THE IDENTIFICATION AND QUANTIFICATION OF LEAF MESOPHYLL OIL

BODIES DURING THE DEVELOPMENT OF NATIVE OR ADAPTED PLANT

SPECIES

A Thesis

by

JULIE CHRISTINE ROTHE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2010

Major Subject: Molecular and Environmental Plant Sciences

The Identification and Quantification of Leaf Mesophyll Oil Bodies During the

Development of Native or Adapted Plant Species

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Approved by:

Chair of Committee,	Dirk B. Hays
Committee Members,	Scott Senseman
	Jean Gould
	Michael A. Arnold
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ABSTRACT

The Identification and Quantification of Leaf Mesophyll Oil Bodies During the
Development of Native or Adapted Plant Species. (August 2010)
Julie Christine Rothe, B.A., University of Pennsylvania
Chair of Advisory Committee: Dr. Dirk B. Hays

Oil bodies have recently been identified in mesophyll cells of several species of angiosperms. These oil bodies are predicted to store triacylglycerides similar to storage oil bodies found in seeds of several plant species. Seed triacylglyceride oil is a common feedstock used for production of biofuels. This study examines the production and composition of these oil bodies in four plant species to determine if leaf oil could be used as a new feedstock for biofuel production. The species studied were species predicted to grow well in different climatic regions of Texas, including regions of marginal land with environmental stress: *Agastache foeniculum* (Pursch) Kuntze (Anise Hyssop), *Asclepias incarnata* L. (Swamp milkweed), *Cynara cardunculus* L. (Cardoon), and *Helianthus maximiliani* Schrad. (Maximilian sunflower). Leaf oil body production was monitored throughout the entire plant over four months of development for each species. Also, extractable oil per leaf dry weight was monitored over development. Extracted oil from each species was separated using thin-layer chromatography (TLC) to determine the lipid composition of leaves from each species and to determine oil body composition. The main results of this research showed that in each plant species oil bodies accumulated in the leaves as the leaves senesced. Also, the amount of extractable lipids from each species varied over time. However, there were few correlations between the presence of oil bodies in leaves and the total amount of extractable lipids from leaves, suggesting that oil bodies are poor predictors of extractable lipids in leaves.

DEDICATION

To my family and friends, particularly my father, William Rothe (1946-2009) who passed from Earth to be in eternity with God, our Father and our Lord Jesus Christ, during the course of my research for this thesis.

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Many thanks also go to my brothers and sisters of my church family from Fellowship Church here in College Station. You have been my best friends and personal support during the course of my time here in College Station.

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NOMENCLATURE

ASE   Accelerated solvent extraction

ANOVA   Analysis of variance

DAP   Days after planting

DGAT   Diacylglycerol acyltransferase

ER   Endoplasmic reticulum

FA   Fatty acids

GC-MS   Gas chromatography-mass spectrometry

PDAT   Phospholipid:diacylglycerol acyltransferase

TAG   Triacylglyceride

TLC   Thin layer chromatography

WS   Wax ester synthase

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CHAPTER I

INTRODUCTION

Plant oils are genetically inherited characteristics that vary widely in composition and properties among crop species (Facciotti and Knauf 1998). In 2006, global production of plant oil was approximately 127 million tons (FAOSTAT 2007) while animal fat production was only 22 million tons (Gunstone and Harwood 2006). Most plant oil is used for food and feed production and 14% is used for chemical purposes (Patel et al. 2006). There is an even greater variety of oils in wild plant species, such as *Vernonia* (Facciotti and Knauf 1998) or *Euphorbia* (Facciotti and Knauf 1998), but these are not manageable for widespread industrial use like current domesticated crops. At present, there are limited studies on the application of oils from wild plant species (Facciotti and Knauf 1998). However, some wild species show promise, and it might be possible to use recombinant DNA and gene transfer technology to implant isolated genes for oil from wild species to domesticated species (Facciotti and Knauf 1998).

Plant oils extracted from grains and seeds of agricultural crops have recently been used for production of biodiesel. Biodiesels are more favorable than diesel because biodiesel is a renewable resource, has a lower carbon monoxide emission, contains little to no sulfur or aromatic compounds, has a higher flashpoint, faster biodegration, and greater lubricity (Durrett et al. 2008). Also, biodiesel has been suggested to have double the energy return compared to the energy input for fertilizer, transportation, and conversion (Hill et al. 2006). However, mass production of biofuels from these crops has been problematic because of competition with other economic sectors such as food production over limited agricultural land and water availability and limited plant oil production (Durrett et al. 2008). Even the world's total plant oil production at a little over 120 million tons would only satisfy 80% of the USA's total diesel demand (USDA 2007; USDOE 2007). Yet, plant oil triacylglycerides (TAGs) are chemically the most similar

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This thesis follows the style of *Plant and Cell Physiology*.

biological product known to be able to replace fossil oil in the chemical industry (Dyer et al. 2008).

