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## Screening for Halosulfuron Tolerance and Identifying Ethylene Response Genes in Sweetpotato[Ipomoea Batatas (Lam) L.]

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SCREENING FOR HALOSULFURON TOLERANCE AND IDENTIFYING  
ETHYLENE RESPONSE GENES IN SWEETPOTATO  
[*IPOMOEA BATATAS* (LAM) L.]

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

in

The Department of Plant, Environmental & Soil Sciences

by  
Michael Francis Polozola II  
B.S., Louisiana State University, 2011  
August 2014

I dedicate this thesis to my grandparents Gerald and Sharon Phares who have made the past few years one of the most joyous times of my life.

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## Abbreviations

1-mcp	1-methylcyclopropene
2, 4-D	2, 4-dichlorophenoxyacetic acid
2, 4, 5-T	2, 4, 5-trichlorophenoxyacetic acid
AGP	arabinogalactan protein
AHAS	acetohydroxy acid synthase
ALS	acetolactate synthase
BAP	benzyl aminopurine
ERF	ethylene response factor
EST	expressed sequence tag
HRGP	hydroxyproline-rich glycoprotein
HT	herbicide tolerance
IBA	indole butyric acid
Kn	kinetin
LS	Linsmaier and Skoog
μM	micromolar
MS	Murashige and Skoog
NAA	naphthalene acetic acid
SSH	suppression subtractive hybridization
TDZ	thidiazuron

## Abstract

Weeds cause significant yield loss in sweetpotato. Post-emergent herbicides cause damage to sweetpotato plant, which necessitates development of herbicide tolerant sweetpotato. An optimal tissue culture system is a prerequisite for in vitro screening of herbicide tolerant sweetpotato. Three Louisiana cultivars, 'Beauregard', 'Orleans', and 'LA 07-146', were tested to develop an efficient callus induction and plant regeneration system. 2, 4-D @ 0.23  $\mu$ M was ideal for 'Beauregard' and 'Orleans' callus induction and 0.45  $\mu$ M 2, 4-D for callus induction in 'LA 07-146'. Phytohormone-free media was found suitable for regeneration. Plants were regenerated from callus tissues that survived in vitro with 2  $\mu$ M, 6  $\mu$ M, 8  $\mu$ M and 10  $\mu$ M of halosulfuron methyl (active ingredient of herbicide Sandea<sup>®</sup>). The culture conditions identified through this study have applicability to wide use in somaclonal selection in sweetpotato.

Adventitious root emergence from original cuttings of sweetpotato marks the initial phase of storage root development. A better understanding of the mechanism of adventitious rooting in sweetpotato will be of immense importance to strategize breeding efforts to maximizing its marketable yield. In the present study, role of ethylene in adventitious rooting was investigated by application of 3.8 ppm of 1-mcp to two sweetpotato cultivars, 'Beauregard' and 'Evangeline', known to have contrasting adventitious rooting in response to 1-mcp. Sequencing of suppression subtractive hybridization libraries from both cultivars indicated that genes involved in the cell wall and sugar biosynthesis pathway were differentially expressed by exogenous application of 1-mcp. Two component response regulator, fructose biphosphate aldolase and arabinogalactan, were present in both cultivars. However, their expression pattern varied at different time points between 'Beauregard' and 'Evangeline'. Genes coding for ascorbate oxidase and oxysterol-binding protein were upregulated in 'Beauregard' whereas two-component response regulator was upregulated in both 'Beauregard' and 'Evangeline'. Except for a slight upregulation of fructose-bisphosphate aldolase in 'Beauregard' and arabinogalactan in 'Evangeline' at 12 h time point, both genes showed downregulation in both

cultivars at all other time points. Most of the differentially expressed genes were shown to be involved in ethylene signaling. This study confirmed previous results that ethylene is involved in adventitious rooting in sweetpotato.

## **Chapter I. General Introduction**

### **Economic Importance of Sweetpotato**

Sweetpotato is an important food crop worldwide. Internationally it is ranked as the 7<sup>th</sup> most important food crop (Kays, 2004). In the United States sweetpotato is consumed primarily as a seasonal vegetable. Sweetpotato is the state vegetable crop of North Carolina. It is estimated that the importance of sweetpotato will continue into the future as the global population grows. Sweetpotato, along with other root and tuber crops, are expected to contribute significantly to the global food security, especially among the poor in developing countries by the year 2020 (Scott et al., 2000). Within the U.S., sweetpotato is widely cultivated in Louisiana where 13 thousand acres were harvested with an economic production value of ~48 million dollars in 2010 to the state (USDA, 2011). Acreage has declined significantly since then. Competition from other high value commodities has been a contributing factor to this. The present investigation was undertaken to study two important factors that contribute to maximizing sweetpotato yield: (1) effective weed management; and (2) a better understanding of storage root formation.

### **Weed Management in Sweetpotato**

Weeds cause a yield reduction of 14% to 68% in sweetpotato depending on the cultivar (LaBonte et al., 1999), and reduce the quality of sweetpotato due to their ability to divert nutrients, water and sunlight away from the crop (Smith and Miller, 2007). Prostrate growth habit of sweetpotato on the ground is a limitation to its weed management. A critical weed-free period of 2 to 6 weeks after transplanting was optimum for maximum yield and quality (Seem et al., 2003). Mechanical measures, including hand weeding, have been used to control weeds in sweetpotato (Welker, 1967). The rising cost of labor is cost-prohibitive, but mechanical cultivation to reduce weed pressure is still widespread. Pre-emergence herbicides have been used in sweetpotato during the first two months of growing. Presently, only three post-emergence herbicides are recommended for sweetpotato in Louisiana (Smith

and Miller, 2007). Post-emergence herbicides for broadleaf weeds can cause damage to the sweetpotato plant (Dittmar and Stall, 2013).

#### Use of Sandea in Sweetpotato Cultivation

The herbicide Sandea<sup>®</sup>, with its ability to control many broadleaf weeds and nutsedges, has been of interest to sweetpotato growers. Sandea, classified as a Sulfonylurea, inhibits acetolactate synthase (ALS) and acetohydroxy synthase (AHAS), and affects the production of branched chain amino acids isoleucine, leucine and valine (Griffin, 2008). When applied before transplanting, Sandea<sup>®</sup> did not cause noticeable damage or yield reduction in sweetpotato while nutsedge control was at least 90% (Main et al., 2005). In Louisiana, the combinations of Sandea<sup>®</sup> with Valor<sup>®</sup> (flumioxazin) or Command<sup>®</sup> (clomazone) as pretransplant increased the yield of more marketable sizes (Smith et al., 2006). Post-emergent application of Sandea<sup>®</sup> caused 12.5% to 22.5% damage to sweetpotato canopy compared to the untreated checks after one week. However, the damage was reduced (6 %) after 2 weeks of growth and no damage was visible after 3 weeks (Shankle et al., 2003). Although there was no significant yield loss, foliage damage diminished interest of the producers from using Sandea<sup>®</sup> as a post-emergent application. Application of Sandea<sup>®</sup> over 13 g ha<sup>-1</sup> damaged sweetpotato storage roots, but the cultivar 'Beauregard' showed much less damage than 'Covington', which was more sensitive to post-emergent applications (Dittmar et al., 2013). Thus, development of cultivars with enhanced resistance to Sandea<sup>®</sup> would provide producers more flexibility in controlling weeds, especially nutsedges that may develop later in the season.

#### Using Tissue Culture Techniques to Acquire Herbicide Tolerance

Tissue culture techniques offer a unique opportunity for acquiring herbicide tolerance (HT) in sweetpotato either by genetic engineering, mutation or creating a system to screen existing cultivars and germplasm for innate HT. Callus-based screening for sethoxydim tolerance was successfully used in seashore paspalum (Heckart et al., 2010). Development of an optimum tissue culture system involving callus induction and regeneration is a prerequisite for in vitro screening, mutagenesis and/or

genetic engineering for HT in sweetpotato. Protocols developed earlier in sweetpotato were genotype-dependent (Kreuze et al., 2009). Therefore, there is a need to develop a reproducible and genotype-independent culture medium-hormone recipe for efficient callus induction and regeneration in sweetpotato. The second chapter of this thesis reports the results of a study that was undertaken with an objective to develop an optimum callus induction and regeneration system for halosulfuron screening with an ultimate goal of developing HT sweetpotato.

### Adventitious Rooting in Sweetpotato

Adventitious root emergence from sweetpotato cuttings (slips) marks the initial phase of storage root development (Togari, 1950; Belehu et al., 2004). A better understanding of the mechanism underlying adventitious rooting in sweetpotato will be useful for development of better cultivars with improved marketable yield. Auxins and cytokinins play an important role in the thickening process of storage root formation from initial adventitious roots (Noh et al., 2010, Tanaka et al., 2005, Tanaka et al. 2008, Solis et al., 2014). Hormones, such as zeatin riboside, dihydro-zeatin riboside and abscisic acid were shown to have important roles in storage root formation (Wang et al., 2005). Recently, gene expression analysis hinted the role of ethylene in sweetpotato root development (Firon et al., 2013). Adventitious root growth and epidermal cell death in rice were shown to be linked to the ethylene signaling pathway (Mergemann and Sauter, 2000). However, first evidence of involvement of ethylene in genotype-specific adventitious rooting of sweetpotato was reported by Villordon et al. (2012). Therefore, an understanding of the regulation of initial adventitious rooting by ethylene will help to improve quality and uniformity of cutting establishment (Villordon et al., 2012).

