

Preliminary Assessment of Lotus (*Nelumbo nucifera* Gaertn.) Rhizomes  
: An Underutilized Aquatic Vegetable Crop

by

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## Abstract

Lotus (*Nelumbo nucifera* Gaertn.) is a perennial aquatic edible plant which offers a wide diversity of potential health benefits including improving learning and cognitive memory, anti-obesity activity, anti-HIV activity, anti-tumor activity, diuretic activity, antipyretic activity, anti-inflammatory activity and anti-diabetic activity. Determination of the optimum physiological maturity of edible lotus rhizome is unclear and problematic. In order to establish a reliable maturity index for two edible lotus species (*N. nucifera* 'E2' and *N. lutea*) we have evaluated the changes in various phytochemical parameters during four consecutive harvest determinations.

Therefore, the global objectives of this study were to determine the effects of harvest maturity on lotus rhizome nutritional quality and to possibly establish a reliable harvest index. The proposed senescence indicators (chlorophyll content index (CCI), dry matter content (DMC), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), malondialdehyde (MDA) and total soluble protein (TSP) content), in conjunction with phytochemical antioxidant metabolites (ascorbic acid, (AsA), proline (Pro), total phenols (TP) and antioxidant capacity (ABTS and DPPH)) in leaves and rhizomes from two edible species of lotus (*N. nucifera* 'E2' and *N. lutea*) were determined at four harvest dates during growing season to establish objective criteria for estimating optimal commercial harvest maturity. Significant differences were found among harvest dates for senescence indicators and phytochemical antioxidant components. A potential

maturity index (chlorophyll content) was established for *N. nucifera* rhizomes to provide optimal nutritional quality and consumer demand. In addition, *N. lutea* was considered as an early maturing species when compare with *N. nucifera* 'E2' based on proposed objective criteria evaluated.

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## List of Abbreviations

AAPH	2,2'-azobis-(2-amidinopropane) HCl
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt
AsA	Ascorbic Acid
CCI	Chlorophyll Content Index
DAP	Days After Planting
DMC	Dry Matter Content
DPPH	2,2-Diphenyl-1-picrylhydrazyl
dw	Dry Weight
EDTA	Ethylenediamine Tetraacetic Acid
fw	Fresh Weight
GAE	Gallic Acid Equivalents
H <sub>2</sub> O <sub>2</sub>	Hydrogen Peroxide
MDA	Malondialdehyde
PAL	Polyphenol Oxidase
PBS	Phosphate-buffered Saline
PGE2	Prostaglandin E2
PPO	Phenylalanine Ammonia Lyase
Pro	Proline
ROS	Reactive Oxygen Species
TBA	Thiobarbituric Acid



TCA	Trichloroacetic Acid
TP	Total Phenolic
TSP	Total Soluble Protein
VCEAC	Vitamin C Equivalent Antioxidant Capacity
WI	Whiteness Index

## CHAPTER I

### EVIDENCE FOR AN ASSOCIATION WITH CONSUMPTION OF LOTUS (*NELUMBO SPP.*) AND REDUCTION IN CHRONIC DISEASE

#### Natural History and Characteristic of Lotus

Lotus (*Nelumbo nucifera* Gaertn.) is perennial aquatic edible plant which produces rhizome that is very popular as a vegetable. In addition, leaves, stems, seeds and other parts are edible and many organs of the plant are thought to have multiple medicinal properties (Ni et al., 1987).

#### Origin and Geographical Distribution of Lotus

Lotus belongs to the *Nelumbolaceae* family and the genus *Nelumbo* (Wang and Zhang, 2004). There are only 2 species in this genus: *Nelumbo nucifera* with pink, red or white flowers, distributed in Asia and Oceania, and *Nelumbo lutea* with yellow flowers, distributed in North and South America (Wang and Zhang, 2004).

The American species is represented as *Nelumbo lutea*, and known as American Lotus in addition to yellow lotus (Sayre, 1963) 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 throughout Central America and North America. Small native populations are found in the West Indian Archipelago and the extreme southeastern portion of Ontario, Canada (Wang and Zhang, 2004).

*N. nucifera* has very diverse common names including lotus, sacred lotus, East Indian lotus, Egyptian lotus, Indian lotus, Chinese lotus, and sacred water lily (Yamaguchi, 1990). Due to the known diversity within *N. nucifera* spp., China is considered to be a natural distribution center of *N. nucifera* (Wang and Zhang, 2004). However, the origin of the sacred lotus is still debated.

### **Value Added Products**

Several organs of *Nelumbo lutea* are edible. The starchy rhizome of American lotus was used as food by Native American Indians in the past. Seeds are gathered and eaten like nuts and also used in soups. What is more, the terminal shoots are once collected and dried and used as winter food (Ni et al., 1987; Wang and Zhang, 2004).

In Asian culture predominantly in China, all plant parts of *N. nucifera* are consumed as food or used for medicinal purposes, including rhizomes, nodes, roots, seeds, young shoots, leaf, stalks, petals, stamens, and pericarps (or fruit receptacles, seedpods) (Wang and Zhang, 2004). Of course, the major edible part of lotus are enlarged rhizomes and seeds, that are nutritionally rich including starch, sugars, proteins, lipids, vitamins, minerals, alkaloids, flavonoids and other biochemical substances. Nutritional compositions in *N. nucifera* rhizomes and seeds have been widely reported and new compounds are continuously found. However, information on nutritional composition in *N. lutea* is very limited (Xueming, 1987).

Nutritional compositions of *N. nucifera* rhizomes were summarized by Xueming (1987). Based on analysis of 33 cultivars, rhizomes contain approximately 8.4-22.7%

starch, 1.4-4.8% total sugar, 0.2-2.4% reductive sugar, 0.1% fat, 0.94-2.44% protein, 0.07-1.02% free amino acids, 0.26-0.35 mg g<sup>-1</sup> Vitamin C, 1.3-1.9 µg g<sup>-1</sup> Vitamin B<sub>2</sub>, 1.4-1.7 µg g<sup>-1</sup> Vitamin B<sub>6</sub>, 0.3% total polyphenols, 1.1% ash, peroxidase, and other minerals.

Lotus rhizomes are sold whole or in cut pieces, fresh, frozen, or canned. Rhizomes are consumed raw or prepared for salad, but mostly cooked in Chinese and Japanese cuisine. Fresh rhizomes may be stirred fried after being sliced, stewed as soup with pork, steak, or chicken or other kinds of meats. They can also be steamed with rice. Rhizomes are typically soaked in syrup or pickled in vinegar. Recently canned salted-rhizomes, fresh-frozen rhizomes and dried rhizome powders are commercially marketed in China. Glazed lotus-rhizomes are also available in the market. Other products such as lotus rhizome juice (beverage), yogurt vinegar and wine are under development in China (Zhu and Xia, 2002; Zhang et al., 2002; Liu, 2007).

### **ROS and Antioxidants**

Reactive oxygen species (ROS) such as singlet oxygen (<sup>1</sup>O<sub>2</sub>), superoxide radical (O<sub>2</sub><sup>·-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and hydroxyl radical (·OH) are generated as by-products during aerobic metabolism in cells (Gutteridge, 1994). Biologically, cells are naturally provided with diverse defensive enzymatic and non-enzymatic mechanism antioxidants. They act synergistically against these potentially harmful oxidizing agents (Halliwell, 1994). However, even this multifunctional protective system cannot

completely counteract the injurious effects of ROS. As a result, oxidative damaged molecules accumulate in cells. It is widely acknowledged that the accumulation of ROS damaged intracellular macromolecules, including DNA, protein and lipid, is an essential factor in the aging process and in certain degenerative diseases such as cancer, immune system decline, brain dysfunction, cataracts and coronary heart disease (Halliwell et al, 1992).

A possible way to prevent ROS-mediated cellular injury is to supplement endogenous natural oxidative defense systems with the dietary intake of antioxidants such as vitamins A, C and E (Block et al., 1992; Di Mascio et al., 1991; Krinsky, 1991). Some natural products have well-proven antioxidant activities. Protective effects of their natural components have been attributed, in large part, to their scavenging free radicals, chelating transition ions and/or modulating antioxidant proteins within cells (Kameoka et al., 1999)

Plant tissues synthesize a wide variety of antioxidants which include ascorbic acids, proline, flavonoids, flavones, anthocyanin, catechin, etc. These compounds exhibit free radical scavenging activity and have received increasing attention for their potential role in the prevention of human disease as well as in food quality improvement (Shahidi and Baczk, 1995; Vinson et al., 2001). Several studies on *Nelumbo nucifera* using different methods have proved that lotus have a high antioxidant content and a strong antioxidant capacity. (Hu and Skibsted, 2002; Khattak et al., 2009; Yang et al., 2007; Park et al., 2009)

## **Main Antioxidant Compounds Related to Lotus**

Ascorbic Acid (ASA) usually serves as a good inhibitor of enzymatic browning due to its antioxidant property. The possibility of preventing enzymatic browning in fresh-cut products through the application of exogenous ASA also suggests the possible involvement of this antioxidant compound in the occurrence of this disorder in wounded tissues (Sapers, 1993). The mechanism by which ASA exerts its protection is still not completely understood. More and more evidence show the possibility of involvement ASA metabolism in enzymatic browning. Moreover, both ASA metabolism and browning have a close relationship with environmental stress, i.e. senescence and wounding (Sapers, 1993). For this discussion, it is possible that both ASA and phenols serve as the protection for plants from environment stresses. Work is in progress for further investigation of the possible role of ASA in the tolerance of plant tissues to cut-induced browning.

Several studies reported the content of ascorbic acid in *Nelumbo nucifera* rhizome. Mukherjee *et al* (1996) reported the vitamins thiamine (0.22mg/100gdw), ascorbic acid (1.5mg/100gdw) and an asparagine-like amino acid (2%) present in the *Nelumbo nucifera* rhizome. Fresh rhizome contains 83.80% water, 0.11% fat, 1.56% reducing sugar, 0.41% sucrose, 2.70% crude protein, 9.25% starch, 0.80% fire, 1.10% ash and 0.06% calcium. Compare with his study, Park *et al* (2009) reported a high content of ascorbic acid ( $10.1\pm 0.9$ ,  $12.6\pm 1.1$ , and  $8.6\pm 0.7$  mg/gdw) for water extracts of three different Korean lotus cultivars (Muan, Chungyang, and Garam). In his study,

contents of ascorbic acid, polyphenols, flavonoids, and flavones are higher in the Chungyang cultivar, which indicated the contents of the nutritional and bioactive compounds varied significantly among different lotus cultivars.

Phenolic compounds are the hydroxylated derivatives of benzoic and cinnamic acids and contribute to overall antioxidant activities in the plants (Vinson et al., 2001). Role of phenolic compounds as antioxidants have been well documented. Structural chemistry of polyphenols suggests their free radical scavenging properties (Rice-Evans et al., 1997). The activity of antioxidants is determined by its reduction potential, ability to stabilize and delocalize the unpaired electrons, reactivity with other antioxidants, and transition metal chelating potential. Polyphenols have all the above characters and thus play a significant role in antioxidative defense systems in plants. Many phenylpropanoid compounds such as flavonoids, isoflavonoids, anthocyanins and polyphenols are induced in response to wounding, nutritional and cold stresses, and high visible light.

An earlier study by Yang et al. (2007) showed 437.5 and 1266.0 mg catechin equivalents/100gdw of phenolic content in methanol and acetone extracts of lotus rhizome, respectively. Hu and Skibsted (2002) reported 31.9 mg GAE/100gdw phenolic content in 50% aqueous ethanol extract of lotus rhizomes. In addition, Jung's group (2008) reported the total phenolic content at 177.70 mgGAE/gdw in *Nelumbo nucifera* leaf tissue and 21.60 mgGAE/gdw in rhizome tissue. Total phenolic content of the lotus leaf was determined to be  $374 \pm 7.5$  mg/gdw gallic acid equivalents and

203±7.0 mg/gdw rutin equivalents, respectively (Huang *et al.*, 2010). Another study from Park *et al.* (2009) reported the content of polyphenols extracted with methanol and water from four cultivars of lotus roots. Yang *et al.* (2007), Hu and Skibsted (2002), Huang *et al.* (2010) and Jung *et al.* (2008) reported total phenolic values varied considerably.

Proline is synthesized from glutamate by a series of reduction reactions. In this synthesis process, proline and pyrroline-5-carboxylate (P5C) may regulate redox and hydride ion-mediated stimulation of pentose phosphate pathway (Hagedorn and phang, 1983; Phang, 1985). During respiration, oxidation reactions produce hydride ions that help reduction of P5C to proline into cytosol. Through the reaction of proline dehydrogenase, proline can enter the mitochondria. Within the mitochondria, instead of NADH, proline acts as a reducing equivalent and can support oxidative phosphorylation. The reduction of P5C in cytosol provides NADP<sup>+</sup>, which is a co-factor for glucose-6-phosphate dehydrogenase (G6PDH). G6PDH plays a crucial role by catalyzing the first rate limiting step of the pentose phosphate pathway. Phang (1985) first proposed this model and stated its role in stimulation of purine metabolism via ribose-5-phosphate in animal cells. On the basis of this hypothesis, Shetty (1997) proposed a model that proline-associated and phenylpropanoid pathways, and therefore, the modulation of this pathway could lead to the stimulation of phenolic phytochemicals. Because of the role of phenolics as antioxidants, proline is also considerable when determining antioxidant properties of plants tissue.