To solve some of the limitations of using seed oil as the primary resource for biodiesel production, the development of plants with higher levels of oils or hydrocarbons in leaf or vegetative tissue has been suggested as an alternative resource for biodiesel production to fruit or seeds (Durrett et al. 2008). One such possibility is to develop plants that accumulate carbohydrates and starch in their leaves or stems by genetically modifying the reduction of carbon export from leaves to new leaves, roots, and fruits (Durrett et al. 2008). This translocation of carbohydrates in plants is a source of total carbon and energy loss for the plant. However, preventing this translocation could be a potential problem because the accumulation of carbon would lead to inhibition of photosynthesis for leaves. As a resource for biodiesel, increasing carbohydrates in leaves is still not as promising as increasing oil content in leaves for several reasons: (1) Oils have twice as much energy content per carbon atom as carbohydrates; (2) Since oils have a higher energy content than carbohydrates they will be a larger sink for photosynthesis so that photosynthesis is not threatened; (3) One-third of the carbon is lost as CO_2 for the conversion of carbohydrates to ethanol but not for the conversion of oil to ethanol; (4) The plants own reducing power availability makes the conversion of carbohydrate to oil quite efficient (Durrett et al. 2008).

Another possibility for the use of vegetative matter for biodiesel production has been oil from perennial grasses (Heaton et al. 2004). Perennial grasses have high biomass yields with low fertilizer and other inputs. A perennial grass with oil content of 20-25% by weight has been predicted to be able to yield 3400 L of biodiesel per hectare which is three to six times the yield of canola or soybean (Durrett et al. 2008). In grasses, extraction and conversion of oil to biodiesel instead of lignocelluloses to ethanol, which is also possible, would require substantially less energy (Hill et al. 2006).

Other plant species with high reported levels of oil in plant tissue include: wild species of tomato *Lycopersicon pennelli* (Fobes et al. 1985) which accumulate a high percentage of acylated sugars, *Cyperus esculentus* (Zhang et al. 1996) which accumulates

oil in tubers, and *Tetraena mongolica* (Wang et al. 2007) which accumulates oil in phloem tissue of stems. Some species have also been shown to accumulate TAGs in leaves during senescence and stress (Kaup et al. 2002, Sakaki et al. 1990a, Sakaki et al. 1990b). As more transcription factors and regulatory networks for synthesis of oil in plant tissues are discovered, the possibility of genetically inducing or increasing oil production in plant tissues is greater. The species that tested positive for oil body content in leaf mesophyll cells by Lersten et al. (2006) add to the possible range of plants that could supply oil to generate more biodiesel.

The ***purpose of this research*** is to further elucidate the presence and chemical composition of oil bodies in plant leaf and stem tissue. The *significance of this purpose* is to see whether plant species with high amounts of oil bodies in leaf or stem tissue could be domesticated and the lipids in their leaves and stem be collected for biodiesel production. The central hypothesis of this study is that *leaf mesophyll oil bodies are composed of triacylglyceride (TAG) oils, the same oil extracted from seed for biofuel production. A high biomass species with high foliar TAG oil production could be domesticated and cropped as a significant new feedstock for biofuel production, particularly if the species is also heat, drought, and salinity tolerant.* Alternatively it could be possible to genetically modify a species to overproduce oil bodies in vegetative tissues and thus increase the amount of extractable lipid content from the species. Such technology would ideally be applied to fast-growing high-biomass species that are acclimated to grow in hot, dry, or saline conditions throughout Texas. Such a species would be suited to give a high yield product that could be of widespread agricultural importance. My hypothesis has been tested using the following specific objectives:

Objective 1. Determine the leaf oil body production of selected native Texas plant species. Species reported positive for oil body production by Lersten et al. (2006) and many other addition species, particularly ones predicted to grow well in Texas environments, will be surveyed.

 Objective 2. Determine whether leaf oil body concentrations change over developmental time in four plant species with high-, mid-, and low-level oil body

content using light microscopy. From the species tested in Objective 1, four species were selected for further analysis of leaf oil body production. Oil body production and lipid extraction levels were quantified from early vegetative development to flowering in younger and older leaves using free-hand sectioning and light microscopy as performed in Objective 1.

Objective 3. Determine whether total lipid leaf content changes during plant development in each species using solvent extraction and its correlation to quantified microscopy images. Accelerated Solvent Extraction (ASE) was used as a quick and efficient method to extract and determine the total lipids from dried leaf samples during the different developmental stages of the four species in 2.

Objective 4. Determine the different classes of leaf lipids present in each species, particularly during moments of high oil body production. Thin Layer Chromatography (TLC) was used to separate the different classes of leaf lipids extracted from each species. Identifying the lipid classes helped to determine any associations of lipid classes such as TAGs to oil bodies.

CHAPTER II

REVIEW OF LITERATURE RELEVANT TO THIS THESIS

Plant oil bodies

Lersten et al. (2006) recently reported the presence of stored oil bodies, likely TAGs, in mesophyll cells of leaves of some species of plants. The report claims that the presence of these oil bodies in leaves of some plant species has been overlooked and lost in past literature, yet if the presence of these leaf TAGs is true, they could serve as a potential new source of TAG oil for increased production of biodiesel. Surveying mostly species from the north-central U.S., Lersten et al. (2006) reported 71 species from 24 families (23.5% positive rate out of all species surveyed) that stained positively with Sudan IV dye for oil bodies in leaf mesophyll cells. Lersten et al. (2006) hypothesized that these are TAG oil bodies, similar to the TAG oil bodies found as energy storage in seeds, embryos, etc. What has not been determined is, is the total oil content in leaves of these species and chemical composition sufficient enough in quantity and quality to warrant their use as a novel feedstock source for production of biodiesel?