### Use of Ethylene Antagonists in Sweetpotato

Ethylene antagonists, such as 1-methylcyclopropene (1-mcp), bind to ethylene receptors and affect plant's ethylene perception. 1-mcp has been approved for use to increase the shelf-life of fruits, vegetables and cut flowers (Cheema et al., 2013; Watkins, 2006), but it has not been studied extensively in vegetables (Bower and Mitcham, 2001). Sprout growth and monosaccharide content in

sweetpotato roots was reduced by 625 nL/L of 1-mcp for 24 h (Cheema et al., 2013). Interestingly, 1-2 ppm of 1-mcp was shown to affect the development and growth of sweetpotato adventitious roots in a genotype dependent fashion (Villordon et al., 2012). ‘Beauregard’ showed significant reduction in the number and growth of adventitious roots, but root count was unaffected in ‘Evangeline’ with slight reduction in root growth. Histochemical analysis indicated that ethylene-induced cell death was inhibited by 1-mcp in ‘Beauregard’. Cell death and rudimentary adventitious root tip development was, nevertheless, persistent (Villordon et al., 2012). However, the genetic basis of the differential response of two cultivars ‘Beauregard’ and ‘Evangeline’ to lateral rooting by the application of 1-mcp is lacking (Villordon, et al., 2012). In apples, mode of the action of 1-mcp was shown to be related to delaying or suppression of the expression profile of genes involved in ethylene biosynthesis and reception (Varanasi et al., 2013). Therefore, molecular investigations into the response of sweetpotato to 1-mcp and its role in ethylene biosynthesis pathway could contribute to a better understanding of genetic regulation of agronomic traits like storage root development, flood tolerance, storage life and disease resistance. The third chapter of the thesis focuses on the study which was conducted with an objective to identify of genes that are differentially regulated by 1-mcp application in two sweetpotato cultivars ‘Beauregard’ and ‘Evangeline’.

## **Chapter II. Development of an In Vitro Screening Method for Herbicide Tolerance in Sweetpotato (*Ipomoea batatas*)**

### **Introduction**

Sweetpotato is an important food crop worldwide. Among other biotic pests, weeds cause 14% to 68% reduction of yield depending on the cultivar (LaBonte et al., 1999) and quality of sweetpotato due to their ability to divert nutrients, water and sunlight away from the crop (Smith and Miller, 2007). Weed management in sweetpotato is difficult due to its prostrate vine growth. It is assumed that little weed control is needed after the first two months because of rapid canopy coverage (Titus, 2008). However, if weeds are not managed in that first two months, it can have a detrimental effect on the ability of the sweetpotato to out-compete weeds. A critical weed-free period of 2 weeks to 6 weeks after transplanting was found to be optimum for maximum yield and quality (Seem et al., 2003). Mechanical measures, often supplemented with hand weeding, have been used to control weeds in sweetpotato (Welker, 1967). The rising cost of labor has made hand pulling prohibitively expensive, but mechanical cultivation to reduce weed pressure is still widespread. Three mechanical cultivations are a part of an average weed control program in sweetpotato. Registered pre-emergent herbicides exist for sweetpotato. Four pre-emergence herbicides: Command<sup>®</sup>, Dacthal<sup>®</sup>, Valor<sup>®</sup> and Devrinol<sup>®</sup> are recommended in Florida for controlling broadleaf and grass weeds (Dittmar and Stall, 2013). Command<sup>®</sup>, Valor<sup>®</sup> and Dual Magnum<sup>®</sup> are additionally registered for use in Louisiana for controlling broadleaf and grass weeds (Smith and Miller, 2007). Post-emergent broadleaf weed control is challenging as most herbicides for broadleaf weeds cause damage to the sweetpotato plant (Dittmar and Stall, 2013).

The herbicide Sandea<sup>®</sup>, with its ability to control many broadleaf weeds and nutsedges, has been of interest to sweetpotato growers. Commercial grade Sandea<sup>®</sup> 75WG contains 75% halosulfuron-methyl as the active ingredient (Gowan Co, LLC, Yuma, AZ). The mode of action of this particular herbicide is the inhibition of acetolactate synthase (ALS) and acetohydroxy synthase



(AHAS) by blocking conversion of  $\alpha$ -ketoglutarate to 2-acetohydroxybutyrate and conversion of pyruvate to 2-acetolactate, respectively (Griffin, 2008 ). It is classified as a Sulfonylurea with 4 d to 12 d half-life, and affects the production of branched chain amino acids isoleucine, leucine and valine, the biosynthetic pathway that occurs within the chloroplast (Griffin, 2008). Sandea<sup>®</sup> is readily absorbed by roots, shoots and foliage, and is easily translocated in the xylem and phloem to meristematic tissues of the plant. When applied as a pre-emergence herbicide, it suppresses weed emergence, whereas growth cessation occurs when applied post-emergent. It also leads to chlorosis or purpling and malformation of immature leaves, necrosis, and abnormal branching (Griffin, 2008).

When applied before transplanting, Sandea<sup>®</sup> did not cause noticeable damage or yield reduction in sweetpotato while nutsedge control was at least 90% (Main et al., 2005). In Louisiana, the combinations of Sandea<sup>®</sup> with Valor<sup>®</sup> (flumioxazin) or Command<sup>®</sup> (clomazone) as pretransplant increased the yield of more marketable sizes (Smith et al., 2006). Post-emergence application of Sandea<sup>®</sup> was shown to cause 12.5% to 22.5% damage to sweetpotato canopy compared to the untreated checks after one week. Damage lessened (6 %) after two weeks and no damage was visible after three weeks (Shankle et al., 2003). Although there was no significant yield loss, foliage damage diminished producer interest from using Sandea<sup>®</sup> as a post-emergent application. In addition, research indicates that application of Sandea<sup>®</sup> over 13 g ha<sup>-1</sup> could damage the sweetpotato storage root, with the cultivar ‘Beauregard’ showing much less damage at higher doses of Sandea<sup>®</sup> than ‘Covington’, which was more sensitive to post emergence applications (Dittmar et al., 2013). Thus, development of cultivars with improved resistance to Sandea<sup>®</sup> would provide producers more flexibility in controlling weeds, especially nutsedges that may develop later in the season.

A culture medium-vitamin recipe for micropropagation and maintenance of virus-free sweetpotato cultivars exists (Hoy and Clark, 2006); however, development of an optimum tissue culture system involving callus induction and regeneration is a prerequisite for in vitro screening, mutagenesis and/or genetic engineering for herbicide tolerance (HT). There are a few reports that

document sweetpotato's response to tissue culture system (Golmirzaie and Toledo, 1998; Ahmed et al., 2011). Both 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 2,4,5-trichlorophenoxyacetic acid (2, 4, 5-T) have been used for callus induction, where the latter produced embryogenic callus in cultivars that did not respond as favorably to 2, 4-D (Al-Mazrooei et al., 1997; Sefasi et al., 2012). 2, 4-D was effective at low rate (2.3  $\mu$ M) and started to have negative effect at rates above 13.57  $\mu$ M (Oggema et al., 2007a). The abaxial surface of the leaf formed more embryogenic callus than the adaxial surface (Liu and Cantliffe, 1984). Callus generation and indirect embryogenesis were considered advantageous over direct embryogenesis, which produces fewer regenerated plants, and abscisic acid was shown to enhance somatic embryogenesis (Liu, et al., 2001; Oggema et al., 2007b).

Compact and organized callus tissue was shown to regenerate better than loose and friable callus (Liu and Cantliffe, 1984). Phytohormone-free medium was generally used for callus regeneration (Liu and Cantliffe, 1984). A comprehensive analysis of various protocols has led to the conclusion that many of the protocols developed were genotype-dependent (Kreuze et al., 2009). Therefore, there is a need to develop a reproducible and genotype-independent tissue culture system i.e., standard medium-hormone recipe for efficient callus induction and regeneration in sweetpotato.

Callus-based screening for sethoxydim tolerance was successfully used in seashore paspalum (Heckart et al., 2010). The present study was undertaken to develop an optimum callus induction and regeneration system for halosulfuron screening with an ultimate goal of developing halosulfuron resistant sweetpotato.

## Materials and Methods

### Plant material

In vitro maintained virus-free plants of three sweetpotato cultivars, 'Beauregard', 'LA 07-146' and 'Orleans', were obtained from the Department of Plant Pathology and Crop Physiology at the Louisiana State University Agricultural Center (Courtesy of Mary Hoy). 'Beauregard' was chosen due to its widespread cultivation in the U.S. and elsewhere (Rolston et al., 1987); 'LA 07-146' was selected

because it is a high yielding cultivar with ideal qualities for the French fry industry; ‘Orleans’ is a new release similar to ‘Beauregard’ but has a better shape (LaBonte et al., 2012).

The plant material was maintained in tissue culture in large magenta vessels (one on top of the other with a connector, Sigma-Aldrich, St. Louis, MO) on 40 ml to 50 ml of LS medium (Linsmaier and Skoog, 1965) using the protocol described by Hoy and Clark (2006). The large container allowed plants to develop larger leaves and thicker stems than when grown in culture tubes that tend to support adequate stem growth but substantially smaller leaves. At least two multiplication cycles were performed with stem cuttings of 2 cm to 3 cm (with at least two nodes) to generate plants with enough leaf and stem materials for use as explants.

### Callus induction

A pilot experiment was conducted with leaf and stem tissues of two cultivars ‘Beauregard’ and ‘LA 07-146’ that were placed in Petri dishes containing 25 mL of MS (Murashige and Skoog, 1962) medium supplemented with the following phytohormones: 2, 4-D ( $\mu\text{M}$ ): 0.05, 0.09, 0.23, 0.45, 3.39, 4.52, or 11.31; NAA (naphthalene acetic acid,  $\mu\text{M}$ ): 2.69, 5.37, 13.43, or 26.85; IBA (indole butyric acid,  $\mu\text{M}$ ): 0.49, 1.23, 2.46, 4.92, 6.15, 7.38, 8.61, 9.84, 14.76, 19.68, 24.60, 29.52, 34.44, 39.36, 44.28, or 49.20; TDZ (thidiazuron,  $\mu\text{M}$ ): 0.11; Combination ( $\mu\text{M}$ ): 0.54 NAA + 9.29 KN (kinetin) + 2.22 BAP (benzyl aminopurine), 0.54 NAA + 8.88 BAP, 0.54 NAA + 0.11 TDZ. The phytohormone concentrations were selected based on literature available on sweetpotato (Liu et al., 2001; Oggema et al., 2007a) and other crops, such as rice (Baisakh et al., 2001).