## **Antioxidant Capacity**

DPPH·(1,1-Diphenyl-2-picryl-hydrazyl) is used as a free radical to evaluate antioxidant activity of some natural compounds. The degree of its discoloration is attributed to hydrogen donating ability of test compounds. Yang et al. (2007) reported a significant DPPH· radical scavenging activity in methanol and acetone extracts of lotus rhizomes. Park et al. (2009) also reported a high antioxidant activity in three lotus cultivars ('Muan', 'Chunyang' and 'Garam'). Several previous researches indicated the correlations were found between concentrations of phenolic compounds and DPPH radical scavenging activity in lotus (Hu and Skibsted, 2002; Choe et al., 2009 and Lee et al., 2005)

## **Evidence for an Association with Consumption of lotus (*Nelumbo nucifera*) and Reduction in Chronic disease**

### **Antiviral Activity and Antimicrobial Activity**

All parts of *N. nucifera* have been used for various medicinal purposes in oriental medicine. In particular, leaves are known for diuretic and astringent properties, and are used to treat fever, sweating, and strangury and as a styptic. During our continuing search for plant-derived anti-HIV agents from natural products, the 95% EtOH extract of the leaves of *N. nucifera* was found to display significant anti-HIV activity ( $EC_{50} < 20 \mu\text{g/mL}$ ,  $TI > 5$ ).

Kashiwada et al (2005) reported the antiviral activity of lotus leaf. In his study, (+)-1(R)-Coclaurine and (-)-1(S)-norcoclaurineliensinine, together with quercetin 3-

*O*- $\beta$ -D-glucuronide, were isolated from the leaves of *Nelumbo nucifera* demonstrated potent anti-HIV activity with EC<sub>50</sub> values of 0.8, <0.8 and 2 $\mu$ g/mL respectively. Other potent anti-HIV bisbenzylisoquinoline alkaloids such as nuciferine, liensinine, negferine and isoliensinine have also been isolated from the leaves of *N. nucifera*, with EC<sub>50</sub> values below 0.8 mg/ml and therapeutic index values of 36, > 9.9, > 8.6 and > 6.5, respectively (Kawashida et al., 2005). After that, Li and Xu (2008) also reported the antibacterial activity of *Nelumbo nucifera* leaf extracts. In this study, the antimicrobial activity of extracts was tested by using *Nelumbo nucifera* leaf extracts against five microorganisms: *Actinobacillus actinomycetemcomitans* Y4, *Actinomyces viscosus* 19246, *Porphyromonas gingivalis* 33277, *Fusobacterium nucleatum* 25586, and *Actinomyces naeslundii* wvl 45. They reported the component that had a greatest antimicrobial activity was determined to be quercetin. The minimum inhibitory concentrations of the most active lotus leaf extracts were 0.63, 1.25, 1.25, 0.63 and 2.50 mg/mL for *A. actinomycetemcomitans*, *A. viscosus*, *P.gingivalis*, *F. nucleatum*, and *A. naeslundii*, respectively. Consumption of lotus rhizomes was also found to have antibacterial activities and antifungal activities (Liu and Xu., 2008). Agnihotri et al (2008) reported antifungal activity for (R)-roemerine, isolated from the leaves of *Nelumbo nucifera* (IC<sub>50</sub>=0.2 and 4.8  $\mu$ g/mL against *Candida albicans*).

Different extracts of rhizomes showed antibacterial effects against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *B. pumilis* and *Pseudomonas aeruginosa*. Chloroform extract was found to be the most effective

when compared with the standard drug chloramphenicol. (Mukherjee PK et al. 1995a)

Antifungal and anti-yeast activities of the rhizome extract were evaluated against five different strains of fungi and yeast, including *Candida albicans*, *Aspergillus niger*, *A. fumigatus* and *Trichophyllum mentagopyhtes*; the extract showed potential activity in all the strains tested and the effect was comparable to that of griseofulvin, used as standard drug for comparison. (Mukherjee PK et al. 1995b)

### **Anti-obesity**

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a negative effect on health, leading to reduced life expectancy and/or increased health problems. *Nelumbo nucifera* has demonstrated anti-obesity activities from both leaf and rhizome tissue extracts. (Liu et al., 2013; Ahn et al., 2013; Ono et al., 2006; Ohkoshi, E. et al., 2007; Wu et al., ; Shi-Ying X and Charles FS., 1986; Lin et al., 2009 and You et al., 2014).

Ono et al. (2006) have examined the effects of *Nelumbo nucifera* leaf extracts on digestive enzyme activity, lipid metabolism and thermogenesis. They also evaluated the anti-obesity effect using mice with obesity induced by a high-fat diet. A concentration-dependent inhibition from *Nelumbo nucifera* leaf extract of the activities of  $\alpha$ -amylase and lipase, and up-regulated lipid metabolism and expression of UCP3 mRNA in C2C12 myotubes was found by Ono et al (2006). Ohkoshi, E. et al (2007) also reported that the dietary supplementation of the leaf of *Nelumbo nucifera* resulted in a suppression of body weight gain in mice fed a high-fat diet. Bioassay-

guided fractionation and repeated chromatography of the leaf of *Nelumbo nucifera* has led to the isolation and identification of quercetin 3-O- $\alpha$ -arabinopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -galactopyranoside, rutin, (+)-catechin, hyperoside, isoquercitrin, quercetin and astragalol. These compounds exhibited lipolytic activity, especially in visceral adipose tissue. The flavonoid-enriched *N. nucifera* leaf extract supplement improves the high fat diet-induced abnormal blood lipids and liver damage in hamster as significantly as the common drugs, such as silymarin and simvastatin (Lin et al., 2009). Wu et al (2010) have also reported the *Nelumbo nucifera* leaf extract target lipid-regulated enzymes, such as fatty acid synthase, glutamic oxaloacetic transaminase and glutamic pyruvic transaminase, and effectively attenuate body lipid accumulation and prevent obesity. This agreed by Liu's study (2013) that *Nelumbo nucifera* leaf flavonoids showed a high inhibitory activity against porcine pancreatic lipase,  $\alpha$ -amylase, and  $\alpha$ -glucosidase with IC<sub>50</sub> values of 0.38 $\pm$ 0.022, 2.20 $\pm$ 0.18, and 1.86 $\pm$ 0.018mg/mL, respectively. It significantly lowered the lipid components, such as the total cholesterol, triglycerides and low-density lipoprotein cholesterol. Moreover, the *Nelumbo nucifera* leaf flavonoids alleviated high-fat diet-induced lipid accumulation in the liver (Liu et al., 2013). Ahn et al (2013) also reported the anti-obesity activity of *Nelumbo nucifera* leaf. In this study, he isolated 13 megastigmanes, a new eudesmane sesquiterpene, eight alkaloids, and 11 flavonoids. Compounds cis-N-feruloyltyramine, trans-N-coumaroyltyramine inhibited pancreatic lipase, whereas

compounds (6R, 6aR)- roemerine-Nb-oxide, liriodenine showed a strong inhibitory effect on adipocyte differentiation. (Ahn et al., 2013)

Anti-obesity activity has also been observed in *Nelumbo nucifera* rhizome that serum total cholesterol and triglyceride levels were decreased in rats by feeding the supplement of *Nelumbo nucifera* rhizome extract. In addition, administration of extract of lotus rhizome resulted in a significant decrease in relative weights of adipose tissues in rats fed a high-fat diet and inhibition of lipid accumulation and attenuated expression of adipogenic transcription factors. (You et al., 2014) Therefore, lotus rhizomes exert antioxidant and anti-obesity effects and could be used as a functional ingredient in obesity-related diseases.

#### **Antidiarrheal Activity**

Antidiarrheal activity has been studied in *Nelumbo nucifera* rhizomes. Animals fed with *Nelumbo nucifera* rhizomes showed a high inhibitory activity against castor oil induced diarrhea and inhibitory activity against PGE<sub>2</sub>-induced enterpooling. In addition, it also showed a reduction in gastrointestinal motility after charcoal meal in rats. (Mukherjee et al., 1995) All the results established the efficacy of *Nelumbo nucifera* rhizome as an antidiarrheal ingredient. This was been further confirmed by Talukder and Nessa's study (1998) that rats with *Nelumbo nucifera* rhizome supplement were observed with a reduction of the frequency of defecation, wetness of fecal dropping and PGE<sub>2</sub>-induced enterpooling. It also showed the reduction of propulsive movements of charcoal meal.

### **Antipyretic Activity:**

Antipyretic activity of methanolic extract of rhizome of *Nelumbo nucifera* was studied on normal body temperature and yeast induced pyrexias in rats by Mukherjee et al (2006). The extract, in doses of 200, 300 or 400 mg/kg (po) produced dose dependent lowering of normal body temperature and yeast provoked elevation of body temperature in rats, which is comparable with the standard antipyretic drug, paracetamol (150mg/kg). (Mukherjee et al., 2006)

### **Antimalarial Activity**

Antimalarial activity have been studied in *Nelumbo nucifera* leaf by Agnihotri et al. (2008) From the leaves of *Nelumbo nucifera*, twelve metabolites were isolated and identified by spectroscopic methods. Antimalarial activity for (R)-roemerine and *N*-methylasimilobine ( $IC_{50}$ =0.2 and 4.8  $\mu$ g/mL for the D6 clone, respectively, and 0.4 and 4.8 $\mu$ g/mL for W2 clone, respectively) was observed. In addition, the substituents in position C-1 and C-2 of sporphine alkaloids are important for antimalarial activity. This was observed by structure-activity relationship analysis. (Agnihotri et al., 2008) Results were confirmed by Kamaraj's (2012) study that also reported antimalarial activities from *Nelumbo nucifera* leaves.

### **Hepatoprotective Activity**

An ethanolic extract of leaves was studied for its hepatoprotective activity against  $CCl_4$ -induced liver toxicity in rats by Huang et al. (2010) Hepatoprotective activity of lotus leaf extract (LLE) at doses of 300 and 500 mg/kg and in vivo

antioxidant activity at 100 mg/kg that was comparable with that of a standard treatment comprising 100 mg/kg of silymarin, a standard hepatoprotective drug (Huang et al., 2010). In his study, lotus leaf extract possessed strong hepatoprotective and antioxidant activity in a rat model of CCl<sub>4</sub>-induced liver toxicity in rats. The hepatoprotective activity of lotus leaf extract due to its antioxidant capacity and result from the high content of flavonoids and phenolic compounds in the leaf extract (Huang et al., 2010).

### **Psychopharmacological Activity**

*Nelumbo nucifera* rhizome extract was found to cause reduction in spontaneous activity, decrease in exploratory behavioral pattern by the head dip and Y-maze test, reduction in muscle relaxant activity by rotarod, 30° inclined screen and traction test and potentiated the pentobarbitone induced sleeping time in mice (Mukherjee et al., 2006)

### **Anti-diabetic Properties**

Methanolic extract of *Nelumbo nucifera* leaf increased insulin secretion from  $\beta$  cells and human islets. Extracts enhanced the intracellular calcium levels in  $\beta$  cells and also enhance phosphorylation of extracellular signal-regulated protein kinases 1/2 and protein kinase C. Catechin dose-dependently enhanced insulin secretion. (Huang et al., 2011)

Lotus leaves resulted in sharp decreases in serum total cholesterol, free cholesterol and phospholipids compared with the high fat-loaded control group. (Onishi E et al., 1984)

Mukherjee et al (1995; 1997) also reported the anti-diabetic properties of *Nelumbo nucifera* rhizome that improved glucose tolerance and potentiated the action of exogenously injected insulin; reduced the blood sugar level of normal, glucose-fed hyperglycaemic and streptozotocin-induced diabetic rats when compared with control animals. This confirmed research results by Shi-Ying and Charles (1986) that the methanolic extract of nodes at a dose of 400 mg/kg and 100 mg/kg of isolated tryptophan showed potential anti-diabetic activities in glucose-fed hypoglycaemic mice.



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## CHAPTER II

### LEAF SENESCENCE, HARVEST MATURITY AND PHYTOCHEMICAL PROPERTIES OF TWO LOTUS SPECIES (*NELUMBO NUCIFERA* GAERTN.)

#### ABSTRACT

Leaves and rhizomes from two edible species of lotus (*N. nucifera* 'E2' and *N. lutea*) were harvested at four harvest dates during the growing season (118, 132, 146 and 160 days after planting (DAP) for *N. nucifera* 'E2' and 125, 139, 153 and 167 DAP for *N. lutea*, respectively) to establish objective criteria for estimating optimal commercial harvest maturity. In this study, senescence indicators (chlorophyll content index (CCI), dry matter content (DMC), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), malondialdehyde (MDA) and total soluble protein (TSP) content), in conjunction with phytochemical antioxidant metabolites (ascorbic acid, (AsA), proline (Pro), total phenols (TP) and antioxidant capacity (ABTS and DPPH)) in leaves and rhizomes were determined as possible reliable indices for estimating optimal maturity at harvest. Results indicate that significant differences were found among harvest dates for senescence indicators and phytochemical antioxidant components evaluated. In addition, CCI was determined to be the most reliable predictor for estimating maximum nutritional quality for *N. nucifera* 'E2'. However, the determination of an appropriate maturity

index is still remains to be determined for *N. lutea*. In addition, *N. lutea* was determined to be early maturing specie when compared to *N. nucifera* ‘E2’.