Oil bodies in plants are defined as spherical TAGs covered by a phospholipid monolayer with oleosin (oil body) proteins embedded in the outer monolayer (Hsieh and Huang 2004, Jolivet et al. 2004, Siloto et al. 2006). The membrane of oil bodies comes from the endoplasmic reticulum (ER) (Harwood and Russell 1984). The TAGs of oil bodies are derived also from the ER in specialized regions (Murphy 2005). Thus, it is hypothesized that oil bodies themselves come from the ER (Galili et al.1998). However, how TAGs become enveloped by an oleosin phospholipid monolayer membrane while leaving the ER remains unclear (Murphy 2005). Studies have thus far shown that oleosins are synthesized and integrated onto the ER and then are targeted to oil bodies via the ER. The TAGs themselves are synthesized between the lipid bilayer of the ER in specialized subdomains until a single layer of oleosins covers the entire oil body surface (Galili et al. 1998) (Figure 1). This proteinaceous membrane prevents oil bodies from fusing. This is advantageous since having many small oil bodies exposes more membrane surface area

to the catalytic enzymes localized in glyoxysomes to break down the membrane and TAGs as energy during seedling development (Facciotti and Knauf 1998, Harwood and Russell 1984). The membrane is composed of oleosins and phospholipids that help keep lipid bodies from fusing via steric hindrance and electronegative repulsion (Tzen et al. 1993, Huang et al. 1993).
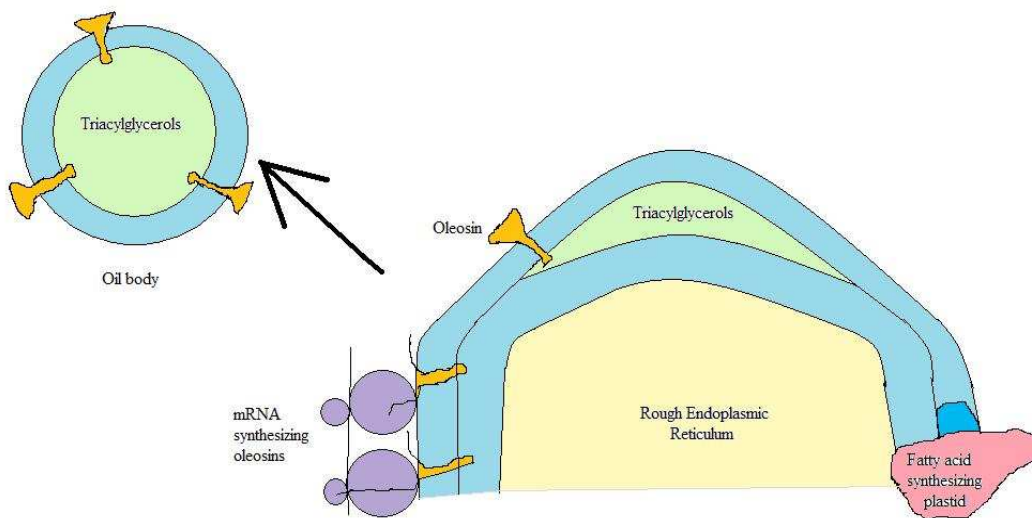
Fig. 1 Oil body production from the rough endoplasmic reticulum. Adapted from Huang et al. (1993). A schematic drawing of the production of an oil body at the rough endoplasmic reticulum (ER) of the cell through the enveloping of TAGs by an oleosin phospholipid monolayer.

Oil bodies are commonly acknowledged to occur in seeds, fruits, embryos, and endosperm in plants, but their occurrence in leaf or other plant cells has been lost in the literature. The size and composition of oil bodies has been reported to be species dependent but falls within a narrow range – commonly 0.65 to 2.0 µm in diameter with composition being of TAGs, phospholipids, and proteins (oleosins) (Tzen et al. 1993).

The TAGs in a seed provide a source of carbon skeletons and energy (ATP) for the germinating embryos as it starts to assemble carbohydrates, proteins, fats, etc. At seed germination there is increased lipase activity that releases fatty acids to be converted to sucrose which is transported to different tissues of the plant (Hitchcock and Nichols 1971). Accumulation of TAGs derived from the ER in seeds can be affected by environmental conditions that may cause changes in lipid synthesis and metabolism in the ER. Such possible environmental effects include light intensity, temperature, mineral nutrient deprivation, osmotic agents and the nature of carbon source (Murphy 2005).

The TAGs composing oil bodies are storage lipids (Facciotti and Knauf 1998). Other lipids in the cell are structural having a role in maintenance of membrane function (Facciotti and Knauf 1998). Structural and storage lipids share a common pathway for synthesis with the difference being whether diacylglycerol acyltransferase esterifies the third fatty acid in diacylglycerol or not (Facciotti and Knauf 1998). The source of production for TAGs is typically imported sucrose from photosynthates to the plant embryo (Figure 2) (Facciotti and Knauf 1998). Partitioning of photosynthates to either oil or protein is affected by both environment and plant genetics (Facciotti and Knauf 1998). In nitrogen rich soils, proteins will accumulate over oil, but in cold temperatures where nitrogen uptake is reduced, oil accumulation is common (Canvin 1965). However, the genetics of a plant variety can be modified so as to favor oil production over protein from photosynthates (Facciotti and Knauf 1998). Other possibilities to increase oil yield would be to increase the flux of photosynthates to the embryo instead of to other photosynthate sinks, such as the seed coat, or to increase the embryo's ability to enzymatically process photosynthates (Facciotti and Knauf 1998).