The cultures were incubated under dark at  $26\pm 1^\circ\text{C}$ . The cultures with TDZ treatment and phytohormone combinations were incubated under a 12:12 h light:dark regime. Pilot experiment suggested that 2, 4-D was the best phytohormone and leaf tissue was better than stem cuttings as explant for callus induction (data not shown). Subsequently, 2, 4-D was used at various concentrations (0.23, 0.45, 1.13, 2.26, 3.39, or 0.45  $\mu\text{M}$ ) to establish its optimal level for callus induction from leaf

discs of 'Beauregard', 'LA 07-146' and 'Orleans'. Five leaf explants were cultured per Petri dish in three replicates for each treatment and cultivar. The cultures were incubated under dark inside a growth room maintained at  $26 \pm 1$  °C. Observations on callus induction were made every week. Compact creamy yellow (embryogenic) sections of the primary callus tissues were excised from the leaf explants and subcultured every three weeks on fresh medium with the same level of 2, 4-D.

#### Screening of callus tissue for halosulfuron tolerance

Embryogenic callus tissues propagated from medium with 0.23  $\mu\text{M}$  or 0.45  $\mu\text{M}$  2, 4-D (dependent upon the cultivar) were used for screening with halosulfuron. Callus tissues from all three cultivars were placed on MS medium supplemented with 0.45  $\mu\text{M}$  2, 4-D and halosulfuron at various concentrations (2, 4, 6, 8 and 10  $\mu\text{M}$ ). Cultures without halosulfuron served as the control. Cultures were incubated under continuous dark at  $26 \pm 1$  °C. The cultures were observed on a daily basis until a clearly visible browning of the tissues was noticed. Half of the surviving callus was subcultured on fresh herbicide supplemented callus induction medium and the other half was transferred to phytohormone-free medium for regeneration. In another experiment, leaf discs were also cultured on Petri dishes with callus induction medium supplemented with 0.23  $\mu\text{M}$  2, 4-D and different concentrations of halosulfuron to determine its effect on callus induction. Calli induced from halosulfuron-free as well as halosulfuron-supplemented medium were subcultured three times on respective fresh medium every 4 weeks. Each treatment was replicated three times with 20 callus tissues or leaf explants per replicate on herbicide-supplemented medium. Data on callus induction was averaged over replications and mean data for each treatment were compared using the least significance difference (LSD) method at  $P = 0.05$  level of significance.

#### Callus regeneration

Three-month-old control callus and callus tissues that survived and grew well on the herbicide supplemented medium were harvested and transferred to phytohormone-free MS medium for

regeneration. In addition, callus tissues not exposed to the herbicide were also placed on regeneration medium that contained halosulfuron. Four callus tissues were placed in magenta vessels with 80 mL of medium. The cultures were kept under 12 h light period at  $26 \pm 1$  °C. Upon regeneration, the green shoots were carefully separated from the callus tissues and transferred to fresh medium for further growth and rooting. The rooted plants were transplanted onto 4-inch pots filled with equal proportion of sand and peat moss following the protocol of Hoy and Clark (2006). The greenhouse was maintained at a day:night temperature regime of 29:22 °C during Spring 2014.

## Results and Discussion

### Callus induction

Both the cultivars, ‘Beauregard’ and ‘LA 07-146’, produced embryogenic as well as non-embryogenic callus from leaf and stem explants. However, leaf explants produced more compact, creamy yellow (embryogenic) callus compared to stem explants, which produced either too hard or too friable callus. Callus from stem tissue was hard and did not proliferate to an embryogenic culture (data not shown). Leaf explants also had the advantage of generating more callus tissue since callus induction occurs at a higher frequency on the cut edges of the explants.

Callus induction was noticed within 7 to 14 days of culture of leaf explants on callus induction medium. Of the different 2, 4-D levels tested, 0.23  $\mu$ M was found best with more than 95% rate of creamy yellow embryogenic callus for ‘Orleans and ‘Beauregard’. 2, 4-D at 0.45  $\mu$ M was better for ‘LA 07-146’ compared with 0.23  $\mu$ M 2, 4-D. The texture and color of callus induced from these two levels of 2, 4-D were superior to others with the resulting callus being embryogenic in nature and creamy yellow in color (Table 2.1; Figures 2.1, 2.2). Most callus tissues formed from treatments above 0.45  $\mu$ M 2, 4-D were non-embryogenic and at 4.52  $\mu$ M 2, 4-D the callus was friable and difficult to handle. Similarly, the color of the treatments at higher levels of 2, 4-D was dull brown in color. These results differ significantly from those observed by Oggema et al. (2007a) where callus

induction was found effective at a rate of 2.26  $\mu\text{M}$  2, 4-D for a local Kenyan cultivar. Similarly, Liu and Cantliffe (1984) used 2.26  $\mu\text{M}$  as the lowest concentration of 2, 4-D. This may explain a 20% rate of generating embryogenic callus (at 4.52  $\mu\text{M}$  2, 4-D) as opposed to high rates achieved in the present study at 0.23  $\mu\text{M}$  and 0.45  $\mu\text{M}$  2, 4-D. However, Sefaski et al (2012) obtained 40% to 75% callus induction at 0.90  $\mu\text{M}$  of 2, 4-D in six Ugandan sweetpotato cultivars, whereas the embryogenic callus was in the range of 10% to 35%. Varietal difference was not significant among the 2, 4-D treatments with respect to leaf weight after a week callus was induced, but the embryogenic nature of the callus differed significantly (Figure 2.1).

Table 2.1 Weight per leaf (g) with the callus induced from it of sweetpotato cultivars ‘LA 07-146’, ‘Orleans’, and ‘Beauregard’ cultured on various concentrations of 2, 4-D.

2,4-D ( $\mu\text{M}$ )	‘LA 07-146’	‘Orleans’	‘Beauregard’
0	0.093 $\pm$ 0.007 <sup>2</sup> a	0.048 $\pm$ 0.016a	0.06 $\pm$ 0.0170a
0.23	0.421 $\pm$ 0.123bc	0.459 $\pm$ 0.103b	0.641 $\pm$ 0.092cd
0.45	0.749 $\pm$ 0.034c	0.986 $\pm$ 0.095d	0.826 $\pm$ 0.118d
1.13	0.810 $\pm$ 0.050cd	0.619 $\pm$ 0.187bc	0.463 $\pm$ 0.083bc
2.26	0.928 $\pm$ 0.166d	0.869 $\pm$ 0.043cd	0.662 $\pm$ 0.137cd
3.39	0.864 $\pm$ 0.104cd	0.618 $\pm$ 0.030bc	0.303 $\pm$ 0.058b
4.52	0.331 $\pm$ 0.026b	0.547 $\pm$ 0.267bc	0.351 $\pm$ 0.117b

<sup>2</sup>Values represent means ( $\pm$  SEM). Means within columns followed by a common letter are not significantly different based on Fisher’s least significant difference mean separation test (P = 0.05).

### Callus regeneration

Previous experiments have shown that ‘Beauregard’ callus has a poor regeneration potential (Mary Hoy, personal communication). The present experiment also confirmed that ‘Beauregard’ had a lower regeneration rate (37.5%) than ‘LA 07-146’ (77.8%), but was higher than ‘Orleans’ (13.9%) (Table 2.2). As in the present study, regeneration has been achieved on hormone free basal media (Liu and Cantliffe, 1984). However, high shoot regeneration frequency was obtained in F9 media that contained 0.91  $\mu\text{M}$  zeatin riboside as compared to hormone-free media in Ugandan cultivars (Sefasi et al., 2012).