## **INTRODUCTION**

Current knowledge of optimum time to harvest lotus rhizomes (*Nelumbo nucifera* Gaertn.) is unclear and problematic due to its aquatic nature and habitat. There are no established objective criteria available to assess rhizome physiological maturity at the time of harvest with the exception of subjective visual cues of complete leaf senescence or defoliation, especially in containerized lotus vegetable production. Detailed knowledge concerning physiological senescence processes of edible lotus rhizomes could contribute to increased quality, consumer demand and the establishment of cultural protocols for domestic markets as a functional food. Maturity indices for fruit and vegetables typically are based on a diverse range of physiochemical, biochemical and quality traits that are classified subjectively and objectively to determine optimal maturity thresholds at harvest (Gross et al, 2004). Development of a maturity index requires collection of phenological measurements (i.e., size, color, firmness, soluble solid and dry matter content, primary and secondary metabolites, protein, etc.) that reflect changes throughout growth, development and senescence and correlate with minimum consumer commodity acceptability. In this context, collection of phenological data in regard to lotus is problematic due to its aquatic nature and habitat. Contrary to many fruit and vegetables currently marketed in the United States, commercial grades or standards of

edible lotus have not been established. Lotus is an aquatic perennial native to many subtropical and temperate zones and is cultivated extensively throughout Asia and Iran (Huang et al., 2003). Recently, lotus has become a potential vegetable crop in Australia (Nguyen, 2001), New Zealand (Follett and Douglas, 2003) and the United States (Tian et al., 2006, 2008, 2009 a, b). Nearly all plant parts of lotus can be consumed as food or used as a nutraceutical source, including rhizomes, seeds, young shoots, leaves, stalks, petals, stamens and pericarp (Wang and Zhang, 2004). As an edible vegetable crop, lotus is one of the most popular vegetables in the world due to known ethnoculinary, pharmacological and nutraceutical applications (Sridhar and Bhat, 2007) and health beneficial properties. Lotus leaves are rich in flavonoids and other secondary metabolites and have been extensively evaluated for their antioxidant, antibacterial, anti-HIV and anti-obesity properties (Ahn, 2013; Choe et al., 2010; Huang et al., 2010; Jung et al., 2008; 2010; Kashiwada et al, 2005; Liu et al., 2013; Ono et al., 2006; Sugimoto et al., 2008). Lotus rhizomes are rich in dietary source of fiber, carbohydrates, vitamins C, B<sub>1</sub>, B<sub>2</sub>, and E, carotenoids, phenolic compounds, amino acids, and macro and micronutrients and are used in soups, salads, desserts and stir-fried food throughout China, Japan, India, Korea and Australia (Chiang, and Luo, 2007; Gong et al., 2008; Hu and Skibsted, 2002; Hertog, 1994; Ke et al., 2000; Mukherjee et al., 2010; Wu, 1987;). Additionally, lotus rhizomes exhibit high antioxidant activity that has been assigned physiological roles such as anti-inflammation, neuroprotection, anti-diabetes, and immune regulation (Bouckenoghe

et al., 2006; Mukherjee et al., 2010; Shad et al., 2012; Tsuruta et al., 2011; Yang et al., 2007). In acknowledging these attributes, the concentration of functional components of lotus is highly dependent on the plant part evaluated (Wu et al., 2011), rhizome pigmentation (Chen et al., 2013), cultivation region and extent of processing (Zhao et al., 2014). It is therefore, relevant to question whether the concentration of functional components in lotus may vary extensively with maturity at harvest and rhizome quality. Cultivated aquatic paddy field or pond production of lotus typically has a growing season of 3 to 6 months for many Chinese rhizome cultivars and 5 to 8 months for Thai cultivars (Bowen, 1982; Lowe-McConnell, 1982; Shrestha and Knud-Hansen, 1994; Yi et al., 2002). The edible lotus rhizome can be harvested after 120 days in warm climates and after 150 to 180 days or after the leaves senescence in cold climates (Yamaguchi, 1990). In Asian cultures, visual cues of complete leaf pattern senescence or defoliation of cultivated edible lotus is used to determine the time to harvest (Yamaguchi, 1990). However, visual cues such as leaf defoliation to indicate maturity at harvest may not reflect optimal lotus rhizome nutritional quality.

Leaf senescence is a highly ordered, genetically regulated oxidative process involving numerous metabolic pathways such as chlorophyll and protein degradation that ultimately lead to loss of cellular function and eventual death of cells, tissue or whole plant (Silva et al., 2010; Hung et al., 2006; Kawakami and Watanabe, 1988; Dhindsa et al., 1981). Initiation and progression of leaf senescence are influenced by both environmental (high or low temperature, drought, ozone, nutrient deficiency and

pathogen infection) and internal factors (phytohormones, developmental and reproductive age) and are characteristic of monocarpic or polycarpic plant species. In monocarpic species or annuals, senescence related changes have extensively been examined and defined (Lim et al., 2007). In contrast, in polycarpic plant species or perennials, senescence related changes have not been examined extensively and are currently under debate (Munné-Bosch, 2007, 2008; Yu et al., 2009). Based on developmental and senescence photosynthetic properties of rotund or orbicular lotus leaves, these plants have been classified as a monocarpic aquatic plant (Misra and Misra, 2010). As such, in monocarpic species, senescence in leaves is highly coordinated with the senescence of the whole plant and influenced by reproductive function and the environment (Combe and Escobar-Gutiérrez, 2009; Fischer, 2012). In both monocarpic plants and perennials, senescence related changes in part are attributed to the involvement of reactive oxygen species (ROS). ROS such as superoxide anion radicals ( $O_2^-$ ), hydroxyl radicals ( $\cdot OH$ ), hydrogen peroxide ( $H_2O_2$ ) and singlet oxygen ( $^1O_2$ ) in plant leaf senescence is well documented (Khanna-Chopra, 2012; Gill and Tuteja, 2010; Sakamoto et al., 2008). Among these ROS,  $H_2O_2$  is an important intermediate signaling metabolite (Lin and Kao, 1998). As an intercellular signal,  $H_2O_2$  mediates a diverse cascade of plant physiological and biochemical processes. During growth and development  $H_2O_2$  is capable of triggering plant cellular decisions and involving cell survival or death in response to abiotic and biotic stresses (Gadjev et al., 2008; Quan et al., 2008; Van Breusegem et al., 2008;

Peng et al., 2005; Mittler et al., 2004). During normal cellular metabolism chloroplast, mitochondria and peroxisomes are sources of ROS in active and senescent cells. In the absence of cellular biochemical and physiological adjustments, if ROS are allowed to accumulate then disruption of normal cellular function, lipid peroxidation, protein denaturation and DNA mutation typically occurs and are indicative of cellular oxidative damage to membranes, proteins and nucleic acids (Hodges et al., 2004; Hodges and DeLong, 2007). Longevity of lotus fruit (seed) during extreme temperature exposure and oxidative stress is correlated with the expression of heat-resistant proteins and enhanced production of cellular antioxidants (Cheng and Song, 2007). Thus, an important feature common to the above senescence related changes is a decline in antioxidant content with a corresponding increase in steady state production of ROS. Senescence related physiological processes involving programmed metabolic decline in leaves and rhizomes of lotus have not been adequately described. At present there is no consensus of specific coordinated physiological and biochemical changes in leaves and rhizomes of lotus which reflect optimal quality, flavor and nutritive value during development, growth and commercial maturity. Due to the aquatic nature of lotus, development of objective criteria for monitoring optimal commercial harvest maturity of lotus rhizomes presents several challenges. However, characterization of senescence process in lotus leaves and rhizomes would provide a better understanding that will lead to improved postharvest technology and handling. Previous studies conducted at Auburn

University provide initial insights on best management practices of containerized lotus production (Tian et al., 2006, 2008, 2009 a, b). Despite these studies, to the best of our knowledge, there is little consideration regarding the influence of leaf and associated senescence changes in edible lotus rhizomes at harvest. If commercialization of container grown or aquaculture managed ponds in the U.S. is to be advanced then further studies are required to address these knowledge deficiencies.

To address the knowledge deficit concerning physiological harvest maturity, this study was conducted to examine the possible relationship of leaf senescence indicators (chlorophyll, dry matter, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)), malondialdehyde (MDA) and total soluble protein (TSP) content, in conjunction with phytochemical antioxidant metabolites (ascorbic acid, (vit C), proline (Pro), total phenols (TP) and antioxidant capacity (ABTS and DPPH)) as possible objective criterion of lotus rhizome harvest maturity. In addition, differences between American species (*N. lutea*) and Asian species (*N. nucifera*) in antioxidant properties and optimal maturity harvest were highlighted.

## **MATERIALS AND METHODS**

**Plant material and growth conditions:** Two species of lotus, *Nelumbo nucifera* 'E2' (a medium sized cultivar of *N. nucifera* with limited number of flowers) and the native American species, *N. lutea*, were selected. The Chinese cultivar *N. nucifera* 'E2' was chosen for this study due to its large and prolific rhizome production and its selected use as an edible cultivar in China. The American species,

*N. lutea* was selected because there have been no selections made for of this species for the edible market in the US. The experiment was conducted outside in full sunlight at the Paterson Greenhouse Complex at Auburn University, AL (32.6°N, 85.48°W) in April 2010. Dormant lotus rhizome- propagules were stimulated to sprout by planting initially in 9 L (2.4 US gal) black plastic containers [bottom 22 cm (8.6 in), diameter 27 cm (10.7 in), and height 19 cm (7.5 in)] without drainage holes on 15 April, 2010 in an enclosed greenhouse under ambient environmental conditions (natural light, 30°C and 95% RH). Black plastic containers (9L) were filled to 7.6 cm (3.0 inch) with a substrate blend consisting of 1:1 combined mixture of natural sandy loam and pine bark and filled with tap water (pH 7.0, EC = 0.13mS cm<sup>-1</sup> measured using a Hanna pH/EC/TDS meter) (Hanna Instruments, Woonsocket, RI). Lotus rhizome- propagules were transferred from the 9 L containers to 100 L (27 US gal) black plastic containers [bottom 50 cm (19.69 in), diameter 60 cm (23.62 in), and height 36 cm (14.17 in)] without drainage holes on 15 May 2010. Each pot contained one propagule with two internodes. Containers were filled to 15.23 cm with the aforementioned substrate. Containers were placed outside on a padded area of 6m x 6m under full sunlight with 25 cm (9.8 in) spacing. The experimental design consisted of a 2 (species) x 4 (harvest date) factorial treatment arrangement with four replications. Each replicate consisted of a single pot. A total of 32 plants were used in the experiment.



All pots were fertilized once following the emergence and establishment of several floating leaves and one or two standing leaves on 24 June 2010 with slow release fertilizer POLYON 20-10-10 (20N-10P-10K) (Harrell's LLC, Lakeland, FL 33801) at 55g per pot. Water levels within each pot were monitored on a daily basis and maintained at the original water level.

**Chlorophyll content index:** All data collection was based on reported estimated dates of maturity from 120 to 180 days after planting for potential harvest maturity (Yamaguchi, 1990). Chlorophyll content of fully intact expanded standing leaves was determined non-destructively using a portable hand-held chlorophyll meter (CCM-200 plus; Opti-Sciences, Hudson, NH). Chlorophyll measurements were performed between the hours of 10:00 - 11:00 am of the designated dates (118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.)) of data collection. Four plants per harvest date were sampled per species with four readings were recorded per leaf based on directional co-ordinates (N, S, E and W). Dependent on species, an average of 4 to 6 leaf samples were measured per plant and species on sampling dates. Care was taken to avoid leaf midrib and leaf marginal areas during sampling. Immediately following chlorophyll meter readings, leaves (fully expanded standing leaves (leaves rising above the water on petioles), and floating leaves) and rhizomes were collected at 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N.*

*nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139 days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.), respectively. Four plants were harvested at each sampling date. After chlorophyll measurements were performed, leaf samples were separated (fully expanded standing leaves and floating leaves) and only fully expanded standing leaves and rhizomes were utilized for further analysis. Debris from leaf and rhizome samples were gently removed by washing with tap water and gently blotted dry with paper towel. Leaves and rhizome samples were placed in perforated plastic containers that were previously labeled according to treatment and replication and transported to the Department of Horticulture Postharvest Physiology Lab. Leaf samples were cut into small millimeter pieces using a laboratory razor blade, frozen with liquid nitrogen and stored in plastic bag at -80°C for further laboratory analysis. Rhizome samples were transversely sliced (3mm thickness) using a food slicer (Waring Pro Food Slicer, East Windsor, NJ) and placed in ultra-low freezer at -80°C until further laboratory analysis.