Photosynthates → sucrose → glucose-6-P → fructose-1,6-P → Glyceraldehyde-3-P

① glycerophosphate-dehydrogenase NAD.+

② dephosphorylase reductase

Glycerol

Dihydroxyacetone-P

aa ← phosphoglycerate

Glycerol-3-P

aa ← phosphoenolpyruvate

aa ← pyruvate → acetyl-CoA → fatty acids synthesis → structural lipids triglycerides

protein synthesis
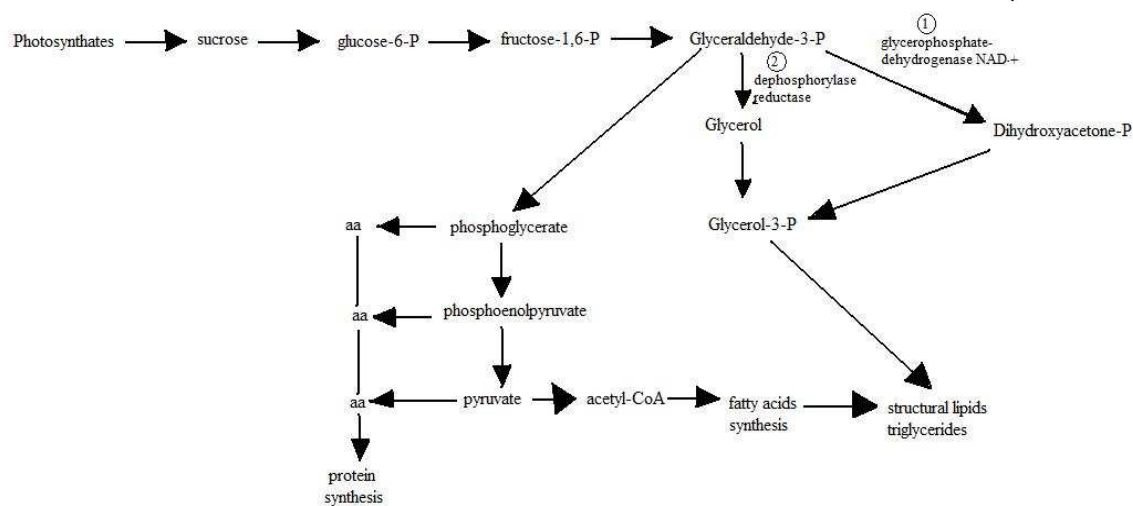
Fig. 2 Production pathway of structural TAGs from photosynthates going to the plant embryo. Adapted from Harwood and Russell (1984).

Some other plants have products similar to oil bodies. Jojoba, *Siimondsia chinensis*, (Harwood and Russell 1984) is one of the few plants known to have wax ester bodies accumulate as energy reserves in internal organs, and the wax bodies are morphologically similar to oil bodies (Harwood and Russell 1984). Steinke et al. (2001) reported the ability of lipase to catalyze the formation of wax esters similar to those of jojoba from seed TAGs. Also wax synthase genes have been transgenically expressed in *Arabidopsis* (Lardizabel et al. 2000) to produce transgenic seeds with a high production of wax esters (Lardizabel et al. 2000). Unrelated to plant DGAT (diacylglycerol acyltransferase) 1 and 2 family genes nor jojoba WS (wax ester synthase) genes, a WS/DGAT gene for the synthesis of wax ester and TAGs has been identified in *Acinetobacter calcoaceticus* (Kalscheuer and Steinbuchel 2003); by manipulating the gene and its substrates in microbes, it should be possible to increase microbial production of TAGs and jojoba-like wax esters of interest (Kalscheuer and Steinbuchel 2003).

Oils in leaf tissue

Oils acknowledged to occur in plant tissue are volatile oils and neutral oils. Volatile oils are terpenes that are isomeric hydrocarbons of the form $C_{10}H_{16}$ and are generally fragrant and volatilize upon exposure to air. Additionally, some terpenes are thought to function in reducing membrane permeability during heat stress. Neutral oils in contrast are TAGs and are not volatile. These TAGs can occur as oil bodies and are found in seeds, fruits, embryos, and endosperm as mentioned.

Harwood and Russell (1984) made a table of the changes in lipid content of a plant during development (Table 1). Included in these plant tissue lipids are structural lipids. According to Harwood and Russell (1984), in leaves some 70% of total proteins and 80% of total lipids are in chloroplasts. Mature leaves will have a constant lipid composition. However flowering and fruiting will bring about changes in leaf lipids. Leaf senescence will bring about a large decrease in lipid components of chloroplasts (Harwood and Russell 1984). In seed development, there is a slow increase of lipids in seeds for the first 10-30 days; then, for the next 2-5 weeks there is a rapid accumulation of lipids; at seed maturity there is only a minor increase in leaf lipids (Harwood and Russell 1984).