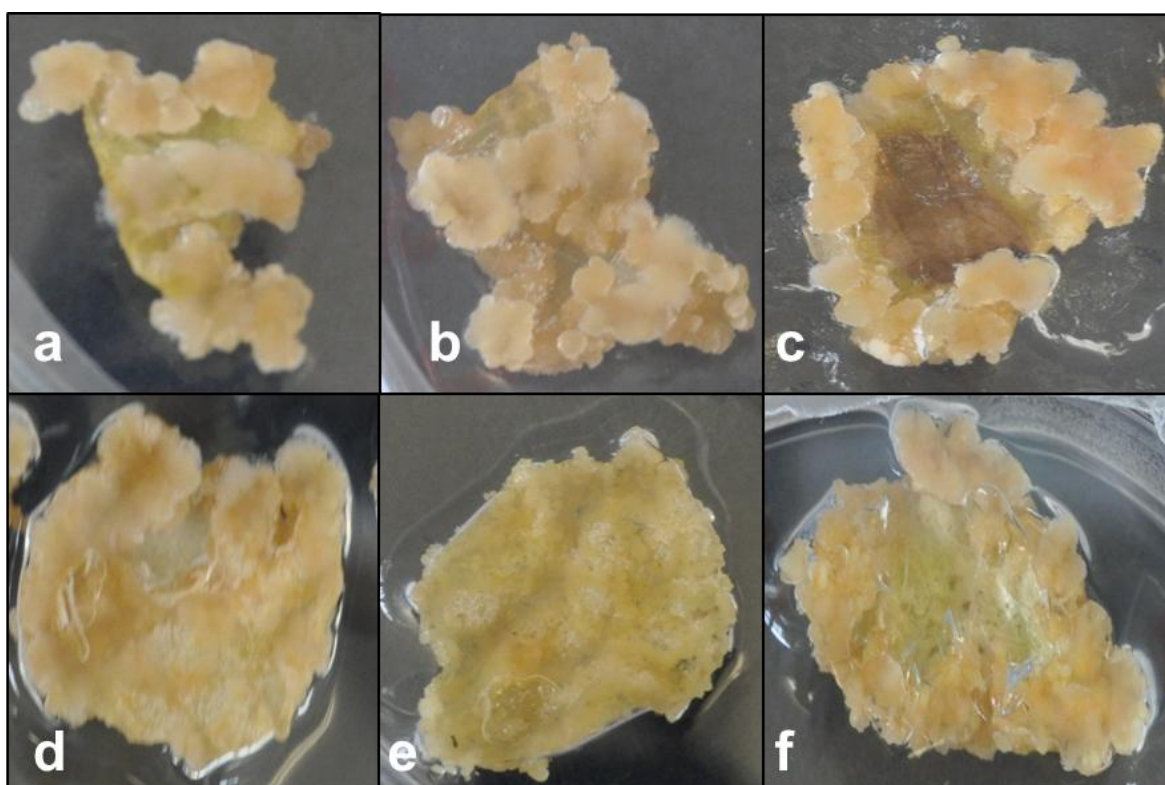


Figure 2.1. Color and texture of callus tissues of three sweetpotato cultivars after 4 weeks of culture under optimal 2, 4-D concentrations. a = 'Orleans', b = 'Beauregard' and c = 'LA 07-146' at 0.23  $\mu\text{M}$  2, 4-D', d = 'Orleans', e = 'Beauregard' and f = 'LA 07-146' at 0.45  $\mu\text{M}$  2, 4-D.

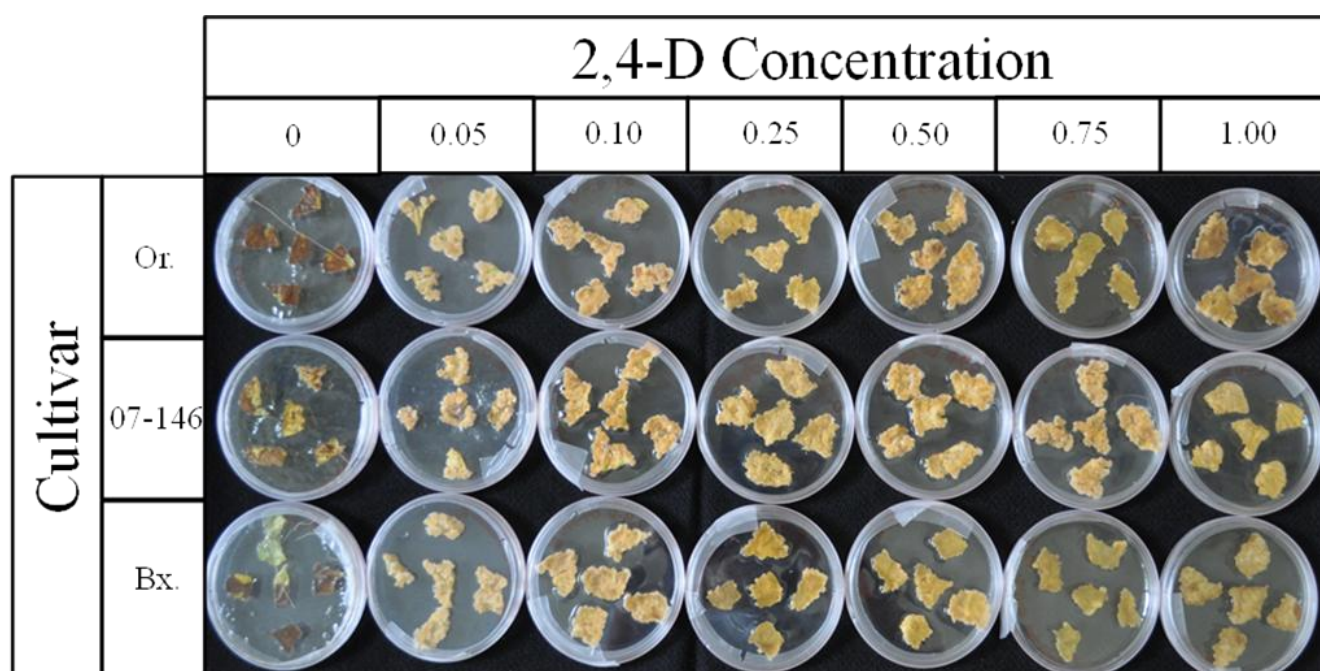


Figure 2.2. Visual differences of the callus of sweetpotato cultivars 'Orleans' (Or), 'LA 07-146', and 'Beauregard' (Bx) in response to 2, 4-D concentrations ranging from 0 to 4.52  $\mu\text{M}$ .

Table 2.2. Callus regeneration rates of sweetpotato cultivars ‘LA 07-146’, ‘Orleans’, and ‘Beauregard’ induced from 0.23  $\mu$ M 2, 4-D supplemented media after four weeks of transfer to phytohormone-free MS medium.

Regeneration	Number of calli	No. of calli regenerated	Percent regenerated
‘LA 07-146’	72	56	77.8
‘Orleans’	72	10	13.9
‘Beauregard’	72	27	37.5

#### Halosulfuron screening

Callus induction and callus weight was not significantly affected when selection was performed on media containing halosulfuron up to a concentration of 10  $\mu$ M (Table 2.3). However, when callus induced on herbicide-free media was subcultured on halosulfuron-containing media for seven weeks, the callus weight started to decline with increasing herbicide concentration, (Table 2.4). Also the callus started to turn brown to dark brown or black after 8 weeks of exposure to herbicide.

Table 2.3. Weight per Leaf (g) of sweetpotato cultivars ‘LA 07-146’, ‘Orleans’ and ‘Beauregard’ on various halosulfuron concentrations after four weeks of inoculation on callus induction medium (MS medium supplemented with 0.23  $\mu$ M 2, 4-D)

Halosulfuron ( $\mu$ M)	‘LA 07-146’	‘Orleans’	‘Beauregard’
0	0.345 $\pm$ 0.054 <sup>z</sup> ab	0.222 $\pm$ 0.002b	0.252 $\pm$ 0.008b
2	0.329 $\pm$ 0.063ab	0.190 $\pm$ 0.001ab	0.230 $\pm$ 0.016ab
4	0.398 $\pm$ 0.003b	0.194 $\pm$ 0.012ab	0.256 $\pm$ 0.040b
6	0.313 $\pm$ 0.016ab	0.176 $\pm$ 0.011a	0.201 $\pm$ 0.030a
8	0.319 $\pm$ 0.017ab	0.210 $\pm$ 0.030ab	0.236 $\pm$ 0.041ab
10	0.294 $\pm$ 0.005a	0.232 $\pm$ 0.041b	0.238 $\pm$ 0.004ab

<sup>z</sup>Values represent means ( $\pm$  SEM). Means within columns followed by a common letter are not significantly different based on Fisher’s least significant difference mean separation test (P = 0.05).

Table 2.4. Weight of callus (g) of the sweetpotato cultivars ‘LA 07-146’, ‘Orleans’, and ‘Beauregard’ after a second subculture on media containing various halosulfuron concentrations.

Halosulfuron ( $\mu$ M)	‘LA 07-146’	‘Orleans’	‘Beauregard’
0	0.521 $\pm$ 0.080 <sup>z</sup> b	0.409 $\pm$ 0.142ab	0.538 $\pm$ 0.104b
2	0.409 $\pm$ 0.065a	0.393 $\pm$ 0.107ab	0.467 $\pm$ 0.004b
4	0.407 $\pm$ 0.023a	0.399 $\pm$ 0.000ab	0.305 $\pm$ 0.001a
6	0.476 $\pm$ 0.025ab	0.218 $\pm$ 0.012a	0.394 $\pm$ 0.060ab
8	0.625 $\pm$ 0.193c	0.269 $\pm$ 0.018ab	0.304 $\pm$ 0.121a
10	0.412 $\pm$ 0.068a	0.239 $\pm$ 0.019a	0.391 $\pm$ 0.139ab

<sup>z</sup>Values represent means of two replications ( $\pm$  SEM). Means within columns followed by a common letter are not significantly different based on Fisher’s least significant difference mean separation test (P = 0.05).



Although effect of halosulfuron was not significant among the cultivars, regeneration was negatively affected. Callus tissues from all the cultivars that survived halosulfuron showed signs of regeneration within a week of transfer to hormone-free media (Figure 2.3). Shoots of 'LA 07-146' callus were able to develop further to full plantlet, whereas no plantlets were obtained from 'Beauregard' and 'Orleans'. The herbicide may have further affected the regenerability of these cultivars, which showed low regeneration potential even without herbicide. Interestingly, all of the plantlets from 'LA 07-146' were obtained from calli that were selected and regenerated under halosulfuron pressure. No viable plantlets were regenerated on halosulfuron-containing hormone-free media from callus tissues that were not previously exposed to halosulfuron during the callus induction or subculture stage. It is possible that the callus, which survived halosulfuron during culture either acquired tolerance or the herbicide led to somaclonal variation in the callus tissue.