**Dry matter content:** Leaf (5 g fresh weight) and rhizome tissue (50 g fresh weight) were weighted immediately after collect and dried to a constant weight (g) in a drying oven (Grieve SC-350, Round Lake, IL) at 76°C for 36 hours, weighed again and dry matter contents calculated as follows:  $\text{Dry matter Content} = 1 - \frac{FW - DW}{FW} \times 100$ ; where FW = fresh weight and DW = dried weight.

**Total soluble protein:** The total soluble protein content was determined using bovine serum albumin (BSA) as a standard following Bradford (1976). One part

of dye reagent (Protein Assay Kit II; Bio-Rad Laboratory, Hercules, CA) was diluted with four parts of Milli-Q water, 18.2 MΩ (Milli-Q distilled water purification system, Millipore Corp., Bedford, MA) and used for protein determination. Frozen leaf samples (0.5g) or partially thawed rhizome samples (2g) were ground in liquid-N<sub>2</sub> with a mortar and pestle at 4°C containing 10 mL phosphate buffered saline (PBS, 50 mM, pH 7.8) . The homogenate was centrifuged at 12,000 g<sub>n</sub> at 4°C for 10 min. The clarified supernatant was collected and 100 μL of extract was added to 1mL of previously diluted dye reagent. After 30 min room temperature incubation, the absorbance was measured at 595nm against a blank (1 mL of reagent and 100 μL of PBS) by using a micro plate reader (Synergy HT, BIO-TEK Instruments, Inc., Winooski, Vermont).

**Ascorbic acid (AsA):** AsA content was determined according to Gossett et al. (1994) with minor modifications allowing adaptations for micro-plate determinations (Hodges et al., 1996). Leaf (300 mg) and rhizome (1g) samples were homogenized in 5 mL of cold m-phosphoric acid- acetic acid solution (30g MPA, 0.5g ethylenediamine tetraacetic acid, EDTA; and 80 mL glacial acetic acid diluted to 1 L with Mili-Q water). The homogenate was centrifuged at 13,000g<sub>n</sub> for 15 min at 4°C, filtered with Miracloth (Calbiochem, La Jolla, CA), and diluted to a final volume of 60 mL. To 100μL of clarified extract, 50μL of Milli-Q water, 250μL of KH<sub>2</sub>PO<sub>4</sub> (150mM, pH 7.4 and 5 mM EDTA), followed by a 10 min room temperature incubation to which 50μL of Milli-Q water was added. A subsequent series of

reagents consisting of 200 $\mu$ L of trichloroacetic acid (TCA), 200 $\mu$ L of O-phosphoric acid, 200 $\mu$ L of 4% (w/v) 2, 2-bipyridyl dissolved in 70% HPLC grade ethanol and 100 $\mu$ L of 3% (w/v) FeCl<sub>3</sub> was added to complete the reaction mixture. Generation of a standard curve was achieved by constructing six different concentrations of L-ascorbic acid (0, 20, 40, 60, 80, and 100  $\mu$ M) that were performed in parallel with appropriate diluted samples. Samples and standards were incubated in a water bath maintained at 40°C for 60 min. The absorbance was read at 525 nm using the micro plate reader. Results were expressed as vitamin C mg /100 gfw.

**Proline content:** Proline content was determined according to the method of Bates et al., 1973. Leaf (300 mg) and rhizome (1g) were homogenized in 5 mL in 3% (w/v) sulphosalicylic acid and centrifuged 10,000 g<sub>n</sub> for 15 min at 4°C. To the 500 $\mu$ L clarified supernatant, 2 mL of acid ninhydrin solution (1.25 g ninhydrin + 30 mL glacial acetic acid + 20 mL 6 M H<sub>3</sub>PO<sub>4</sub>) was added and incubated for 1h at 100°C. Transferring the reaction mixture to an ice bath terminated the reaction and 5 mL of toluene was added to the mixture and vortexed for 20 sec. Absorbance of the pink upper toluene phase (200 $\mu$ L) was measured at 520 nm against pure toluene as a reference blank using a micro plate reader. A reference standard curve was prepared using proline to determine proline concentration in corresponding samples. Results were expressed as mg /100 gfw.

**Total phenolic:** Total phenolic content was determined with the Folin-Ciocalteu method (Singleton and Rossi, 1965). Frozen leaf tissue (300 mg) or

partially thawed rhizome tissue (2g) with 10 mL or 15 mL of 80% HPLC grade methanol, respectively, and were shaken on a platform orbital shaker Barnstead Lab-Line Max-Q 2508 (Fisher Scientific, Raleigh, NC) overnight at 5°C. Forty microliters of ethanolic extract (diluted with 1.5 mL of Milli-Q water), 200 µL of Folin-Ciocalteu's reagent (Folin: Milli-Q water, 50:50, v/v) mixed together. The mixture was allowed to stand for 6 min at room temperature before adding 160 µL of 7.5% sodium carbonate solution. After 30 min incubation at 37°C the absorbance was measured at 765 nm at room temperature using the micro plate reader. Total phenolic content was calculated using a standard curve of gallic acid (0, 20, 40, 60, 80 and 100 mg /L). A blank consisted of Milli-Q water instead of sample. The results were expressed as mg gallic acid equivalent (GAE) /100 gfw.

**Antioxidant capacity:** Antioxidant capacities of lotus leaf and rhizome were determined by colorimetric measurements using the ABTS method of Lee et al. (2009) Methanolic extracts obtained from total phenolic procedure were utilized for ABTS antioxidant capacity determination. The ABTS radical cation (ABTS<sup>•+</sup>) was generated by reacting 2.5 mM of ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] with 1.0 mM of AAPH [2,2'-azobis-(2-amidinopropane) HCl] in 100 mL phosphate-buffered saline (PBS) solution [100mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl]. The ABTS radical solution was heated in 68 °C water bath for 1 h with frequent agitation and filtered at reduced pressure through a ZAPCAP<sup>®</sup>-CR filter unit (Whatman Inc., Florham Park, NJ). The blue-

green ABTS radical solution was adjusted with fresh PBS to an absorbance of  $0.650 \pm 0.020$  prior to use. The blue-green ABTS radical solution was adjusted with fresh PBS to an absorbance of  $0.650 \pm 0.020$ . Aliquots (4  $\mu\text{L}$ ) of sample solution or blank (80% HPLC grade methanol) were mixed with 196  $\mu\text{L}$  of ABTS radical solution and immediately read at 734 nm at 37 °C for duration of 30 minutes in the micro-plate reader. Generation of a standard curve was achieved by constructing six concentrations of L-ascorbic acid (0, 20, 40, 60, 80 and 100 mg/L) and six concentrations of Trolox (0, 40, 80, 120, 160, and 200 mg/L). The ABTS radical scavenging capacities of sample extracts were expressed on the fresh weight basis as mg vitamin C equivalent/100gfw (VCEAC) and mg Trolox equivalents/100gfw.

**Free radical scavenging activity:** The modified DPPH free radical scavenging activity was determined according to Brand-Williams et al. (1995) as modified by Kim et al., (2002). For DPPH free radical scavenging activity determination, methanolic extracts obtained from total phenolic procedure were utilized. The stable 0.02 mM of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH $\cdot$ ) dissolved in 200 mL of 80% HPLC grade methanol. The radical solution was stirred at room temperature for 20 min and then adjusted to  $0.650 \pm 0.020$  absorbance values at 517nm. Generation of a standard curve was achieved by constructing six concentrations of L-ascorbic acid (0, 40, 80, 120, 160 and 200 mg/L). Aliquots (6.67  $\mu\text{L}$ ) of clarified methanolic extract, blank, or L-ascorbic acid standard were mixed with 193.33  $\mu\text{L}$  of DPPH radical solution. The decrease in absorbance of the resulting solution was monitored

at 517 nm for 30 min duration in a micro-plate reader. The DPPH free radical scavenging activity of methanolic lotus rhizome was expressed as vitamin C equivalent (VCEAC)/100gfw.

**Hydrogen peroxide:** Concentration of H<sub>2</sub>O<sub>2</sub> was spectrophotometrically determined following the method of Jana and Choudhuri (1981). H<sub>2</sub>O<sub>2</sub> was extracted by homogenizing 300 mg leaf or 2.5g rhizome tissue with 3 mL or 5 mL of phosphate buffer (50mM, pH 6.5) respectively. The homogenate was centrifuged at 6000 g<sub>n</sub> at 4°C for 15 min. To determine the H<sub>2</sub>O<sub>2</sub>, 3 mL of the clarified supernatant was mixed with 1 mL of 0.1% titanium chloride solution in 20% (vol /vol) H<sub>2</sub>SO<sub>4</sub> and the mixture was centrifuged at 6000 g<sub>n</sub> at 4°C for 15 min. The absorbance of the clarified extract was measured at 410 nm and H<sub>2</sub>O<sub>2</sub> content was calculated using the extinction coefficient 0.28 μM<sup>-1</sup>cm<sup>-1</sup>, and expressed as nM/gfw.

**Malondialdehyde (MDA):** MDA content, a measure of lipid peroxidation, was determined by measuring the amount of content according to the method of Du and Bramlage (1992). Frozen leaf (500mg) or partially thawed 1g rhizome tissue samples were ground in liquid-N<sub>2</sub> with a mortar and pestle at 4°C in 5 mL of 0.5% trichloroacetic acid (TCA). The homogenate was centrifuged at 20,000 g<sub>n</sub> for 15 min at 4°C. A 2 mL aliquot of the supernatant was combined with 2 mL of 20% TCA and 1 mL of 0.67% thiobarbituric acid (TBA) and heated at 95°C for 15 min, cooled in a water bath and centrifuged at 10,000 g<sub>n</sub> for 15 min. The absorbance of clarified supernatant (200 μL) was measured at 532 nm and 600 nm, MDA concentration was

determined using the equation:  $\text{MDA (nmol/mL)} = \left[ \frac{A532 - A600}{157000} \right] 10^6$  . Lipid

peroxidation was expressed as MDA content in  $\text{nmol gfw}^{-1}$  .

**Statistical analysis:** The homogeneity of variance assumption was tested for all responses using Levene's test and the ABS option in PROC GLM in SAS version 9.2 (SAS Institute, Cary, NC). An analysis of variance was then performed on data using PROC MIXED with a completely randomized design and a factorial treatment design of specie and date. Appropriate corrections for heterogeneity of variance were applied where Levene's test was significant with the GROUP option on the REPEATED statement in PROC MIXED. Orthogonal contrasts were used to determine linear or quadratic trends over date, and paired contrasts were used to determine difference in species. Pearson Correlation test was also conducted to determine the correlations among treatment means. All significances were at  $\alpha=0.05$ .

## **RESULTS:**

**Chlorophyll content index (CCI):** There was no significant difference observed in the interaction of harvesting Weeks and lotus species for CCI (Table 1). During the first three harvests (Weeks 0, 2, and 4), CCI values were lower ( $P<0.05$ ) in *N. nucifera* 'E2' when compared to *N. lutea*. CCI values linearly declined in both species until the last harvest. CCI values ranged from 14.95 to 2.53 for *N. nucifera* 'E2' and 25.28 to 1.31 for *N. lutea*, respectively. In both species, there was an absence of maximal predicted CCI and associated optimal harvest determination due to a linear decline.



**Dry matter content:** There was a significant interaction ( $P < 0.001$ ) between harvesting Weeks and rhizome % dry matter content (DMC) (Table 1). Rhizome DMC ranged from 22.92 to 27.87 % for *N. nucifera* 'E2' and 22.78 to 27.19 % for *N. lutea* respectively. DMC increased linearly throughout harvest period for *N. nucifera* 'E2' and therefore, no observed optimal level of DMC was noted. In contrast, a quadratic trend of DMC was observed for *N. lutea* with a maximum peak (35.65 %) during the fourth Week of determination and a corresponding predicted optimal harvest of 3.6 Weeks.

**Total soluble protein:** There were harvest and species interactive effects ( $P < 0.001$ ) in leaves and rhizomes for total soluble protein content (Table 2). Total soluble protein content changed quadratically in leaves of *N. nucifera* 'E2' whereas leaf tissues of *N. lutea* declined linearly for all determinations. Initial leaf total protein content was similar for both *N. nucifera* 'E2' and *N. lutea* during the first Week of harvest. During Weeks 2 and 4, total soluble protein content was highest in leaves of *N. nucifera* 'E2', when compared to *N. lutea* but were similar during the last harvest period. Contrasting trends in protein accumulation were noted in rhizome tissues with respect to species. Total soluble protein content in rhizomes of *N. nucifera* 'E2' accumulated linearly throughout all harvest determinations whereas in *N. lutea* the pattern of accumulation was quadratic. During the first two harvest periods (Weeks 0 and 2), both *N. nucifera* 'E2' and *N. lutea* were similar in protein content however, during the last two harvests (Weeks 4 and 6) *N. lutea* was higher in total soluble

protein content. In both species, there was an absence of maximal predicted total soluble protein content and associated optimal harvest determination.