Table 1 The changes in lipid content and metabolism of a plant over developmental time. Adapted from Facciotti (1998).

Period	Lipid content and metabolism changes
Germination	Seed composition reflects stored lipid; may be 90% of seed dry weight in oil-rich seeds; cuticular lipids and waxes produced first, then leaf lipids, especially those for chloroplast membrane (MGDG [diacylgalactosylglycerol], DGDG [diacyldigalactosylglycerol], SQDG [diacylsulphoquinovosylglycerol], polyunsaturated fatty acids, trans-Δ3-hexadecenoic acid, chlorophylls, carotenoids); oil-rich seeds show disappearance of triacylglycerol reserves as glyoxysomes are present briefly
Mature plant	Steady-state metabolism takes place with typical leaf, shoot and root compositions maintained
Flowering	Many specialized pigments, e.g. carotenoids, produced for coloring petals
Seed development	In oil-rich seeds, lipid accumulation in three phases
Senescence	Rapid degradation of chloroplast acyl lipids and pigments

Other studies have looked at the different classes of lipids contained in leaf tissues. At first it was believed that the major class of leaf lipids was TAGs, but a study by Wintermans (1960) asserted that the major classes of lipids in leaves he collected from *Spinacia oleracea* (Wintermans 1960) (spinach), *Beta vulgaris* (Wintermans 1960) (sugarbeet), *Sambucus nigra* (Wintermans 1960) (elderberry), and *Phaseolus vulgaris* (Wintermans 1960) (bean) were galactosyl diglyceride, sulpholipid, phosphatidyl glycerol, phosphatidyl inositol, phosphatidyl choline, and phosphatidyl ethanolamine (Hitchcock and Nichols 1971). The study also found that green chloroplasts were particularly enriched in phosphatidyl-glycerol (GPG) and in mono- and digalactosyl lipids (Wintermans 1960). The study did find that yellow leaves compared to green leaves had significantly lower phosphatide-P lipids mainly due to a lack of phosphatidyl glycerol (GPG) (Wintermans 1960). Another study was published by Roughan and Batt (1969) that extended Wintermans work on lipid content of leaves from observing 4 species to observing 20 different species. Results showed that concentrations of different classes of lipids varied among plant species (Roughan and Batt 1969). Additional lipid

groups that were present were phophatidyl serine, phosphatidic acid, and phytoglycolipid; then there were also trace amounts of TAG, diglyceride, glucocerebroside, and sterol esters (Roughan and Batt 1969).

Other studies reporting TAGs in plant tissues

The primary study that re-introduced the idea of TAG oil bodies being present in leaves and stems of some plant species was that of Lersten et al. (2006) that has already been discussed. Lin and Oliver (2008) did a follow-up study on the TAGs in leaves of plants, surveying 25 species that were tested positive for oil bodies by Lersten et al. (2006). In addition to this survey, Lin and Oliver studied TAG levels of *Malus* (Lin and Oliver 2008) leaves from morning to evening to see whether photosynthetic activity affected leaf TAG levels; also TAG levels were analyzed from budding to senescence in *Malus*. Their results showed that TAG levels in *Malus* increased after a full day of photosynthesis. Also, TAG levels were shown to decrease in leaves after budding, but then TAG levels sharply increased after 3 months as the leaves senesced. Lin and Oliver were able to confirm a relative amount of TAG in 13 of the 25 species reported as positive for oil bodies by Lersten et al. (2006). Of these, *Cirsium vulgare* (Lin and Oliver 2008) and *Lactuca serriola* (Lin and Oliver 2008) showed the greatest amount of TAG over even *Malus*, and thus fatty acid profiles of these three species were made. These profiles show that fatty acid composition is similar for all three species and that these leaf TAGs are from a separate metabolic pool than membrane lipids, thus serving different physiological functions. Exceptions to this conclusion were TAGs that seemed to accumulate over leaf senescence since these TAGs had similar fatty acid profiles to chloroplastic galactolipids. These senescence-associated TAGs are not the same as the cytosolic oil body TAGS that are of interest. From the results of this study, it can also be hypothesized that since not all species reported positive for oil bodies by Lersten et al. (2006) also reported positive for detectable TAG amounts, then what has been stained as oil bodies in leaves may not be TAGs but another type of oil since the Sudan IV dye used by Lersten et al. (2006) can stain other lipids.

A few other studies have reported the presence of TAGs in non-leaf tissues of plants. TAGs have been reported in phloem sap of stems in *Brassica napus* (Madey et al. 2002) plants, stems of *Pinus silvestris* (Piispanen and Saranpaa 2002), phloem cells and xylem parenchyma of stems in *Tetraena mongolica* (Wang et al. 2007), and in the anther and tapetum of *Arabidopsis* (Hsieh and Huang 2004) and *Brassica* (Hsieh and Huang 2004). The latter are thought to serve the same function as in seed (i.e., energy for anther germination).