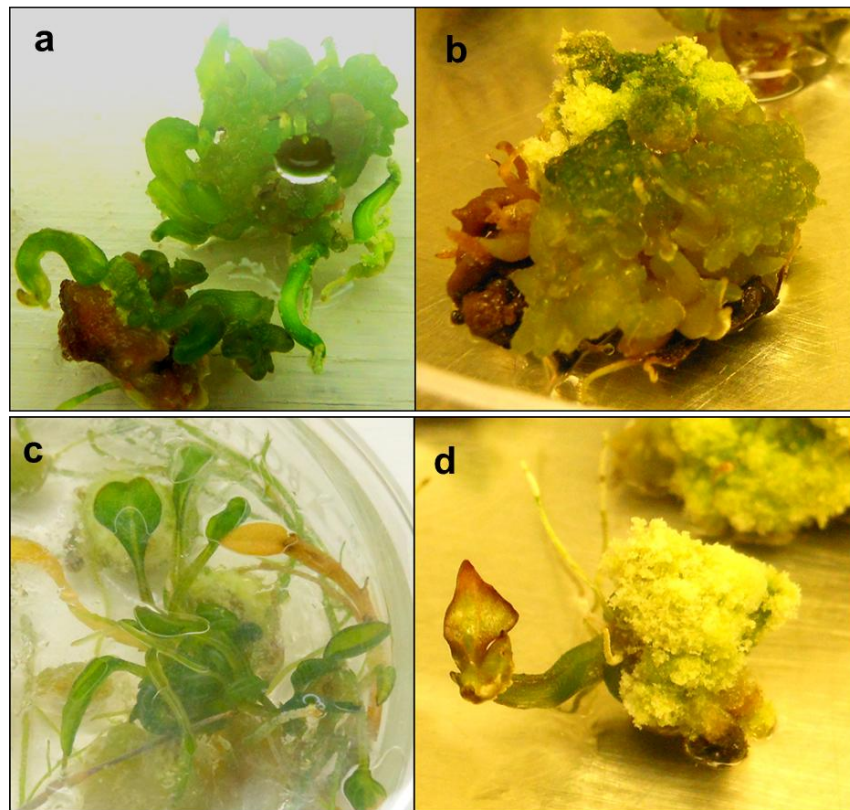


Figure 2.3. Regeneration of 'LA 07-146' after 4 and 8 weeks of transfer to regeneration medium with no halosulfuron (a, c) compared to halosulfuron (b, d) in the media.

Of the plantlets obtained from 'LA 07-146' under halosulfuron pressure, three were from 2  $\mu$ M, and one each from 6  $\mu$ M, 8  $\mu$ M and 10  $\mu$ M. No plantlet could be produced from the callus under 4 $\mu$ M halosulfuron since all calli apparently turned non-embryogenic. This is a significant reduction when compared to the regeneration experiment without the herbicide (Table 2.2). All plantlets produced roots when transferred to fresh medium (Figure 2.4). The plantlets were multiplied subsequently on herbicide-free media. At least 15 putative herbicide tolerant plants were generated for each herbicide treatment and were subsequently transferred to the greenhouse. The plants in the greenhouse will be tested for herbicide screening to determine if in vitro tolerance to halosulfuron corresponds to in vivo tolerance of plants to Sandea<sup>®</sup>. Clones of the plant materials are also being multiplied at the Sweetpotato Research Station, Chase, La. for field trials.



Figure 2.4. Complete plantlets of 'LA 07-146' with roots produced from shoots regenerated on halosulfuron-supplemented media. a = 2 $\mu$ M, b = 6  $\mu$ M, c = 8  $\mu$ M, d = 10  $\mu$ M.

## Conclusion

A successful protocol for callus induction was established for three cultivars, 'Orleans', 'Beauregard' and 'LA 07-146' using an approach to developing somaclonal variants as an adjunct to improving existing cultivars. Nominal amounts of 2, 4-D (0.23  $\mu$ M to 0.45  $\mu$ M) were found sufficient for optimum callus induction. These levels of 2, 4-D were not tried or documented in prior reports. The present protocol was further demonstrated as an effective approach to somaclonal selection for herbicide tolerance in sweetpotato.

### **Chapter III. Identifying Ethylene Response Genes in Sweetpotato in Relation to Exogenous Application to 1-methylcyclopropene (1-mcp)**

#### **Introduction**

Sweetpotato is an important commodity worldwide and is an important part of the diet in the Southeast U. S. Sweetpotato has been a focus of research to delineate the complexity of the mechanisms underlying the formation of its storage roots. Adventitious root emergence from original cuttings (slips) is an indicator of plant establishment and initial phase of storage root development (Togari, 1950; Belehu et al., 2004; Villordon et al., 2009). Therefore, a better understanding of the mechanism of adventitious rooting in sweetpotato will help devise strategies to maximizing its marketable yield.

Multiple hormones, including cytokinins and auxins, have been shown to play a role in root development in several plant species (Ruzicka et al., 2009). It was shown that auxins play an important role in the initial thickening of the storage root by regulating the expression of SDR1 gene (Noh et al., 2010). Cytokinins were also shown to be involved in the thickening of the storage root at the primary cambium and meristems in the xylem, which consist of actively dividing cells (Tanaka et al., 2005) through the modulation of gene expression at the proximal end, around the primary vascular cambium and in the thickest part of the root (Tanaka et al., 2008). Upregulation of genes responsive to auxin and cytokinins in developing storage root also implicate their role in adventitious rooting (Solis et al., 2014). Other hormones, such as zeatin riboside, dihydro-zeatin riboside and abscisic acid have been shown to have important roles in tuberous roots formation and thickening in sweetpotato (Wang et al., 2005). Recently, gene expression analysis also hinted the role of ethylene in sweetpotato root development (Firon et al., 2013). Adventitious root growth and epidermal cell death in rice were shown linked to the ethylene signaling pathway (Mergemann and Sauter, 2000). An understanding of the regulation of initial adventitious rooting by ethylene will be useful to improve quality and uniformity of cutting establishment (Villordon et al., 2012).

The gaseous phytohormone ethylene is known to play important roles in regulation of many physiological and biochemical processes of plants including seed germination, root initiation, abscission, senescence and fruit ripening, depending on the phenological state of the tissues it is acting on (Abeles et al., 1992). It also regulates cellular processes such as cell elongation and root hair formation (Tanimoto et al., 1995). In addition, it is one of the most important signaling molecules in plant's defense response against biotic stresses (insects and microorganisms) and abiotic stresses, such as mechanical wounding, water and temperature stresses (Wang et al., 2002). Generally, ethylene reduces the storage life of fruits and vegetables. In potato, ethylene was shown to extend tuber dormancy and inhibit sprout growth (Prange et al., 1998) for which it is used as a commercial sprouting inhibitor during postharvest storage. However, in sweetpotato ethylene induced multiple sprout initiation, but affected sprout growth, consistent with the results observed in potato (Prange et al., 2005). Involvement of ethylene in genotype-specific adventitious rooting was reported by Villordon et al. (2012).

Ethylene perception in the plant cell can be negated by inhibitors that inhibit synthesis of a key enzyme in ethylene biosynthesis or by antagonists that bind to ethylene receptors. Inhibitors, such as aminoethoxyvinylglycine (AVG) and antagonists, such as 1-methylcyclopropene (1-mcp) are used to increase the shelf-life of fruits and vegetables (Cheema et al., 2013). It extends storage life as well as prevents physiological disorders, such as superficial scald development in apples (Calvo and Candan, 2001) and retains fruit firmness by suppression of ethylene biosynthesis (Varanasi et al., 2013). 1-mcp has been approved for use on various fresh fruits, vegetables and cut flowers (Watkins, 2006), but it has not been studied extensively in vegetables (Bower and Mitcham, 2001). Recently, Cheema et al. (2013) observed that sprout growth in sweetpotato roots was inhibited after their exposure to 625 nL/L of 1-mcp for 24 h, and 1-mcp-treated roots had reduced monosaccharide content in comparison to the untreated roots. 1-mcp at a concentration of 1 ppm to 2 ppm was shown to affect the development and growth of sweetpotato adventitious roots (Villordon et al., 2012). The response to 1-mcp was

genotype-specific where both the number and growth of adventitious roots were significantly reduced in ‘Beauregard’, but root count was unaffected in ‘Evangeline’ with slight reduction in root growth. Histochemical analysis of the nodal tissue with trypan blue indicated that ethylene-induced cell death was inhibited by 1-mcp in ‘Beauregard’ although there was evidence of continual cell death and rudimentary adventitious root tip development (Villordon et al., 2012). This is significant since the majority of storage roots (86%) formed by ‘Beauregard’ have been shown to have their origin from adventitious roots that were extant at 5 to 7 DAT (Villordon et al., 2009).

Regulation of expression of genes involved in various metabolic and cellular processes of plant cell by exogenous ethylene is well known (Zhong and Burns, 2003). However, studies on the effect of 1-mcp on gene expression are limited. In apples, mode of the action of 1-mcp was shown to be related to the delay or suppression of the expression profile of genes involved in ethylene biosynthesis and reception (Varanasi et al., 2013). Molecular investigations into the response of sweetpotato to 1-mcp and its role in ethylene biosynthesis pathway could contribute to a better understanding of genetic regulation of agronomic traits like storage root development, flood tolerance, storage life and disease resistance. Genetic basis of the differential response of two cultivars ‘Beauregard’ and ‘Evangeline’ to lateral rooting by the application of 1-mcp is lacking (Villordon, et al., 2012). The present study reports on identification of genes that are differentially regulated by 1-mcp application in these two sweetpotato cultivars.

## Materials and Methods

### Growing sweetpotato cultivars in the greenhouse

Storage roots of two sweetpotato cultivars, ‘Beauregard’ and ‘Evangeline’ were obtained from the sweetpotato research station, Chase, LA and planted on one gallon pots filled with Miracle-Gro<sup>®</sup> (Scotts<sup>®</sup>: Marysville, OH) potting mix. The pots were grown inside greenhouse facility maintained at a day:night temperature regime of 29:22 °C. The pots were irrigated every other day to complete



saturation and fertilized with OsmocotePlus (N:P:K::15:9:12; Scotts Company LLC) ~3 g/pot once every 3 week or as necessary.