**Ascorbic acid:** Significant harvest by species interaction ( $P < 0.001$ ) was observed in leaves and rhizomes dependent on time of harvest and specie for ascorbic acid content (Table 3). In leaf tissues, ascorbic acid content accumulated quadratically for both species and ranged from 75.54 to 52.54 mg/100gfw for *N. nucifera* 'E2' and from 120.54 to 20.59 mg/100gfw for *N. lutea*. Initial ascorbic acid content was lowest in *N. nucifera* 'E2' during Week 0 when compared to *N. lutea*. In leaf tissues of both species with continued harvest, ascorbic acid content progressively declined quadratically until Week 6. Leaf tissues of *N. nucifera* 'E2' and *N. lutea* were similar in predicted maximum values of ascorbic acid content ( $137.08 \pm 15.7$  mg/100gfw and  $134.88 \pm 14.97$  mg/100gfw respectively) and had similar predicted optimal time to harvest. In contrast, rhizome tissues accumulated ascorbic acid content linearly for *N. nucifera* 'E2' and quadratically in *N. lutea*. Rhizome ascorbic acid content ranged from 49.71 to 74.07 mg/100gfw for *N. nucifera* 'E2' and 112.43 to 116.11 mg/100gfw for *N. lutea*. There was no observable maximal level of ascorbic acid content in rhizomes of *N. nucifera* 'E2'. By contrast, highest content of ascorbic acid (174.59 mg/100gfw ) occurred during Week 2 harvest in rhizomes of *N. lutea* and having statistical predicted maximum values of  $161.8 \pm 14.86$  mg/100gfw of ascorbic acid content and predicted optimal harvest time of 2.8 Week.

**Proline content:** Dependent on time of harvest and species, there were significant interactions ( $P < 0.001$ ,  $P < 0.05$ , in leaves and  $P < 0.01$  in rhizomes) for proline content (Table 4.) In leaf tissues, proline content accumulated quadratically from 8.32 to 4.31 mg/100gfw for *N. nucifera* 'E2' and linearly declined from 4.09 to 1.05 mg/100gfw for *N. lutea*. Throughout all harvest determinations, *N. nucifera* 'E2' contained higher leaf proline content when compared to *N. lutea*. Maximal proline content (14.81 mg/100gfw) occurred during Week 4 and predicted optimal harvest time of 2.8 Weeks was noted. There was no observable maximal level of proline content in leaf tissues of *N. lutea*. Throughout all harvest determinations, *N. nucifera* 'E2' contained lower rhizome proline content when compared to *N. lutea*. In rhizome tissues, proline content accumulated quadratically from 1.72 to 5.27 mg/100gfw in *N. nucifera* 'E2' and linearly from 6.09 to 9.04 mg/100gfw in *N. lutea*. There was no observable maximal level of proline accumulated in rhizome tissues for either species evaluated.

**Total phenolic content:** There was a significant harvest by species interaction ( $P < 0.001$ ) for leaf and rhizome ( $P < 0.01$ ,  $P < 0.001$ ) for total phenolic content (Table 5). Leaf extracts were higher in total phenolic content than extracts from rhizome in both species. Initial total phenolic content in leaf tissue was higher in *N. lutea*; however, with continued harvest periods both species were similar. In leaf tissues, there was a quadratic decreasing trend in total phenolic content from 1629.44 to 490.21 mg GAE/100gfw for *N. nucifera* 'E2' and from 2611.34 to 2191.45 mg GAE / 100gfw for

*N. lutea*. Total phenolic content was highest during Week 4 of harvest in leaf tissues of *N. nucifera* 'E2', (2032.19 mg GAE/100gfw) and noted statistical predicted maximum value of  $1909.24 \pm 306.01$  mg GAE /100gfw with corresponding predicted optimal harvest time of 2.2 Weeks.

In rhizome tissues, total phenolic content decreased linearly from 143.29 to 198.01 mg GAE/100gfw for *N. nucifera* 'E2' and quadratically from 487.72 to 285.5 mg GAE / 100gfw for *N. lutea* and had a predicted maximum value of 501.12 mg GAE /100gfw with a predicted optimal harvest time of 1.9 Weeks.

**Antioxidant capacity and free radical scavenging activity:** In this study, in order to assess how various bioactive compounds may contribute to the overall total antioxidant capacity and free radical scavenging activity of lotus leaf and rhizome, two distinct assays utilizing standards representative of water soluble fraction (vitamin C) or hydrophilic antioxidants and lipid soluble fraction (Trolox) or hydrophobic antioxidants were utilized for comparative purpose.

**Antioxidant capacity:** There were significant variation in hydrophobic antioxidant capacities of leaf and rhizome extracts (Table 6). A significant harvest by species interaction ( $P < 0.01$ ,  $P < 0.001$ ) for leaf and rhizome ( $P < 0.001$ ) for hydrophobic antioxidant capacity values was observed. Leaf hydrophobic extracts were approximately six to thirteen-folds higher than extracts from rhizome dependent on species observed. Initial leaf hydrophobic antioxidant capacities were similar during the first harvest however, in both species, a decreasing quadratic trend for

subsequent harvest periods was observed. *N. nucifera* 'E2' had the highest hydrophobic antioxidant capacity during Week 4 (11927.0 mg Trolox/100gfw) and predicted maximum value of  $10579.2 \pm 1055.6$  mg Trolox/100gfw and corresponding optimal harvest time of 3.4 Weeks, while *N. lutea* did not have a discernable peak. In rhizome tissues, *N. nucifera* 'E2' exhibited a linear increasing trend in hydrophobic antioxidant capacity (865.93 to 1264.62 mg Trolox/100gfw) with and predicted maximum value of  $1267.63 \pm 116.31$  mg Trolox/100gfw and corresponding predicted optimal harvest time of 5.2 Weeks. In contrast, *N. lutea* exhibited an increasing quadratic trend in hydrophobic antioxidant capacity (1952.19 to 1302.42 mg Trolox/100gfw) and predicted maximum value of  $2038.5 \pm 103.14$  mg Trolox/100gfw with corresponding predicted optimal harvest time of 1.4 Weeks.

There was a significant harvest by species interaction ( $P < 0.001$ ) for leaf and rhizome ( $P < 0.05$ ,  $P < 0.001$ ) for hydrophilic antioxidant capacity (Table 7). Leaf hydrophilic antioxidant capacity extracts were approximately eight-fold higher than extracts from rhizome in both species. Leaf hydrophilic antioxidant capacity extracts from *N. nucifera* 'E2' were higher than those of *N. lutea*. In general, in *N. nucifera* 'E2' leaf tissue, hydrophilic antioxidant capacity exhibited a quadratic decreasing trend from 4826.31 to 4260.37 mg VCEAC /100gfw. In *N. nucifera* 'E2' the highest hydrophilic antioxidant capacity occurred during Week 4 with a predicted maximum value of  $5909.25 \pm 613.67$  mg VCEAC/100gfw and predicted optimal harvest time of 3 Weeks. In leaf tissues of *N. lutea* the hydrophilic antioxidant capacity declined

linearly from 4614.62 to 720.00 mg VCEAC/100gfw and therefore, deficient of attainable maximal value. In contrast, rhizome tissue extracts from *N. nucifera* 'E2' were lower than those of *N. lutea* with the exception of the last harvest in which both species were similar in hydrophilic antioxidant capacity.

Interestingly, the hydrophilic antioxidant capacity for both species rhizome tissues declined in a quadratic trend with distinct maximal peaks detected. In rhizomes of *N. nucifera* 'E2' the highest hydrophilic antioxidant capacity occurred during Week 4 (689.31 mg VCEAC/100 gfw) with a predicted maximum value of  $705.22 \pm 54.58$  mg VCEAC/100gfw and predicted optimal harvest time of 4.4 Week, while the maximal predicted time peak for *N. lutea* was predicted at 1.2 Weeks and an estimated maximum value of  $1151.13 \pm 51.53$  mg VCEAC/100gfw.

**Free radical scavenging activity:** There were significant variations in leaf and rhizome hydrophobic free radical scavenging activity (Table 8). In both species, leaf tissues exhibited a quadratic trend in hydrophobic free radical scavenging activity. Initial free radical scavenging activities were similar during the first Week of harvest however, for all subsequent harvest determinations *N. nucifera* 'E2' leaf tissues were consistently higher. Both species noticeably failed to attain a maximal predicted hydrophobic free radical scavenging activity and associated optimal harvest. In rhizome tissues, contrasting significant increasing linear ( $P < 0.001$ ) and decreasing quadratic ( $P < 0.001$ ) trends for *N. nucifera* 'E2' and *N. lutea* respectively. In rhizome tissues, there was a noticeable absence of maximal predicted hydrophobic free radical

scavenging activity and associated optimal harvest determination for *N. nucifera* 'E2'. However, in *N. lutea* rhizome tissues, the maximal predicted hydrophobic free radical scavenging value was ( $775.59 \pm 38.06$  mg Trolox/100gfw) with an optimal harvest period of 2.2 Weeks.

Maturity at harvest significantly influenced free radical scavenging activity dependent on species observed and in both leaf and rhizome tissues (Table 9). There was a significant maturity at harvest by species interaction ( $P < 0.001$ ,  $P < 0.01$ ) for leaf hydrophilic free radical scavenging activity. In both species, leaf tissues exhibited a quadratic trend in hydrophilic free radical scavenging activity. Initial leaf hydrophilic free radical scavenging activities were similar for both species. With further harvest determinations *N. nucifera* 'E2' consistently exhibited higher leaf hydrophilic free radical scavenging activity when compared to *N. lutea*. In leaf tissues of *N. nucifera* 'E2' the maximal predicted hydrophilic free radical scavenging activity was  $3922.12 \pm 462.31$  mg VCEAC/100gfw and optimal harvest period 3.2 Weeks. While in leaf tissues of *N. lutea* the maximal predicted hydrophilic free radical scavenging activity was  $3022.83 \pm 462.52$  mg VCEAC/100gfw and optimal harvest period of 0.4 Weeks. In both species, rhizome tissues exhibited a quadratic trend in hydrophilic free radical scavenging activity. In all harvest determinations, *N. nucifera* 'E2' consistently contained lower hydrophilic free radical scavenging activity when compared to *N. lutea*. Furthermore, in both species, there was a noticeable absence of

maximal predicted hydrophilic radical scavenging DPPH activity and associated optimal harvest determination.

**Pro-oxidant Content:** Pro-oxidants are chemicals that induce oxidative stress, either by generating reactive oxygen species (ROS) or by inhibiting enzymatic or non-enzymatic antioxidant systems.

Leaf hydrogen peroxide (H<sub>2</sub>O<sub>2</sub> content) was approximately twenty to forty-fold higher when compare to rhizome tissue (Table 10). In leaf tissue, there were no significant interactive effects of harvest and species for H<sub>2</sub>O<sub>2</sub> content. However, in both species, significant linear reductions in leaf H<sub>2</sub>O<sub>2</sub> content for all subsequent maturity at harvest determinations following Week O. In *N. nucifera* 'E2' leaf, H<sub>2</sub>O<sub>2</sub> content declined from 97.18 to 11.08 nM/gfw and *N. lutea* from 93.57 to 67.62 nM/gfw. In addition, there was no discernable maximal predicted or associated optimal harvest period for H<sub>2</sub>O<sub>2</sub> content in leaf tissues. In rhizome tissues, there were significant differences (P<0.05) observed between the interaction of harvesting Weeks and species for H<sub>2</sub>O<sub>2</sub>. In rhizome tissues of *N. nucifera* 'E2', the H<sub>2</sub>O<sub>2</sub> content linearly declined and was significantly higher than *N. lutea* until the final harvest in which both species were similar in H<sub>2</sub>O<sub>2</sub> content. In both species, there were no maximal predicted H<sub>2</sub>O<sub>2</sub> content or optimal harvest period noted.

In leaf tissues, both *N. nucifera* 'E2' and *N. lutea* MDA content were similar throughout all harvest determinations and remained stable until Week 4 and dramatically declined thereafter (Table 11). In both rhizome species, there was a



quadratic trend in accumulation in MDA content. Throughout the first three harvest determinations *N. nucifera* 'E2' significantly ( $P < 0.05$ ) accumulated higher MDA content until the fourth harvest in which *N. lutea* significantly ( $P < 0.05$ ) accumulated higher MDA content. In both species, there were no significant maximal predicted MDA content or optimal harvest period noted.