Another study on carnation *Dianthus caryophyllus* (Hudak et al. 2000) petals reported the presence of lipid-protein particles (20-250 nm) in the cytosol of cells (Hudak et al. 2000). Notable about these lipid-protein particles are that they contain the same classes of lipids as oil bodies – phospholipids, diacylglycerol, TAGs, free fatty acids, and steryl/wax esters. However the level of TAGs is lower than that of oil bodies since lipid-protein particles are not meant for storage. Rather, Hudak et al. (2000) hypothesizes that these lipid protein-particles bud off from the surface of the plasma membrane in regions where there are high free fatty acid and steryl/wax ester concentrations. The purpose for the budding off of these lipid-protein particles from the plasma membrane is hypothesized to be for the movement of H^+-ATPase catabolites and free fatty acids that might destabilize the membrane. Lipid-protein particles tested positively both for H^+-ATPase catabolites and phospholipids which suggests their origin from membranes. Since oil bodies also consist of the budding off of masses of lipids from within the cell, the formation of oil bodies from the ER may be similar in purpose or function as that of lipid-protein particles from the plasma membrane.

CHAPTER III

MATERIALS AND METHODS

Leaf sectioning

Many plant species were purchased as seed or whole plants and grown in the greenhouses at Texas A&M University. Species purchased as seed were planted in one-gallon pots filled with Metro-Mix 700 growing medium. Greenhouse conditions were controlled by a pad evaporative cooling system. Average temperatures during the winter were approximate 80 º F during the day and 73 º F during the night. Average temperatures during the winter were approximately 92 º F during the day and 75 º F during the night. Seeds and plants were watered every other day with water treated by reverse osmosis (RO) filtration. Species purchased include many of the same genus or species that tested positive for oil in the study by Lersten et al. (2006). Some additional species tested were high biomass or fast growing plants predicted to grow well under normal to high stress conditions such as in hot, arid, or saline regions of Texas. In addition to these greenhouse species, more species were collected from the field. Some species were collected from and around the area of College Station, Texas. Others were collected from the Kingsville and Corpus Christi areas of Texas. These species collected from the field were wild species with seeming high foliage and oil or fast-growth in various environments of Texas (see Table 2). From both greenhouse and field species, two to three leaves were collected from plants of each species to be sectioned. Leaves that were to be immediately sectioned were stored in 15-mL or 50-mL collections vials filled with water. Leaves that were not to be immediately sectioned were stored in 15-mL or 50-mL collection vials filled with fixative (0.1 M Potassium phosphate buffer at pH 6.8, 2.5% glutaraldehyde, 1.6% paraformaldehyde) for preservation. Collection vials were placed in an ice chest until being stored at 8-10 ˚C. The method for staining and identifying the presence of oil bodies in each species is as follows. Leaves were free-hand sectioned perpendicular to the surface of the leaf and mounted on a microscope slide. Section thickness corresponded to the thickness of the leaf with one to a few cell layer depth though one cell layer would have been preferred but difficult to obtain by free-hand

sectioning. Sections were stained with a few drops of Sudan IV (Lersten et al. 2006). Sudan IV dye preparation was adapted from Kay and Whitehead (1935) but without the dye-alcohol solution being boiled as a safety precaution since alcohol is highly flammable. First, a stock solution of Sudan IV dye was prepared by adding 1 g Sudan IV powder (Sigma Chemicals, St. Louis, MO) to 0.5 L of absolute alcohol (Texas A&M University West Campus Stockroom, College Station, TX). Then a second solution of 62.5 mL of absolute alcohol was mixed with 50 mL of distilled water. To this second solution, 87.5 mL of the stock solution of Sudan IV was added to make the final dye. A Zeiss Standard 14 microscope (Carl Zeiss Co., Oberkochen, Germany) was used to observe samples under bright field illumination at 200x or 500x magnification. Multiple digital images of leaf sections positively staining for oil were taken with a Nikon COOLPIX 4500 Digital Camera (Nikon Inc., Melville, NY) at approximately a 4x or 5.1x zoom on the camera. Images taken at a 500x microscope magnification and 4x camera zoom were approximately 360 micrometers by 260 micrometers. Images taken at a 200x microscope magnification and 5.1x camera zoom were approximately 600 micrometers by 460 micrometers. Lengths could be determined in sectioning images because they were compared to corresponding images of a slide micrometer taken at the same microscope magnification and camera zoom. Digital images were labeled and compiled as data. For each leaf that positively stained for oil, approximately 2 to 10 digital images were taken. These images were later reviewed to confirm the presence of oil bodies in the images. Select high quality images would undergo further analysis with the software program ImageJ (National Institute of Health). An approximately 5000-μm^2 area of each image was chosen, and the percent oil body surface area within the selected area was calculated. Comparisons of the percent leaf oil body surface area of images for each species were used as a quantitative method to determine significant differences among species in their leaf oil body production.

Table 2 All plant species surveyed for oil body production and their sources. Many species were ordered as seed or whole plants and grown in the greenhouses of Texas A&M University. A few species were sampled from the field or other outdoor area. The retailer of species from seed is included. Species are identified as either annual, biennial, or perennial. Species' native growth habitat is identified.