#### Treatment with 1-mcp and tissue sampling

Six inches long slips with six to eight nodes were collected from one-month-old plants of ‘Beauregard’ and ‘Evangeline’ grown inside the greenhouse. The selected slips for treatment were placed within closed 26 liter Sterilite® (Sterilite Corporation: Townsend, MA) plastic storage containers with the Smartfresh® activator solution, Smartfresh® activator and 1-mcp pills (Rohm and Haas, Philadelphia, PA) that would release 3.8 ppm of 1-mcp. The containers were completely sealed with adhesive tape to avoid escape of 1-mcp in its gaseous form (Figure 3.1). Five slips from each cultivar were collected after 6 h, 12 h, 24 h and 48 h of 1-mcp treatment. Five slips placed inside a closed container with no 1-mcp gas exchange served as the control. Control and treated samples were immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction. All treatments and control slips were collected in three biological replications.

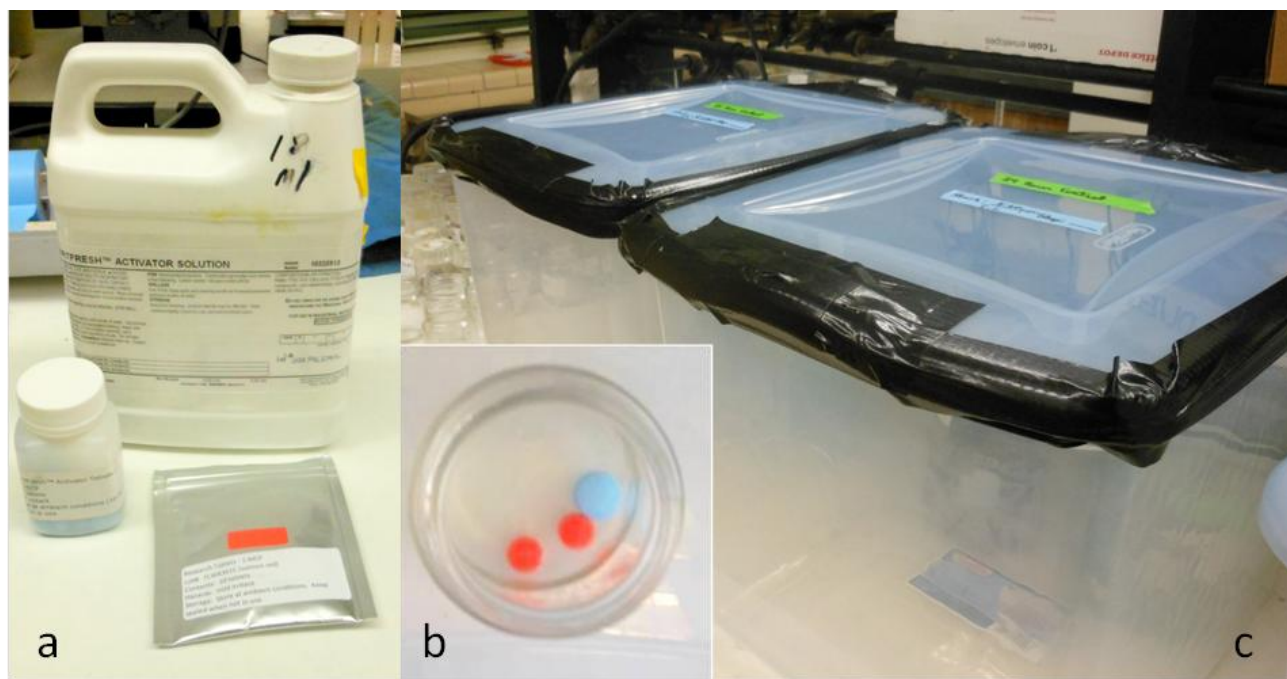


Figure 3.1. Treatment of sweetpotato slips to gaseous 1-mcp. a = 1-mcp kit consisting of activator solution, activator tablet and 1-mcp pill (inside pouch); b = activator solution with 1-mcp pill (purple) and activator tablet (blue); c = sealed plastic containers containing slips exposed to 1-mcp gas.

### RNA isolation and cDNA subtraction

Total RNA was extracted from nodal tissue of treated (different time points) and control slips of ‘Beauregard’ and ‘Evangeline’ using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. The quality and quantity of RNA were assessed using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Equal amounts (5 µg) of RNA from individual time points were pooled for each replication and cultivar. Double strand cDNA was synthesized from 25 µg of control and treated RNA of the two cultivars using Superscript<sup>TM</sup> double-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) and its instructions. Two cDNA subtraction libraries (one each for ‘Beauregard’ and ‘Evangeline’) were prepared as per the method detailed in Khan et al. (2013). Briefly, 5 µg of double stranded cDNA was digested with *RsaI*. The digested cDNA from the 1-mcp-treated samples was ligated with two different adaptors and used as tester. Two rounds of forward subtractions were performed using cDNA from the control samples as a driver. Differentially expressed upregulated genes were amplified by primary PCR with 27 cycles of 94 °C for 30 s, 66 °C for 30 s and 72 °C for 1.5 min. The primary PCR product were enriched by secondary PCR with 12 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 1.5 min.

### Cloning and sequencing of differentially expressed genes

The subtracted cDNA clones were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and transformed into *Escherichia coli* DH5a cells following the method described by Ramanarao et al. (2011). One hundred ninety two white colonies containing the cDNA inserts were confirmed positive with M13 forward and reverse primers. Plasmids were extracted from the positive clones and single-pass sequenced using an ABI 3130xl sequencing platform as described by Khan et al (2013).

### Sequence processing and bioinformatics analysis

The vector and adapter sequence contaminants were cleaned from the sequences manually. The clean sequences, after excluding duplicated sequences, were assembled using the CAP3 program with

default parameter settings (Huang and Madan 1999). The resulting unigenes were functionally annotated through BLASTx and BLASTn-based (Altschul et. al. 1997) homology search against NCBI protein and nucleotide database (<http://www.ncbi.nlm.nih.gov/>) at e-value cut-off of 1e-06. The GOslim terms for biological process, molecular function, and cellular component associated with significant BLASTx hits were assigned to the unigenes using GOslim viewer ([http://agbase.msstate.edu/cgi-bin/tools/goslimviewer\\_select.pl](http://agbase.msstate.edu/cgi-bin/tools/goslimviewer_select.pl)). Pathway analysis was performed using KEGG as described earlier (Khan et al., 2013).

### Transcript profiling of differentially expressed genes

The expression pattern of 10 genes was validated by semi-quantitative reverse transcription polymerase chain reaction (sqRT-PCR) following the method described earlier (Effendy et al., 2013). Gene-specific primers (Table 3.1) were designed using Primer3Plus web resource (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and were synthesized by Integrated DNA technologies (IDT Inc, Coralville, IA). First-strand cDNA was synthesized from 1 µg of the total RNA isolated from the control and treated (all time points) slips of both cultivars using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). One µl of the first-strand cDNA was amplified using gene-specific primers with the thermal profile as described earlier (Effendy et al., 2011). The amplification products were separated on 2 % agarose (Amresco, Solon, OH).

Table 3.1. Primer sequences of the genes used for expression analysis of sweetpotato cultivars ‘Beauregard’ and ‘Evangeline’ in response to 3.8 ppm 1-mcp application. Genes common between ‘Beauregard’ and ‘Evangeline’ are shown in red font.

Gene name	Forward primer	Reverse primer
Two component response regulator	CATATACACGGCCCGAACGA	AATGCAATGACACCGGCAAC
Oxysterol-binding protein	TTGAACTCTGTCTGACTCGGC	CGAGCTGGAAGTGTATCGCT
Fructose-bisphosphate aldolase	GGCAATCTGAAGTGGAGGCT	GCCTGTGCAGAAGTTTGGTG
Hydrolase	CCTCGGCGACTGACCATTTA	CAGTTCCGGCTGAATCACCT
Arabinogalactan protein	TGAAATTCGGGCACGGAGAA	ACTGCATGCGACTTCCATCA
Ascorbate oxidase	CATGCACTCTAGTCCTGCC	ACAGGCAGTGTTTCATCTGGT
Fructokinase	AAAGGAATCACCAGCTCCCG	ATGAGTCCGCTTTGTCCCTG



The relative abundance of three genes that were common in both cultivars was determined using quantitative real-time PCR. qRT-PCR was carried out in triplicate in a 20 µl final reaction volume containing 2 µl of 10x diluted first-strand cDNA, 10 µl of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and 0.2 µM of each primer in a MyiQ real-time PCR analysis system (Bio-Rad, Hercules, CA) as per Solis et al. (2014). A serial dilution of 100, 10, 1, 0.1 and 0.01 ng of 1<sup>st</sup> strand cDNA was used for all genes to generate a standard curve by plotting the Ct (threshold cycle) values against log (ng) 1<sup>st</sup> strand cDNA, and to ensure that the efficiencies of the individual transcripts were equal. The mRNA expression was normalized against the sweetpotato elongation factor (*IbEF1α*; Effendy et al., 2013) and calculated as a ratio using the  $2^{-\Delta\Delta C_t}$  method (Pfaffl, 2001).

## Results and Discussion

### Phenotypic response of ‘Beauregard’ and ‘Evangeline’ to 1-mcp

Phenotypic differences were observed among ‘Beauregard’ and ‘Evangeline’ slips treated with 1-mcp. A visible necrosis occurred on the young emerging shoot of ‘Beauregard’ slips as early as 6 h after exposure to 3.8 ppm 1-mcp, while Evangeline did not show this response (Figure 3.2). However, the older mature leaves of both cultivars experienced mild wilting after 24 h of 1-mcp application. Older leaves in both cultivars showed chlorophyll bleaching (chlorosis) after 48 h of 1-mcp application. Initiation of adventitious roots was observed in slips as early as 24 h after 1-mcp treatment. However, rooting was pronounced at 48 h in both 1-mcp-treated cultivars, where ‘Evangeline’ had six adventitious roots compared to ‘Beauregard’, which only had one adventitious root (Figure 3.3)

### Sequence characterization of 1-mcp-responsive genes in ‘Beauregard’ and ‘Evangeline’

All sequenced subtracted cDNAs of ‘Beauregard’ and ‘Evangeline’ (192 each), after removing vector sequences and adaptors, produced quality sequences without any ambiguous or uncalled sequence. The average EST (Expressed Sequence Tag) length in ‘Beauregard’ was 567.8 bp (ranging between 236 bp and 1139 bp) whereas in ‘Evangeline’ it was 532.1 bp (range between 78 bp and 1078 bp).