**Correlation analysis:** Pearson's correlation was used to determine the relationship between selected phytochemical attributes associated with leaf and rhizome maturity at harvest Table 12. There were significant ( $P \leq 0.05$ ) correlations observed. These include significant ( $P \leq 0.05$ ) negative correlation between leaf CCI values and rhizome dry matter content (DMC) ( $r = -0.78$ ), total soluble protein (TSP) ( $r = -0.58$ ), hydrophilic antioxidant capacity ( $r = -0.79$ ), hydrophobic antioxidant capacity ( $r = -0.83$ ), ( $r = -0.60$ ) hydrophilic free radical scavenging activity, ( $r = -0.75$ ) hydrophobic free radical scavenging activity, rhizome ascorbic acid ( $r = -0.53$ ), rhizome proline content ( $r = -0.57$ ), rhizome total phenolic (TP) content ( $r = -0.64$ ), rhizome  $H_2O_2$  content ( $r = 0.74$ ) and rhizome MDA content ( $r = 0.68$ ) for *N. nucifera* 'E2'. In contrast, *N. lutea*, CCI values were not correlated with rhizome DMC, AsA, TSP, proline, TP content, rhizome hydrophobic free radical scavenging activity, rhizome  $H_2O_2$ , or MDA content. However, in *N. lutea* CCI was moderately positively correlated with rhizome ABTS hydrophilic antioxidant capacity ( $r = -0.69$ ), ABTS hydrophobic antioxidant capacity ( $r = -0.64$ ) and rhizome ( $r = -0.63$ ) hydrophilic free radical scavenging activity.

In contrast, there was no correlation between leaf CCI values of *N. lutea*, and rhizome DMC, TSP, ASA, PRO, and TP content. However, positive correlation was observed between CCI values and hydrophilic antioxidant capacity ( $r = 0.69$ ), hydrophobic antioxidant capacity ( $r = 0.64$ ), and hydrophilic free radical scavenging activity ( $r = 0.63$ ) in lotus rhizomes.

In *N. nucifera* 'E2' rhizomes, TSP was positively correlated with ASA ( $r = 0.55$ ), proline ( $r = 0.83$ ), hydrophilic antioxidant capacity (ABTS,  $r = 0.53$ ) and hydrophobic free radical scavenging activity (DPPH,  $r = 0.55$ ). AsA was positively correlated with proline ( $r = 0.53$ ), hydrophilic antioxidant capacity (ABTS,  $r = 0.82$ ), hydrophobic antioxidant capacity (ABTS,  $r = 0.85$ ), hydrophilic free radical scavenging activity (DPPH,  $r = 0.86$ ), hydrophobic free radical scavenging activity (DPPH,  $r = 0.69$ ) and negatively correlated with  $H_2O_2$  ( $r = -0.83$ ). Proline content was positively correlated with hydrophilic antioxidant capacity (ABTS,  $r = 0.51$ ) and negatively correlated with  $H_2O_2$  ( $r = -0.62$ ) and MDA ( $r = -0.53$ ). Total phenolic content was negatively correlated with  $H_2O_2$  ( $r = -0.84$ ) and MDA ( $r = -0.68$ ). Hydrophilic antioxidant capacity (ABTS) was negatively correlated with  $H_2O_2$  ( $r = -0.91$ ). Hydrophobic antioxidant capacity (ABTS) was negatively correlated with  $H_2O_2$  ( $r = -0.83$ ) and MDA ( $r = -0.72$ ). Hydrophilic free radical scavenging activity (DPPH) was negatively correlated with  $H_2O_2$  ( $r = -0.71$ ) and MDA ( $r = -0.58$ ). Hydrophobic free radical scavenging activity (DPPH) was negative correlated with

H<sub>2</sub>O<sub>2</sub> (r = -0.84) and MDA (-0.68). *N. nucifera* 'E2' rhizome H<sub>2</sub>O<sub>2</sub> content was positively correlated with MDA content (0.96).

In *N. nucifera* 'E2' leaves, TSP was positively correlated with proline content (0.54). ASA was positively correlated with TP (0.63), hydrophilic free radical scavenging activity (DPPH, 0.62) and H<sub>2</sub>O<sub>2</sub> (0.55). Proline content was positively correlated with hydrophilic antioxidant capacity (ABTS, r = 0.81), hydrophobic antioxidant capacity (ABTS, r = 0.73), hydrophilic free radical scavenging activity (DPPH, r = 0.72) and H<sub>2</sub>O<sub>2</sub> (0.54). Total phenolic content was negatively correlated with H<sub>2</sub>O<sub>2</sub> (-0.51). Hydrophobic antioxidant capacity (ABTS) was negatively correlated with H<sub>2</sub>O<sub>2</sub> (r = -0.62). Leaf H<sub>2</sub>O<sub>2</sub> was positively correlated with MDA content (r = 0.67).

## **DISCUSSION**

Development of a maturity index requires collection of phenological measurements that reflect changes throughout growth, development and senescence and correlate with minimum consumer commodity acceptability. As an edible vegetable crop, lotus is one of the most popular vegetables in the world due to known ethnoculinary, pharmacological and nutraceutical applications (Sridhar and Bhat, 2007) and health beneficial properties. There are two distinct species of lotus *N. lutea* (North American, with light yellow floral petals) and *N. nucifera* (Asian, with white pale or pink floral petal colors) Hayes et al., 2000. To date, there is no information available with regard to coordinated physiological and biochemical

changes that impart optimal consumer flavor, texture and nutritional quality of either *N. nucifera* or *N. lutea*. Due to the aquatic nature and habitat of lotus, determination of the optimum rhizome physiological maturity is still unclear and problematic. In practice, the accepted harvest time for edible lotus is estimated to be 120 DAP in warm climates and after 150 to 180 days or after the leaves senesce in cold climates (Yamaguchi, 1990). There are no established harvest criteria to assess rhizome commercial maturity. Contrary to many fruit and vegetables currently marketed in the United States, commercial grades or standards of lotus has not been established. The present study determined relationship of diverse leaf senescence indicators with various phytochemical properties in order to establish a reliable harvest index for lotus.

Leaf senescence is a highly ordered, genetically regulated oxidative process involving numerous metabolic pathways such as chlorophyll and protein degradation that ultimately lead to loss of cellular function and eventual death of cells, tissue or whole plant (Silva et al., 2010; Hung et al., 2006; Kawakami and Watanabe, 1988; Dhindsa et al., 1981). In order to establish a reliable maturity index for two edible lotus species (*N. nucifera* 'E2' and *N. lutea*) we have evaluated the changes in various phytochemical parameters during four consecutive harvest determinations.

In the present study, chlorophyll content index provided a reliable indication of changes in physiological senescence indicators across all harvest dates for *N. nucifera* 'E2'. However, it was not possible to accurately provide a reliable estimation of

optimal harvest maturity for all senescence indicators for *N. lutea*. A plausible explanation for this observed difference between the two species may be due to *N. lutea* appearing to be an earlier maturing species and selected dates of harvest may not accurately reflect optimal commercial maturity. Further research will be needed to determine a suitable maturity index for *N. lutea*.

Haga et al. (2009) reported chlorophyll content was the best predictor able to separate the late maturity class from the early-medium and medium-late classes of potatoes. The late maturity class generally had lower chlorophyll content throughout the growing season as compared to the other two classes. This supports the speculation that *N. lutea* can be classified as an early maturing species when compared to *N. nucifera* 'E2' due to the higher content of chlorophyll in *N. lutea* leaf tissue.

No significant correlation appeared between leaf dry weight and antioxidant parameters, which indicated leaf dry weight could not be used to accurately estimate the physiological maturity in both *N. nucifera* 'E2' and *N. lutea* rhizomes.

Ascorbic acid was reported to be closely associated with the senescence process that is initiated by ROS and it is assumed to contribute substantially to the antioxidant capacities of antioxidants in fruit and vegetables (Ramirez et al., 2014; Borraccino et al., 1994). This was further confirmed by our study that AsA was highly correlated with antioxidant capacity in *N. nucifera* rhizome, and a similar range of AsA content (between  $15.8 \pm 1.1$  and  $22.3 \pm 1.7$  mg/gdw) from four different cultivars (Inchisa,

Muan, Garam, and Chungyang) selected in *N. nucifera* was reported by Park et al (2009). The optimal harvest time was not addressed in *N. nucifera* 'E2' rhizome due to the continued accumulation of AsA. However, the predicted optimal harvest date for containerized *N. lutea* was achieved at 2.8 Weeks (146 days DAP) in warm climate.

Proline accumulation occurs in diverse plant species and is believed to play adaptive role in abiotic and biotic stress tolerance. Salinity and drought are known to induce oxidative stress. Early in vitro studies showed that proline can be a ROS scavenger (Smirnoff and Cumbes 1989). During development of *Arabidopsis thaliana*, levels of proline vary among plant organs and also dependent on plant age, leaf age (Verbruggen et al., 1993; Chiang and Dandekar., 1995). In development of *Arabidopsis* plants, higher expression of proline was found in young leaves than the old leaves. In young leaves, high proline expression could be detected all over the leaf blade. While in old leaves, expression of proline was restricted to be veins and hydathodes (Hua et al., 1997). In the current study, higher proline content was also found in young leaves instead of old leaves. The accumulation of proline in *N. nucifera* 'E2' rhizome may suggest the development of the storage organ. The optimal harvest time for both species was not determined because the continuous accumulation of proline content in the rhizome.

Phenolic compounds are the hydroxylated derivatives of benzoic and cinnamic acids and contribute to overall antioxidant activities in the plants (Vinson et al., 2001).

The phenolic compounds, found in medicinal plants as well as lotus, play important roles in preventing detrimental health issues, including inflammation, cancer, and arteriosclerosis, by virtue of their antioxidant activity by chelating redox-active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversion into ROS (Sato et al., 1996; Li et al., 2008). An earlier study by Yang et al., (2007) showed 437.5 and 1266 mg catechin equivalents/100 gdw phenolic content in methanol and acetone extracts of lotus rhizome, respectively. Hu and Skibsted (2002) reported 31.90 mg GAE/100gdw phenolic content in 50% aqueous ethanol extract of lotus rhizome. While, from Jung's research at 2008, total phenolic content at 177.7 mg GAE/gdw were found in leaf tissue, and 21.60 mg GAE/gdw were found in rhizomes. All these results indicated that lotus is one of the phenolic enriched plants, which can be consumed as functional food for health benefits. Optimal nutritional value for *N. nucifera* 'E2' was still not achieved during harvest time. However, as an earlier maturing species, *N. lutea* reached optimal maturity at 1.9 Weeks (138 DAP).

ABTS radical method is an excellent tool for determining the ability of antioxidants to quench free radicals both in aqueous and organic phases (Kim et al., 2002a, b; Leong and Shi, 2002). ABTS assay is more sensitive than DPPH method for estimating the water-soluble antioxidants (Pellegrini et al., 2003), which explained the higher value of antioxidant capacity in ABTS method.

DPPH was used as a stable free radical donor to evaluate free radical scavenging effects (Shimada et al., 1992). Park et al (2009) reported similar results for

hydrophobic antioxidants capacity (DPPH) in lotus rhizomes when compared with our results. A strong correlation was reported between antioxidant capacity (DPPH) with TP content by previous studies (Hu and Skibsted, 2002; Choe et al., 2009 and Lee et al., 2005), where is contrasting with our results and the differences may due to the different maturity stage of lotus rhizome and containerized lotus cultivation technique.

Ripening and senescence change in *N. lutea* and *N. nucifera* 'E2' can be reflected from antioxidant capacity determination. The optimal maturity for Asian species, *N. nucifera* 'E2' was achieved around 150 DAP. However, the American species, *N. lutea* is an earlier maturing species at 135 DAP.

MDA-Lipid peroxidation refers to the oxidative degradation of lipids. It is the process in which free radicals (including  $H_2O_2$ ) "steal" electrons from the lipids in cell membranes, resulting in cell damage. Lipid peroxidation is considered as a membrane damage indicator. During the senescence process,  $H_2O_2$  (linearly) and MDA (quadratically) were all decreased in *N. nucifera* 'E2' rhizome tissue might due to the rhizome formation during development, which further indicated *N. nucifera* 'E2' did not reach the optimal maturity stage. However, in *N. lutea* rhizome tissue,  $H_2O_2$  remained same and MDA content quadratically increased during the harvest date.

Antioxidants content and capacity were reported to associate with oxidative stress and senescence process that initiated by ROS (Ramirez et al., 2014). , An important feature common to the above senescence related changes is a decline in



antioxidant content with a corresponding increase in steady state production of ROS. In the current study, antioxidant content and capacity was negatively correlated with pro-oxidants in *N. nucifera* 'E2' rhizome. H<sub>2</sub>O<sub>2</sub> and MDA content declined across the harvest dates, and with a corresponding accumulation in all kinds of antioxidant parameters, including TSP, AsA, proline, total phenolic content and antioxidant capacity (ABTS, DPPH). The increasing antioxidant properties and decreasing pro-oxidants content suggest the rhizome of *N. nucifera* 'E2' was still in developmental stage at the end of harvest date (160 DAP), and further indicated that the future research will be needed on determination of the optimal harvest date. Compare with *N. nucifera* 'E2', *N. lutea* was considered as an early maturing species, concerning the change of pro-oxidants content and antioxidant properties during harvest. In *N. lutea* rhizome tissue, H<sub>2</sub>O<sub>2</sub> remained unchanged and triggered the accumulation of MDA throughout the harvest season, suggested the senescence process have already initiated in *N. lutea* rhizome. The estimated optimal harvest of *N. lutea* rhizome was predicted at 2.8 Weeks for AsA, 1.9 Weeks for TP, and 1.4 Weeks for lipid soluble antioxidant activity (ABTS), 1.2 Weeks for water soluble antioxidant activity (ABTS) and 2.2 Weeks for lipid soluble fraction of radical scavenging activity (DPPH). We speculate the optimal physiological maturity for containerized *N. lutea* rhizome could be between 1 Week (132 DAP) and 3 Weeks (146 DAP) in warm climate for maximum antioxidant properties, even though the proper maturity predictor for *N. lutea* was not determined in this study.

