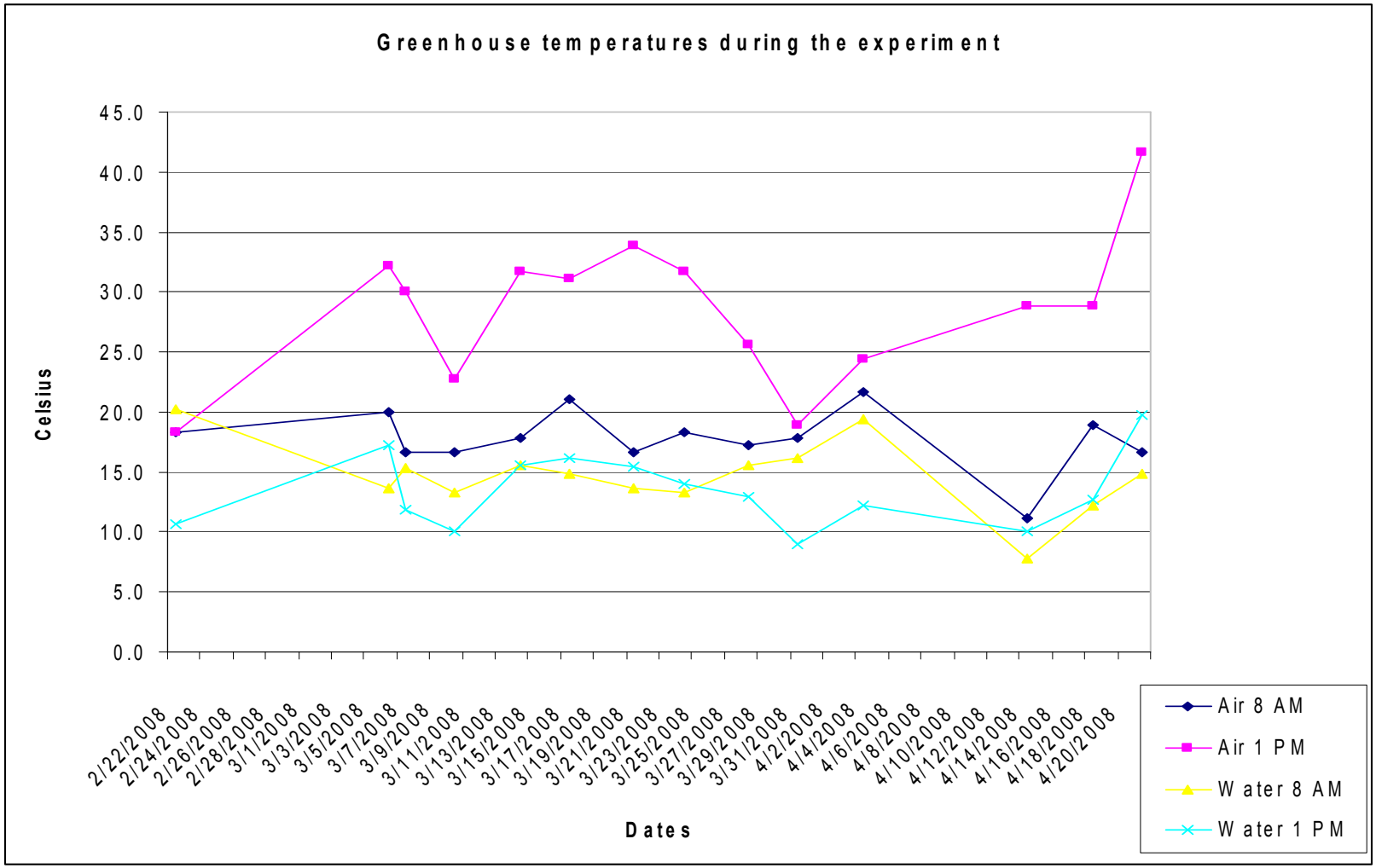

Fig.A.2. Air and water temperature fluctuations measured in the greenhouse where lotus cultivars were forced during 2008 study at Mobile, Al.

APPENDIX 2

Auburn University L O T U S Project Survey

Please take a minute to help us evaluate the value of Lotus as an ornamental plant for our Alabama Gardens. Use the poster to help you answer a few questions. Thank you for taking some of your gardening time to help us add to your gardening pleasure!

1. Have you ever seen a lotus plant/flower?
   _____ Yes _____ No

2. Have you ever had one in your garden?
   _____ Yes _____ No

3. After looking at the pictures/plants, how interested would you be in purchasing one?
   _____ definitely yes
   _____ probably yes
   _____ maybe
   _____ probably not
   _____ definitely not

Please look at the poster on the right to answer the following questions:

4. Which color flower appeals to you the most?
   _____ Pink
   _____ White
   _____ Yellow
   _____ Red

5. Which form of flowers appeal to you the most, doubles or singles?
   _____ single flower form (single row of petals)

   _____ double flower form (multiple rows of petals)

PLEASE CONTINUE ON THE BACK (ONLY 3 MORE)

6. Look at the poster on the right. Would you prefer the:

_____Large plants

_____Medium plants

_____Small plants

7. Of the 16 flowers posted, please pick the top 5 and rank them 1-5 with 1 being your favorite flower and 5 your least favorite. Please place the numerical rankings, 1 through 5, in the space before **(TO THE LEFT)** the number of the flowers you like.

_____1	_____ 9
_____2	_____ 10
_____3	_____ 11
_____ 4	_____ 12
_____ 5	_____ 13
_____ 6	_____ 14
_____ 7	_____ 15
_____ 8	_____ 16

8. To help with our demographic research may we ask:

_____ Male _____ Female

AGE: _____15-25
_____ 26-35
_____ 36-45
_____ 46-55
_____ 56-65
_____ 66-75 and
_____ over 75: WOW, we welcome your wise counsel

Thank you for your support of Auburn University Horticulture – Stop by to see us when you are in Auburn.