Figure 3.2. Necrosis in sweetpotato cultivar 'Beauregard' with burning on the tip (left) and leaf (right)



Figure 3.3. Difference in adventitious rooting of sweetpotato cultivars 'Beauregard' (left) and 'Evangeline' (right) after 48 h of exposure to 3.8 ppm 1-mcp

Assembly of the 'Beauregard' sequences resulted in 142 sequences forming 28 contigs and 50 singlets, thus totaling to 78 unigenes. Similarly, 139 out of 192 'Evangeline' sequence assembled to form contigs whereas 53 were singlets, which totaled 94 unigenes. The average unigene length in 'Beauregard' and 'Evangeline' were 625.1 bp and 544.8 bp, respectively.

Functional annotation of the 'Beauregard' and 'Evangeline' unigenes through BLAST analysis categorized 17 (out of 78) and 20 (out of 94) unigenes with no known function, which were described as either unknown proteins, expressed proteins or hypothetical proteins. Gene ontology analysis of the unigenes from 'Evangeline' showed overrepresentation of genes in the cellular processes (17%) followed by genes in biosynthetic process (11%). Of these, 34% had nucleic acid binding activities followed by catalytic activity (13%), transferase and hydrolase activity (12% each). A majority (22%) of the unigenes were localized in the plastids whereas 14% were membrane bound, 12% localized to plasmamembrane and 11% in the cytosol. In 'Beauregard', genes in the cellular processes (16%) and biosynthetic processes (9%) were comparable to 'Evangeline'. Similarly, 35% of 'Beauregard' unigenes had nucleic acid binding activity, where the genes involved in catalytic (21%) and hydrolase (16%) activities were higher compared to 'Evangeline'. Most unigenes (14%) were plastid localized but the coverage was much smaller compared to 22% in 'Evangeline'. While plasmamembrane bound genes of 'Beauregard' (11%) were comparable to 'Evangeline', 12% of unigenes in 'Beauregard' coded for proteins with extracellular localization, which was much higher than that of 'Evangeline' (5%). However, analysis of the unigenes against the KEGG database revealed interesting results. In 'Beauregard', 56 unigenes coded for enzymes that were represented in 39 different metabolic pathways. But, in 'Evangeline', 77 unigenes were involved in synthesis of enzymes in 35 metabolic pathways. Only 15 pathways were common between 'Beauregard' (24 unigenes) and 'Evangeline' (33 enzymes). This indicated that while a common set of genes were affected by 1-mcp application, the two cultivars showed differential representation of genes possibly being modulated by exogenous application of an ethylene antagonist.

### Temporal expression of 1-mcp responsive genes in ‘Beauregard’ and ‘Evangeline’.

Expression of six 1-mcp induced ESTs from cDNA subtraction library of ‘Beauregard’ and four ESTs from cDNA subtraction library of ‘Evangeline’ was analyzed at different time points by (semi)quantitative PCR (Figures 3.4, 3.5). It also included three ESTs that were common in the two cDNA subtraction libraries. Among the common ESTs, two-component response regulator was induced within 6 h of 1-mcp treatment in both cultivars. However, its expression reduced after 12 h whereas it was maintained in ‘Evangeline’ with the highest upregulation at 48 h. Two component response regulators are known to be associated with different hormonal responses. Arabidopsis response regulators were shown to be responsible for fine tuning ethylene signaling pathway by triggering ethylene response factor (ERF) and downstream genes in Arabidopsis (Hass et al., 2004). Also, mutants of two component regulators such as ARR10 and ARR12 showed inhibition of root elongation in Arabidopsis (Yokoyama, 2007). The accumulation of mRNA of fructose-bisphosphate aldolase was similar in both ‘Beauregard’ and ‘Evangeline’, where the accumulation was upregulated at 12 h of 1-mcp treatment and was downregulated at other time points. Fructose 1,6-bisphosphate aldolase is involved in glycolysis, gluconeogenesis, and calvin cycle. Out of eight fructose 1,6-bisphosphosphate aldolase genes in Arabidopsis, two were shown to be induced by ethylene (Lu et al., 2012). However, fructose bisphosphate aldolase was unregulated in Arabidopsis root in response to ethylene treatment (Mang et al., 2004). An EST encoding for arabinogalactan protein was downregulated in both cultivars upon exposure to 1-mcp. However the expression level was much higher at any given time in ‘Beauregard’ compared to ‘Evangeline’. Arabinogalactan proteins (AGPs) are highly glycosylated members of the superfamily of hydroxyproline-rich glycoproteins (HRGPs) found in cells throughout the plant kingdom and are mostly expressed in fruits (Fragkostefanakis et al., 2012). In tomato, the AGP genes were shown to be upregulated in response to ethylene, abiotic stress, and during fruit ripening. These results indicated that although common genes are induced in response

to 1-mcp in ‘Beauregard’ and ‘Evangeline’, the difference in their expression kinetics could play an important role in the differential adventitious rooting of the two cultivars in response to 1-mcp.

Hydrolase showed upregulation of its mRNA at 6 h of 1-mcp treatment and was downregulated at 12 h and thereafter the mRNA concentration was either same or higher than the 0 h control (Figures 3.4, 3.5).

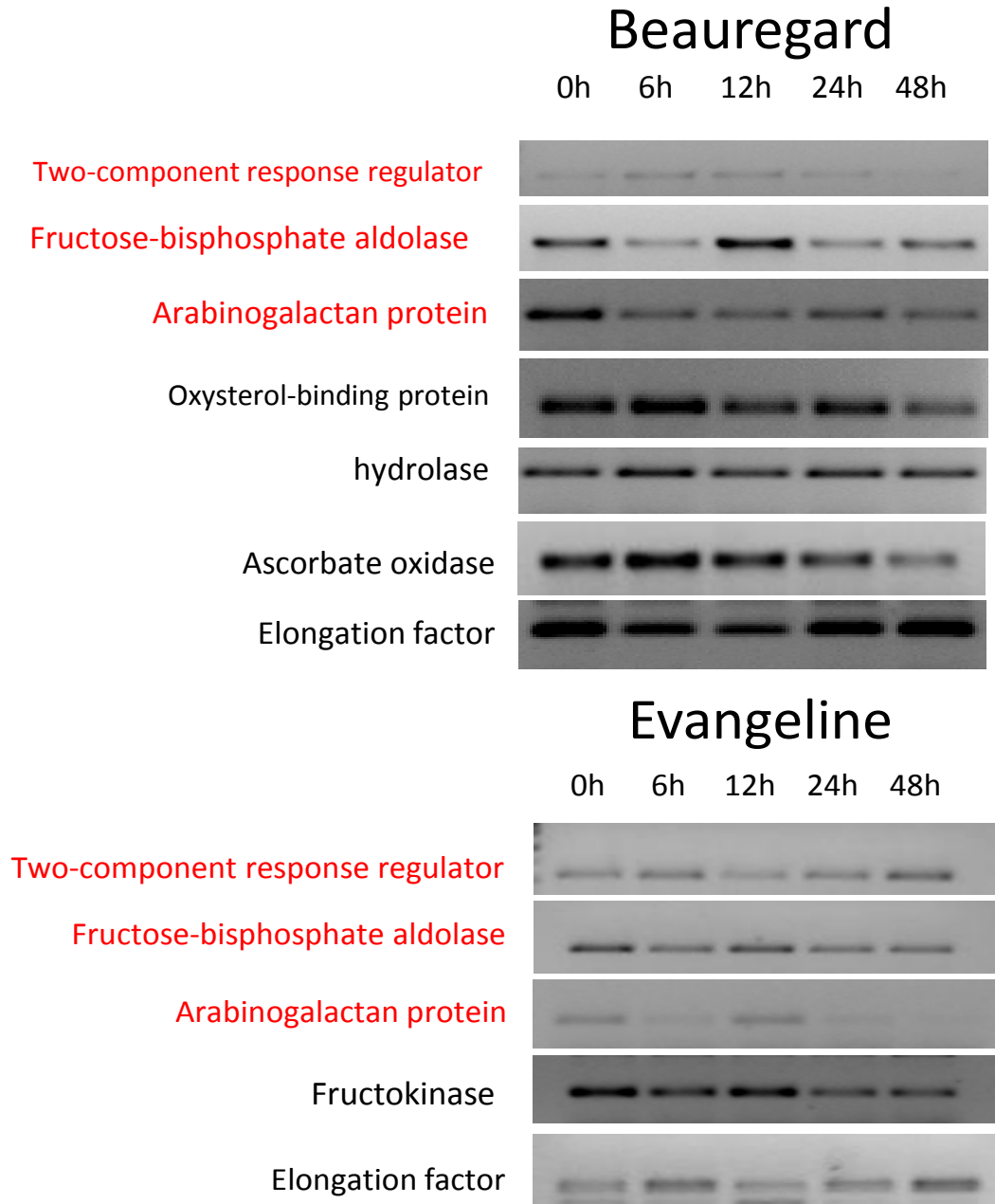


Figure 3.4. Expression analysis of 1-mcp induced genes at different time points after exposure to 3.8 ppm 1-mcp in ‘Beauregard’ (upper panel) and ‘Evangeline’ (lower panel). Genes common to both cultivars are in red font.