In *N. nucifera* 'E2' leaf tissue, a quadratic trend was observed consistently in TSP, ASA, proline, total phenolic content and antioxidant capacity (ABTS-Trolox, ABTS-VCEAC, DPPH-Trolox and DPPH-VCEAC). All antioxidant parameters increased from the first three harvests (0, 2, 4 Weeks) and decreased during the last harvest (6 Weeks) in *N. nucifera* 'E2' leaf tissue. In the meanwhile, a strong linear or quadratic accumulation was showed in all kinds of antioxidant parameters, which suggest the nutrients from leaf tissue may translocate downwards to the storage organ at the end of the growing season. This observation did not displayed in *N. lutea* may due to *N. lutea* was considered as an early maturing species that physiological senescence process has already initiated in rhizome after 3 Weeks. This observation has also been made in several other studies on other rhizomatous perennial plant (Dykyjova and Hradecka, 1976; Schierup, 1978; Graneli et al., 1992; Asaeda et al., 2006).

## **CONCLUSION**

The present study provides preliminary insights of physical and chemical changes of two lotus species during growth, development and commercial maturity. The results of this study highlight the difficulty in attempting to establish reliable harvest maturity index of lotus using both non-destructive and destructive methods indicated within the current study. Results of all tests for *N. nucifera* 'E2' indicate antioxidant content and capacity continued to accumulate after leaves senesced therefore, the optimal time to harvest was not identified. However, in this study, *N.*

*lutea* was determined to be an earlier maturing species when compared with *N. nucifera* 'E2'. All measured factors reached their highest levels between the second and third harvests. Optimal harvest time between 1 Weeks (132 DAP) and 3 Weeks (146 DAP) in warm climate for containerized *N. lutea* is recommended for growers to assess the maximum nutritional value. Monitoring of CCI throughout the harvest season can be used as maturity indices to accurately assure the maximum antioxidant level for harvesting *N. nucifera* 'E2'. Chlorophyll measurements can be considered as a practical method of measuring maturity. However, a more comprehensive understanding of the physiological responses associated with plant senescence in lotus is required. Further research on determines the optimal harvest dates of *N. nucifera* 'E2' and establishes a reliable objective maturity index for *N. lutea* is still needed.

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Table 1. Chlorophyll and dry matter content of leaves and rhizome dry matter content as indicators of senescence in containerized production of lotus (*Nelumbo spp.*)<sup>z</sup>

Harvest (Weeks)	Maturity Index			
	Chlorophyll Content Index		Dry Matter Content	
	CCI		%	
	Leaf		Rhizome	
	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	14.95b <sup>y</sup>	25.28a	22.92ns	22.78
2	10.99b	17.24a	20.88b	27.67a
4	4.45b	11.75a	27.78b	35.65a
6	2.53ns	1.31	27.87ns	27.19
	L***	L***	L***	Q***
Predicted Maximum values				32.86±2.30 <sup>x</sup>
Predicted Optimal Harvest (Weeks)				3.6

<sup>z</sup> Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.), respectively.

<sup>y</sup>The harvest by species interactions were significant at p = 0.05. Significant linear (L) or quadratic (Q) trends using orthogonal polynomials at p = 0.001 (\*\*\*). Mean comparisons within rows using Tukey's test at p = 0.05, ns=non-significant among treatments.

<sup>x</sup>Maximum CCI, dry matter content and optimal harvest period were estimated by using regression analysis at p = 0.05

Table 2. Total soluble protein content of leaves and rhizomes as indicators of senescence in containerized production of lotus (*Nelumbo spp.*).<sup>z</sup>

Harvest (Weeks)	Total Soluble Protein			
	mg/100gfw			
	Leaf		Rhizome	
	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	13.98ns <sup>y</sup>	11.46	10.02ns	12.66
2	14.10a	10.80b	10.14ns	11.70
4	17.88a	11.52b	10.44b	16.14a
6	11.94ns	9.66	12.18b	14.4a
	Q***	L***	L***	Q*

<sup>z</sup> Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.), respectively.

<sup>y</sup>The harvest by species interactions were significant at  $p = 0.05$ . Significant linear (L) or quadratic (Q) trends using orthogonal polynomials at  $p = 0.05$  (\*), or 0.001 (\*\*\*). Mean comparisons within rows using Tukey's test at  $p = 0.05$ , ns=non-significant among treatments.



Table 3. Ascorbic acid content in leaves and rhizomes as indicators of senescence in containerized production of lotus (*Nelumbo spp.*)<sup>z</sup>

Harvest <sup>y</sup> (Weeks)	Antioxidant Content <sup>y</sup>			
	Ascorbic Acid mg/100gfw			
	Leaf		Rhizome	
	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	75.54b <sup>x</sup>	120.54a	49.71b	112.43a
2	114.58ns	124.01	43.86b	174.59a
4	143.62a	117.82b	58.08b	138.41a
6	52.54a	20.59b	74.07b	116.11a
	Q***	Q***	L*	Q***
Predicted Maximum values	137.08±15.7	134.88±14.97		161.8±14.86
Predicted Optimal Harvest (Weeks)	2.8	1.8		2.8

<sup>z</sup>Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.), respectively.

<sup>y</sup>The harvest by species interactions were significant at  $p = 0.05$ . Significant linear (L) or quadratic (Q) trends using orthogonal polynomials at  $p = 0.05$  (\*) or 0.001 (\*\*\*). Mean comparisons within rows using Tukey's test at  $p = 0.05$ , ns=non-significant among treatments.

<sup>x</sup>Maximum ascorbic acid content and optimal harvest period were estimated by using regression analysis at  $p = 0.05$

Table 4. Proline content in leaves and rhizomes as indicators of senescence in containerized production of lotus (*Nelumbo spp.*)<sup>z</sup>

Harvest (Weeks)	Antioxidant Content			
	Proline mg/100gfw			
	Leaf		Rhizome	
	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	8.32a <sup>y</sup>	4.09b	1.72b	6.09a
2	8.54a	3.02b	1.91b	4.48a
4	14.81a	4.20b	2.43b	11.87a
6	4.31a	1.05b	5.27b	9.04a
	Q***	L*	Q**	L**
Predicted Maximum values	12.39±2.31 <sup>x</sup>			
Predicted Optimal Harvest (Weeks)	2.8			

<sup>z</sup>Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.), respectively.

<sup>y</sup>The species by harvest interactions were significant at  $p = 0.05$ . Significant linear (L) or quadratic (Q) trends using orthogonal polynomials at  $p = 0.05$  (\*), 0.01 (\*\*) or 0.001 (\*\*\*). Mean comparisons within rows using Tukey's test at  $p = 0.05$ .

<sup>x</sup>Maximum proline content and optimal harvest period were estimated by using regression analysis at  $p = 0.05$

Table 5. Total phenolic acid content in leaves and rhizome as indicators of senescence in containerized production of lotus (*Nelumbo spp.*)<sup>z</sup>

Harvest (Weeks)	Antioxidant Content			
	Total Phenolic mg GAE/100gfw			
	leaf		Rhizome	
	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	1629.44b <sup>y</sup>	2611.23a	143.29b	487.72a
2	1504.11ns	1498.85	182.89b	514.43a
4	2032.19ns	2191.45	207.60b	374.02a
6	490.21	missing	198.01b	285.50a
	Q***	Q***	L**	Q***
Predicted Maximum values	1909.24±306.01 <sup>x</sup>		501.12±126.00	
Predicted Optimal Harvest (Weeks)	2.2		1.9	

<sup>z</sup>Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.), respectively.

<sup>y</sup>The harvest by species interactions were significant at  $p = 0.05$ . Significant linear (L) or quadratic (Q) trends using orthogonal polynomials at  $p = 0.01$  (\*\*) or 0.001 (\*\*\*). Mean comparisons within rows using Tukey's test at  $p = 0.05$ , ns=non-significant among treatments.

<sup>x</sup>Maximum total phenolic content and optimal harvest period were estimated by using regression analysis at  $p = 0.05$

Table 6. Leaf and rhizome hydrophobic antioxidant capacity as indicators of senescence in containerized production of lotus (*Nelumbo spp.*)<sup>z</sup>

<b>Antioxidant Capacity</b>				
<b>ABTS</b>				
mg TROLOX/100gfw				
	Leaf		Rhizome	
<b>Harvest</b> (Weeks)	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	9052.94ns <sup>y</sup>	8532.02	865.93b	1952.19a
2	8831.31a	7115.58b	1096.57b	2103.54a
4	11927.01a	6320.62b	1268.37b	1706.39a
6	8769.09a	81.43b	1264.62ns	1302.42
	Q**	Q***	L***	Q***
Predicted Maximum values	10579.20±1055.56 <sup>x</sup>		1267.63±116.31	2038.5±103.14
Predicted Optimal Harvest (Weeks)	3.4		5.2	1.4

<sup>z</sup>Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.), respectively.

<sup>y</sup>Harvest by species interactions were significant at p = 0.05. Significant linear (L) or quadratic (Q) trends using orthogonal polynomials at p = 0.01 (\*\*) or 0.001 (\*\*\*). Mean comparisons within rows using Tukey's test at p = 0.05, ns=non-significant among treatments.

<sup>x</sup>Maximum antioxidant capacity and optimal harvest period were estimated by using regression analysis at p = 0.05

Table 7. Leaf and rhizome hydrophilic antioxidant capacity as indicators of senescence in containerized production of lotus (*Nelumbo spp.*).<sup>z</sup>

	Antioxidant Capacity <sup>y</sup>			
	ABTS			
	mg VCEAC/100gfw			
	Leaf		Rhizome	
Harvest (Weeks)	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	4826.31ns <sup>x</sup>	4614.62	451.70b	1117.6a
2	4981.52a	3529.08b	645.03b	1157.12a
4	6541.01a	3377.27b	689.31b	1015.72a
6	4260.37a	720.00b	678.50ns	784.60
	Q***	L***	Q*	Q**
Predicted Maximum values	5909.25±613.67 <sup>w</sup>		705.22±54.58	1151.13±51.53
Predicted Optimal Harvest (Weeks)	3		4.4	1.2

<sup>z</sup>Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.), respectively.

<sup>y</sup>VCEAC - vitamin C equivalent antioxidant capacity; ABTS - 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt.

<sup>x</sup>Harvest by species interactions were significant at  $p = 0.05$ . Significant linear (L) or quadratic (Q) trends using orthogonal polynomials at  $p = 0.05$  (\*), 0.01 (\*\*), or 0.001 (\*\*\*). Mean comparisons within rows using Tukey's test at  $p = 0.05$ , ns=non-significant among treatments.

<sup>w</sup>Maximum antioxidant capacity and optimal harvest period were estimated by using regression analysis at  $p = 0.05$

Table 8. Leaf and rhizome hydrophobic free radical scavenging activity as indicators of senescence in containerized production of lotus (*Nelumbo spp.*)<sup>z</sup>

	Free Radical Scavenging Activity <sup>y</sup>			
	DPPH			
	mg TROLOX/100gfw			
	Leaf		Rhizome	
Harvest (Weeks)	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	5076.22ns <sup>y</sup>	5145.75	500.54b	709.57a
2	5104.18a	3669.33b	615.57b	823.12a
4	5410.91a	4013.63b	681.10ns	689.08
6	3573.97a	559.15b	682.89ns	625.19
	Q***	Q***	L***	Q***
Predicted Maximum values				775.59±38.06 <sup>x</sup>
Predicted Optimal Harvest (Weeks)				2.2

<sup>z</sup>Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.) respectively.

<sup>y</sup>Harvest by species interactions were significant at p = 0.05. Significant linear (L) or quadratic (Q) trends using orthogonal polynomials at p = 0.001 (\*\*\*). Mean comparisons within rows using Tukey's test at p = 0.05, ns=non-significant among treatments.

<sup>x</sup>Maximum antioxidant capacity and optimal harvest period were estimated by using regression analysis at p = 0.05

Table 9. Leaf and rhizome hydrophilic free radical scavenging activity as indicators of senescence in containerized production of lotus (*Nelumbo spp.*).<sup>z</sup>

Free Radical Scavenging Activity <sup>y</sup>				
DPPH				
mg VCEAC/100gfw				
		Leaf	Rhizome	
Harvest (Weeks)	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	3105.08ns <sup>x</sup>	3157.54	325.78b	809.12a
2	3384.00a	2358.84b	441.32b	903.41a
4	4229.65a	2393.45b	471.09b	717.36a
6	2298.02a	49.49b	407.40b	611.05a
	Q***	Q**	Q***	Q***
Predicted Maximum values	3922.12±462.31 <sup>w</sup>	3022.83±462.52		
Predicted Optimal Harvest (Weeks)	3.2	0.4		

<sup>z</sup>Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.) respectively.