Seed	Prairie Moon Nursery Winona, MN	*Artemisia ludoviciana* Nutt. *(*White sagebrush*)* [perennial - USA, Mexico, Canada]*; Helianthus maximiliana* Schrad. (Maximilian Sunflower) [perennial, USA, Canada]
	Wildseed Farms Fredericksburg, TX	*Coreopsis lanceolata* L. (Tickseed) [perennial – USA, Canada]*; Rudbeckia hirta* L. (Black-eyed susan) [annual/biennial/perennial - USA, Canada]*; Rudbeckia amplexicaulis* Vahl (Clasping coneflower) [annual -USA]
	Territorial Seed Farms Cottage Grove, OR	*Cosmos bipinnatus* Cav. (Garden cosmos) [annual – Southwest USA, Mexico, Mesoamerica]*; Cynara Cardunculus* L. (Cardoon) [perennial – USA, Canada]*; Helianthus annuus* L. (Common sunflower) [annual - USA, Mexico, Western Canada]*; Helianthus debilis* Nutt. ssp. *cucumerifolius* (Torr. & Gray) Heiser (Cucumberleaf sunflower) [annual/perennial - South-central USA]*; Zinnia elegans* Jacq. (Zinnia) [annual – Mexico]; *Agastache foeniculum* (Pursch) Kuntze (Anise hyssop) [perennial – North USA; Canada]*; Mentha pulegium* L. (PennyRoyal) [perennial – Northern Africa, Macronesia, Western and middle Asia, Europe]
	Native American Seed Company Junction, TX	*Echinacea angustifolia* DC. *(*Narrow-leaf coneflower*)* [perennial – USA, Western Canada]*; Desmanthus illinoensis* (Michx.) MacMill. ex B. L. Rob. & Fernald (Illinois bundleflower) [perennial – USA]
	Everwilde Bloomer, WI	*Liatris ligulistylis* (A. Nelson) K. Schum. (Meadow blazing star) [perennial – USA, Western Canada]*; Silphium perfoliatum* L. (Cup plant) [perennial - USA, Eastern Canada]
	Dianne's Flower Seed Ogden, UT	*Tithonia rotundifolia* (Mill.) S. F. Blake (Mexican sunflower) [annual/perennial - Mexico, Mesoamerica]

Table 2 continued

Seed continued	Horizon Herbs Williams, OR	*Agastache rugosa* (Fisch. & C.A. Mey.) Kuntze (Purple giant hyssop) [perennial – Far east Russia, China, Eastern Asia, Indo-China]; *Mentha longifolia* (L.) Huds. (Horse mint) [Perennial – Africa, Temperate and tropical Asia, Europe];*Mentha piperita* L. (Peppermint) [perennial – Asia, Europe]; *Mentha spicata* L. (Spearmint) [perennial – Northern Africa, western Asia, Southeastern Europe]*; Pycnanthemum virginianum* (L.) T. Dur. & B.D. Jacks. ex B.L. Rob. & Fernald (American mountain mint) [perennial – USA, Eastern Canada]
	Kika de la Garza Plant Materials Center Kingsville, TX	*Ratibida columnaris* (Nutt.) Wooton & Standl. (Mexican Hat) [perennial – USA, Canada, Mexico]*; Argemone albiflora* Hornem. (Bluestem pricklypoppy) [annual/biennial – Central and eastern USA]
Field		*Ambrosia trifida* L. (Giant Ragweed) [annual - USA, Canada, Northern Mexico]*; Cirsium vulgare* (Savi) Ten. (Common thistle) [Biennial – Northern Africa, Temperate and tropical Asia, Europe]*; Brassica napus* L. X *B. campestris* L. (Canola) [annual/biennial – USA, Canada]; *Myrica pusilla* Raf. (Dwarf wax myrtle) [perennial – USA]; *Nerium oleander* L. (Oleander) [perennial – Southern Europe, Africa, Arabian Peninsula, Western Asia, China, India]; *Sorghum halepense* (L.) Pers. (Johnsongrass) [perennial – Northern Africa, Asia, India]; *Malus* Mill. hybrid (Crabapple) [perennial – USA, Europe, Asia]*; Spirea vanhouttei* (Briot) Zabel (Vanhoutte spirea) [perennial - Europe, Asia]