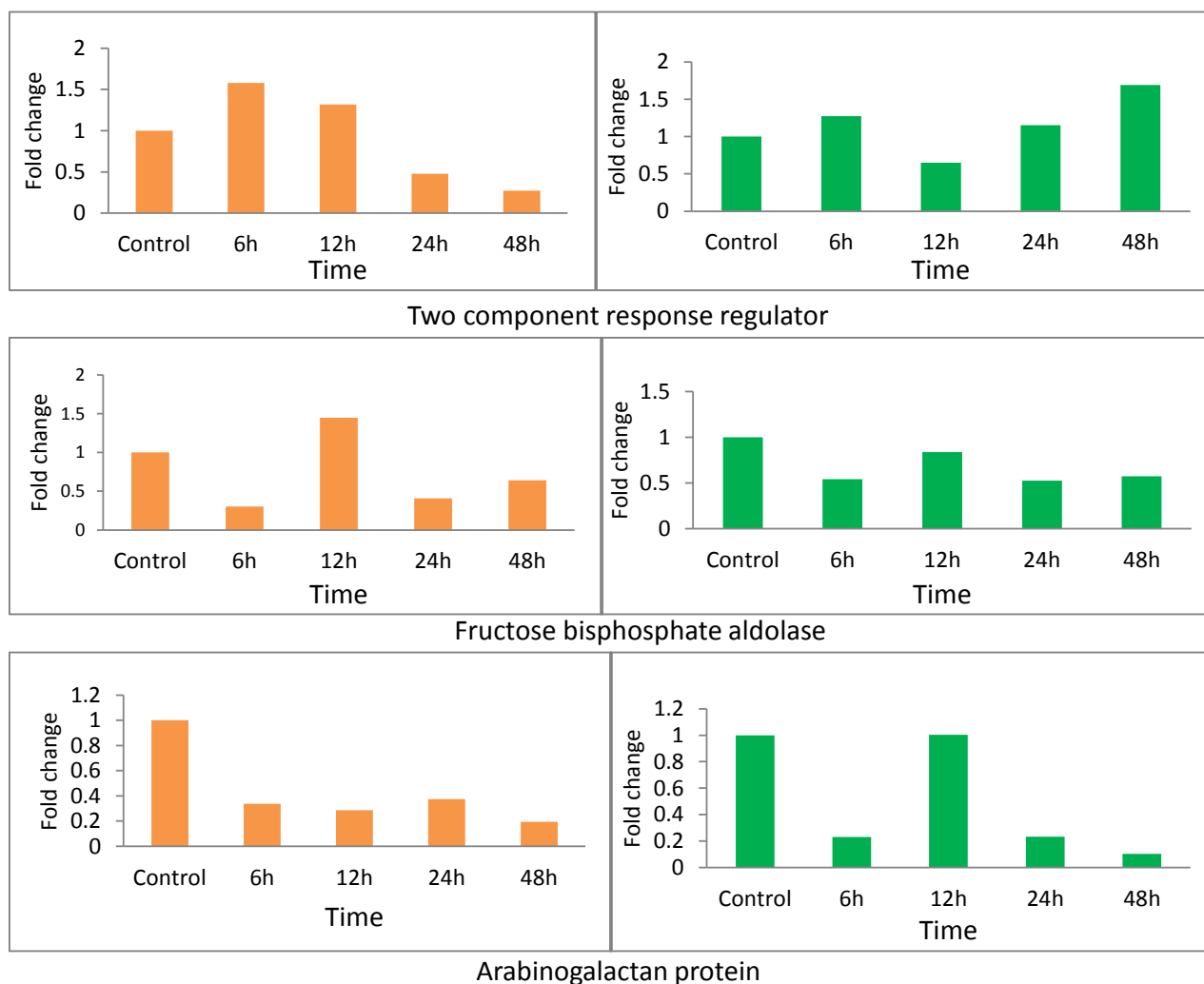


Figure 3.5. Quantitative expression analysis of three genes that were represented in libraries of both 'Beauregard' (left panel) and 'Evangeline' (right panel).

Hydrolase (xyloglucan endotransglycosylase) is involved in cell wall loosening and cell elongation.

Hydrolase was shown to be induced in maize in response to ethylene produced during flooding stress (Saab et al., 1996). At the same time, shoot elongation occurred in *Sagittaria pygmaea* in response to ethylene-induced expression of hydrolase (Ookawara, 2005). Oxysterol binding protein and ascorbate oxidase were highly upregulated after 6 h of 1-mcp treatment and then showed a gradual declining trend in their mRNA accumulation over time. Ascorbate oxidase oxidizes ascorbic acid to monodehydro ascorbate. Spinach leaves treated with ethephon, an ethylene inhibitor, showed rapid reduction in ascorbic acid content, while ethylene insensitive mutants of *Arabidopsis* showed higher level of ascorbic acid content than wild plants upon treatment (Gergoff et al., 2010). More importantly,

ascorbate oxidase catalyzes the oxidative decarboxylation of auxin and thus upregulation of ascorbate oxidase may have some effects on root development (Kerk and Feldman, 2000).

In cultivar 'Evangeline' the EST coding for fructokinase showed downregulation in its mRNA concentration at all the time points after exposure to 1-mcp except at 12 h where it was slightly upregulated. Cross talk between fructose content and ethylene has been reported in many fruits (Mashmichi, 1995). Ethylene treatment induces enzyme of glycolysis like Fructose-2,6-bisphosphate, fructose-6-phosphate-2-kinase in Kiwi fruit (Stitt et al., 1986). However, in *Arabidopsis* ethylene-responsive genes were shown to be suppressed by glucose/fructose treatment (Price et al., 2004). These results also showed that the genes involved in sugar biosynthesis were also affected by the ethylene antagonist, which can influence the adventitious root development by downregulating ethylene responsive genes .

## Conclusion

Despite increasingly accumulating evidence, specific role of plant hormones in adventitious rooting in sweetpotato is still not clear. Plant's response to ethylene is in multitude, so it is likely that a complex interaction of gene regulation and expression may occur when sweetpotato is exposed to exogenous application of 1-mcp that interfere its perception to ethylene. Thus, the genes involved in adventitious rooting in response the ethylene antagonist will likely outnumber those attributed to ethylene-related processes described in the present investigation. The identification of more novel genes through highthroughput sequencing and gene expression strategies will enable a more complete picture of the physiological and molecular aspects of ethylene-adventitious regulation, and additional candidate genes for future manipulation for genetic manipulation in sweetpotato.

## Chapter VI. Summary and Conclusion

The following conclusions were deduced from the experiments conducted:

- ❖ A successful protocol for callus induction and regeneration was established for three sweetpotato cultivars: 'Beauregard', 'LA 07-146' and 'Orleans'.
- ❖ 2, 4-D at a concentration of 0.23  $\mu\text{M}$  and 0.45  $\mu\text{M}$  was found sufficient for optimum callus induction.
- ❖ Although callus induction was successfully achieved in all three test cultivars, the low frequency of regeneration in 'Beauregard' and 'Orleans' suggested that other combinations of culture medium-vitamin recipe needed to be tested to identify an optimal regeneration medium.
- ❖ The utility of the protocol was further demonstrated as an effective approach to somaclonal selection for herbicide tolerance in sweetpotato especially in 'LA 07-146'.
- ❖ Although plants were regenerated from callus that showed in vitro resistance to halosulfuron methyl (Sanda<sup>®</sup>), further testing of plant materials generated from this experiment need to be tested under field conditions to determine if tolerance exhibited in vitro is still expressed in the field.
- ❖ The tissue culture protocol developed in the present study would have practical application in in vitro screening, mutagenesis and gene manipulation for improvement of sweetpotato cultivars.
- ❖ Two sweetpotato cultivars, 'Beauregard' and 'Evangeline', differed in their response to 1-mcp with 'Beauregard' producing no or minimum adventitious root while 'Evangeline' was unresponsive with adventitious roots as early as 24 h after 1-mcp application.
- ❖ A number of genes involved in sugar biosynthesis pathway, cell wall biogenesis, hormone signaling and general response regulation were identified to be differentially regulated in two sweetpotato cultivars 'Beauregard' and 'Evangeline' through a small scale suppression subtractive hybridization strategy.



- ❖ An EST encoding arabinogalactan protein was downregulated in both cultivars upon exposure to 1-mcp. However its expression level was much higher in ‘Beauregard’ compared to ‘Evangeline’ except at 12 h time point.
- ❖ Genes involved in sugar biosynthesis were also affected by the ethylene antagonist, which can influence the adventitious root development by downregulating ethylene responsive genes.
- ❖ Although common genes were induced in response to 1-mcp in ‘Beauregard’ and ‘Evangeline’, the difference in their expression kinetics could play an important role in the differential adventitious rooting of the two cultivars in response to 1-mcp.
- ❖ Considering the cascade of physio-biochemical processes ethylene is involved in plant, it is likely that the genes involved in adventitious rooting in response to the ethylene antagonist are many more than those attributed to ethylene-related processes described in the present investigation. This calls for highthroughput sequencing and gene expression strategies to identify more novel genes to understand the physiological and molecular aspects of ethylene-adventitious regulation, and for future genetic manipulation in sweetpotato.

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## Vita

Michael Polozola II was born in Baton Rouge and has spent most of his adult life up to this point in the Baton Rouge/ Greenwell Springs area of Louisiana. His interest in plants blossomed from a shared hobby of camellia shows and propagation that he developed with his grandparents Sharon and Gerald Phares at a young age. He went on to become the youngest camellia judge in the country in his youth and his passion for plants flourished as he grew along with his plants.

In 2008, Michael started at LSU for his undergraduate education in Horticulture which he completed in 2011. It was during this time that his interest in plants came to fruition and he learned the joys of many facets of Horticulture. He started his graduate education at LSU in 2012 and is currently a candidate for a Master's of Science in Plant Environmental and Soil Sciences to be awarded in August 2014.