<sup>y</sup>VCEAC - vitamin C equivalent antioxidant capacity; DPPH - 2,2-Diphenyl-1-picrylhydrazyl.

<sup>x</sup>Harvest by species interactions were significant at  $p = 0.05$ . Significant linear (L) or quadratic (Q) trends using orthogonal polynomials at  $p = 0.01$  (\*\*) or 0.001 (\*\*\*). Mean comparisons within rows using Tukey's test at  $p = 0.05$ , ns=non-significant among treatments.

<sup>w</sup>Maximum antioxidant capacity and optimal harvest period were estimated by using regression analysis at  $p = 0.05$

Table 10. Leaf and rhizome hydrogen peroxide content as indicators of senescence in containerized production of lotus (*Nelumbo spp.*)<sup>z</sup>

Harvest (Weeks)	Prooxidant Content			
	H <sub>2</sub> O <sub>2</sub> nM/gfw			
	leaf		Rhizome	
	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	97.18ns <sup>y</sup>	93.57	4.32a	2.73b
2	50.06b	76.69a	3.01a	1.81b
4	75.27b	98.33a	3.11a	2.20b
6	11.08b	67.62a	2.22ns	2.39
	L**	L**	L***	NS

<sup>z</sup>Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.), respectively.

<sup>y</sup>The harvest by species interactions were significant at  $p = 0.05$ . Non-significant (NS) or significant linear (L) trend using orthogonal polynomials at  $p = 0.01$  (\*\*) or 0.001 (\*\*\*). Means comparisons within rows using Tukey's test at  $\alpha = 0.05$ , ns=non-significant among treatments.



Table 11. Leaf and rhizome malondialdehyde (MDA) content as indicators of senescence in containerized production of lotus (*Nelumbo spp.*)<sup>z</sup>

Harvest (Weeks)	Prooxidant Content			
	MDA μM/gfw			
	leaf		Rhizome	
	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>	<i>N. nucifera</i> 'E2'	<i>N. lutea</i>
0	10.00ns <sup>y</sup>	11.72	3.94a	3.01b
2	10.10ns	11.73	4.10a	2.44b
4	11.81ns	10.32	3.64a	3.17b
6	6.12ns	4.78	3.07b	3.60a
	L*	NS	Q**	Q***

<sup>z</sup>Harvest were conducted 118 days (10 Sept.), 132 days (24 Sept.), 146 days (8 Oct.) and 160 days (22 Oct.) for *N. nucifera* 'E2' and for *N. lutea* 125 days (17 Sept.), 139days (1 Oct.), 153 days (15 Oct.) and 167 days (29 Oct.), respectively.

<sup>y</sup>The harvest by species interactions were significant at  $\alpha = 0.05$ . Non-significant (NS) or significant linear (L) or quadratic (Q) trend using orthogonal polynomials at  $p = 0.05$  (\*), 0.01 (\*\*), or 0.001 (\*\*\*). Means comparisons within rows using Tukey's test at  $\alpha = 0.05$ , ns=non-significant among treatments.

Table 12. Pearson correlation coefficients (r) among selected leaf and rhizome phytochemical properties of lotus (*Nelumbo spp.*) associated with maturity.

Content <sup>z</sup>	DMC-rhizome	TSP-rhizome	AsA-rhizome	Proline-rhizome	TP-rhizome	ABTS		DPPH		H <sub>2</sub> O <sub>2</sub> -rhizome	MDA-rhizome
						VCEAC-rhizome	Trolox-rhizome	VCEAC-rhizome	Trolox-rhizome		
E2' CCI	*-0.78 <sup>y</sup>	*-0.58	*-0.53	*-0.57	*-0.64	*-0.79	*-0.83	*-0.60	*-0.75	*0.74	*0.68
<i>N. lutea</i> CCI	NS	NS	NS	NS	NS	*0.69	*0.64	*0.63	NS	NS	NS

<sup>z</sup>CCI-chlorophyll content index, DMC-dry matter content, TSP-total soluble protein, AsA - ascorbic acid, TP – total phenolics, ABTS – 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt, DPPH – 1,1-diphenyl-2-picrylhydrazyl radical, VCEAC-vitamin C equivalent antioxidant capacity, MDA-malondialdehyde.

<sup>y</sup>\*Significant at  $p \leq 0.05$ ; -Negative correlation; NS = non-significant

Table 13. Pearson correlation coefficients (r) among selected phytochemical indices of lotus (*Nelumbo nucifera* 'E2') rhizome maturity.

Content <sup>z</sup>	TSP	AsA	Proline	TP	ABTS VCEAC	ABTS Trolox	DPPH VCEAC	DPPH Trolox	H <sub>2</sub> O <sub>2</sub>	MDA
TSP	1	*0.55 <sup>y</sup>	*0.83	NS	*0.53	NS	NS	*0.55	NS	NS
AsA		1	*0.53	NS	*0.82	*0.85	*0.86	*0.69	*-0.83	NS
Proline			1	NS	*0.51	NS	NS	NS	*-0.62	*-0.53
TP				1	NS	NS	NS	NS	*-0.84	*-0.68
ABTS VCEAC					1	*0.94	*0.93	*0.74	*-0.91	NS
ABTS Trolox						1	*0.91	*0.78	*-0.83	*-0.72
DPPH VCEAC							1	*0.78	*-0.71	*-0.58
DPPH Trolox								1	*-0.84	*-0.68
H <sub>2</sub> O <sub>2</sub>									1	*0.96
MDA										1

<sup>z</sup> TSP-total soluble protein, AsA - ascorbic acid, TP – total phenolics, ABTS – 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt, DPPH – 1,1-diphenyl-2-picrylhydrazyl radical, VCEAC-vitamin C equivalent antioxidant capacity, MDA-malondialdehyde.

<sup>y</sup>\*Significant at  $p \leq 0.05$ ; NS = non-significant

Table 14. Pearson correlation coefficients (r) among selected phytochemical indices of lotus (*Nelumbo spp.*) leaf maturity at harvest.

Content <sup>Z</sup>	TSP	AsA	Proline	TP	ABTS VCEAC	ABTS Trolox	DPPH VCEAC	DPPH Trolox	H <sub>2</sub> O <sub>2</sub>	MDA
TSP	1	NS	*0.54	NS	NS	NS	NS	NS	NS	NS
AsA		1	NS	*0.63 <sup>y</sup>	NS	NS	*0.62	NS	*0.55	NS
Proline			1	NS	*0.81	*0.73	*0.72	NS	NS	*0.54
TP				1	NS	NS	NS	NS	*-0.51	NS
ABTS VCEAC					1	*0.94	*0.92	NS	NS	NS
ABTS Trolox						1	*0.90	NS	*-0.62	NS
DPPH VCEAC							1	NS	NS	NS
DPPH Trolox								1	NS	NS
H <sub>2</sub> O <sub>2</sub>									1	0.67*
MDA										1

<sup>Z</sup> TSP-total soluble protein, AsA - ascorbic acid, TP – total phenolics, ABTS – 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt, DPPH – 1,1-diphenyl-2-picrylhydrazyl radical, VCEAC-vitamin C equivalent antioxidant capacity, MDA-malondialdehyde.

<sup>y</sup>\*Significant at  $p \leq 0.05$ ; NS = non-significant

## Appendix

Part	Growth Response	Conclusions
	Leaf dynamics	Floating leaves appear first and remain prominent through longer than standing leaves, and floating leaves senesced earlier than the standing leaves. <sup>1-2</sup>
	Major Flavonoid Identified	Twenty flavonoids belonging to six groups (Myricetin <sup>1-3, 5-6</sup> , quercetin <sup>1-8</sup> , kaempferol <sup>1-2 5-6</sup> , isohammetin <sup>1-2</sup> , diosmetin <sup>1-2</sup> derivatives) were identified in lotus leaf.
e	Phytochrome	Rhizome girth is dependent on photoperiod <sup>1-3</sup> . Increase in girth is between 12 and 13 h photoperiod <sup>1-3</sup> . Rhizome transition to storage organ is under phytochrome control <sup>1</sup> . Short photoperiod induces dormancy <sup>3</sup> .
e	Circadian rhythms	Circadian rhythmic response is involved in the responsiveness to light-break in rhizome enlargement of lotus.
e	Endogenous Ethylene Production	Ethylene is not associated with rhizome enlargement <sup>1-2</sup>
e	Gene	Differentially expressed genes in lotus roots were constructed for different growth stages.
e	Photosynthetic Quantum Yield (F <sub>v</sub> /F <sub>M</sub> ) properties	Lotus have been classified as a monocarpic aquatic plant. There is a gradual decline in photosynthetic efficiency from vegetative to flowering stage.
e	Estimated Physiological Maturity	Estimated dates of maturity from 120 to 180 days after planting for potential harvest maturity
e	Estimated Physiological Maturity	Lotus typically has a growing season of 3 to 6 months for many Chinese rhizome cultivars and 5 to 8 months for Thai cultivars <sup>1-4</sup>
Desiccation	Estimated Physiological Maturity	In North America, several weeks prior to harvest, the tops are typically killed by mechanical means or by the application of chemical desiccants. Desiccation or destruction of the vines triggers maturation of the tuber periderm, which reduces skinning during harvest and moisture loss during subsequent storage
Tuber	Estimated Physiological Maturity	Leaf chlorophyll content was the most accurate predictor of maturity between early, mid and late season potato cultivars. Estimated tuber maturity is attained when 70% plants emerged and 70% of leaves turned yellow.
	Developmental Changes	After tuber initiation dry matter content initially increases exponential and thereafter increases linearly followed by a gradual decline until shoot senesces. Maximal dry matter content is observed between 75 and 105 days after emergence. Ascorbic acid content continuously increase from 15 to 75 days after emergence and gradually declines until shoot senesces. Maximal ascorbic acid content occurred at 75 days of emergence.
	Modification of dietary antioxidant concentration in five native Andean potato cultivars in response to drought (oxidative stress)	Response to drought (oxidative stress) was highly cultivar-specific and related to flesh pigment. Hydrophilic antioxidant capacity (Folin-Ciocalteu assay) was highly correlated with polyphenol content. In purple-skinned cultivar, a 62% increase in chlorogenic acid (5-CQA) was noted in comparison to other cultivars and control. Vitamin C content increased by 39% in purple-fleshed cultivar when compared to other companion cultivars and control irrigation treatment 2009a. The hydrophilic antioxidant capacity observed in potato extracts is the result of various and complex interactions that could be attributed to phenolic compounds, to other non-identified molecules, and to synergistic as well as antagonistic effects between all these components 2009b.
	Partial root-zone drying (PRD), or water restriction effect on chlorophyll content.	PRD at 25 and 50% were imposed two water restriction initiation timings were tested at: 6 weeks (WRIT6w) and 8 weeks (WRIT8w) after planting and chlorophyll concentration assessed. PRDs treatments promoted higher osmotic adjustment particularly in WRIT6w. Significant irrigation treatments effects on chlorophyll content (ChlSPAD) were observed, regardless of WRIT.
Tuber	Chlorophyll concentration as indicator of potato tuber yield	Chlorophyll degradation was negatively correlated with senescence process and also associated with oxidative stress that ultimately reduced yield.
	Photoperiod; Temperature	Photoperiod did not affect absolute tuber growth rate at lower temperatures. Higher temperature and longer photoperiod results in lower relative rates of partitioning of dry matter to the tubers
Tuber	Morphological Characteristics	Descriptive developmental aboveground morphological characteristics was utilized to define growth stages based on the Number of Fully Expanded Leaves (NFEL). The development of various tuber characteristics of three commercial potato cultivars (cvs. "Cara", "Nicola", and "Spunta") was monitored for two growing seasons. The three cultivars represent late, medium and early maturity classes. For individual cultivar, the NFEL appeared to be a reliable aboveground parameter for estimating the size of the largest tuber during the early stages of growth.

Table 13. Pearson Correlation Coefficient between leaf dry weight, rhizome dry weight and antioxidant Content in *N. nucifera* 'E2' and *N. lutea*.

Content <sup>Z</sup>	dw- rhizome	vit C- rhizome	Proline- rhizome	TP- rhizome	ABTS		DPPH	
					VCEAC- rhizome	Trolox- rhizome	VCEAC- rhizome	Trolox- rhizome
<i>N. nucifera</i> 'E2' dw leaf	NS	0.73* <sup>y</sup>	0.59*	NS	NS	NS	NS	NS
<i>N. lutea</i> dw leaf	NS	NS	NS	NS	NS	NS	NS	NS

<sup>Z</sup>dw-dry weight, Vit C - ascorbic acid, TP – total phenolics, ABTS – 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt, DPPH – 1,1-diphenyl-2-picrylhydrazyl radical.

<sup>y</sup>\*Significant at  $p \leq 0.05$ ; -Negative correlation; NS = non-significant