Table 2 continued

Whole plants	Eurybia divaricata (L.) G. L. Nesom (White wood aster) [perennial – USA, Eastern Canada]*; Helianthus tuberosus* L. (Jerusalem artichoke) [perennial – USA, Canada]*; Solidago rugsoa* Mill. (Rough goldenrod) [perennial – USA, Eastern Canada]*; Verbesina microptera* DC. (Crownbeard) [perennial – South USA]*; Euonymus atropurpureus* Jacq. (Eastern wahoo) [perennial – USA, eastern Canada]*; Euonymus alatus* (Thunb.) Siebold (Burning bush) [perennial – China, Eastern Asia]*; Euonymus americanus* L. (Strawberry bush) [perennial – USA]; *Hydrangea macrophylla* (Thunb.) Ser. (Bigleaf hydrangea) (perennial – China, eastern Asia, India]; *Sambucus nigra* L. (Elderberry) [perennial - Europe, Northern Africa, Temperate Asia]*; Viburnum cinnamomifolium* Rehder (Cinnamon leaf viburnum) [perennial – China]*; Viburnum opulus* L. (High cranberry) [perennial – USA, Canada, Temperate Asia, Europe]*; Viburnum propinquum* Hemsl. (Taiwanese viburnum) [perennial – China, Taiwan, Philippines]*; Viburnum plicatum* Thunb. *var. tomentosum* Miq. (Shasta viburnum) [China, Japan, Taiwan]; *Weigela florida* (Bunge) A. DC. (Old fashioned weigela) [perennial – China, Japan, Korea]; *Baptisia alba*(L.) Vent. (White wild indigo) [perennial – USA]*; Baptisia tinctoria* (L.) R. Br. (Yellow false indigo) [perennial – USA, Eastern Canada]*; Indigofera heterantha* (Himalayan indigo); *Asclepias incarnata* L. (Swamp milkweed) [perennial – USA, Canada]*; Asclepias tuberosa* L. (Butterfly milkweed) [perennial – USA, Northern Mexico]; *Monarda didyma* L. (Scarlet beebalm) [perennial – Eastern USA]*; Euphorbiaamygdaloides* (L.) x characias (L.) (Cushion spurge) [perennial - Mediterranean, Southern Europe]*; Euphorbia niciana* x *nicaeensis* All. (Blue haze) [perennial – Africa, Europe]*; Euphorbia stygiana* H. Schäf. (Honey spurge) [perennial - Europe]

Determination of leaf oil body levels over plant development

The criteria for choosing each species that was characterized in more detail were:

1) The species needed to germinate and grow from seed to a sizeable plant within 3 or 4 months from planting.
2) The species needed to be high in foliage.
3) The species needed to survive in variable climatic conditions, particularly stress conditions such as drought or heat.

From these criteria, four species were chosen of differing levels of oil body production: either low-, mid-, or high level oil body production. Oil body production levels were compared using the images collected in experiments addressing Objective 1. The species chosen were *Asclepias incarnata*, *Helianthus maximiliani*, *Agastache foeniculum*, and *Cynara cardunculus*.

Seeds from each of the chosen species were planted and grown in the greenhouse. Seeds were planted in 1.593-gallon pots filled with Metro-Mix 700 growth medium (Sun Gro Horticulture Canada Ltd., Bellevue, WA). Fifty days after planting, plants were moved from 1.593-gallon pots to 10.664-gallon pots. Thirty-four days after planting (DAP), leaves were collected from five plants of each species. For each of these plants, a basipetal, intermediate, and acropetal leaf of either a stem or branch was collected so that three leaves were collected of different sizes and maturation. The basipetal, intermediate, and acropetal leaves collected from each whole plant were selected by the visual estimation of the basipetal leaves to be the oldest and acropetal leaves to be youngest. Approximate sizes for the leaf types collected from each species towards the end of the research time period are as follows: *Agastache foeniculum*: acropetal – 2.4 cm length and 1 cm width; intermediate – 3.0 cm length and 1.9 cm width; basipetal – 5.2 cm length and 3.2 cm width. *Asclepias incarnata*: acropetal – 5.0 cm length and 0.5 cm width; intermediate – 8.6 cm length and 1.4 cm width; basipetal – 12.5 cm length and 2.2 cm width. *Cynara cardunculus*: acropetal – 9.0 cm length and 1.8 cm width; intermediate – 42.5cm length and 7 cm width; basipetal – 53.5 cm length and 13.9 cm width. *Helianthus*

maximiliani: acropetal – 4.8 cm length and 1.1 cm width; intermediate – 6.6 cm length and 1.3 cm width; basipetal – 8.1 cm length and 2.1 cm width. Five plants from each species were sampled to ensure diversity and ample data at each time point.

Each leaf collected was sectioned and stained according to the method previously described in Objective 1. Digital images with a Nikon COOLPIX 4500 Digital Camera (Nikon Inc., Melville, NY) were taken of each leaf section. A method was developed to uniformly try to quantify the level of oil bodies in leaf sections of plant species through image analysis. Such a method would make a numeric comparison of oil body production levels among species possible. Also this method allowed the comparison of leaf image oil body levels to leaf lipid solvent extraction levels as described for Objective 3 for each plant species. The method is as follows: From the digital images of each leaf section, a representative image was selected for analysis with the imaging software ImageJ (National Institute of Health) to quantify the amount of oil bodies present in a given leaf cross-section area. To quantify, the surface area of oil bodies was measured in an area of each image representing 5000-μm^2 of leaf surface, and then the percent surface area oil bodies over the 5000-μm^2 leaf surface area was calculated. From these calculations, an average oil body surface area for images of acropetal, intermediate, and basipetal leaves of each plant species was determined. Most of the images analyzed were taken with a 500x microscope magnification and a 4x camera zoom that produced images of 360 micrometers by 260 micrometers.

The same method was repeated 58, 83, and 103 DAP. During this period, some species completed a full life cycle while others grew to be sizeable plants but had not flowered. For example, *Helianthus maximiliani* had already started flowering before the sampling date of 58 DAP. *Agastache* foeniculum had started budding by 83 DAP and had started flowering by 103 DAP. Neither *Asclepias* incarnata nor *Cynara* cardunculus reached budding or flowering stage during this research time frame. During this period of development for the plants though, leaf oil body variation in each species could be recognized over time.

Determination of total lipid leaf content with ASE

As leaves were collected for sectioning and staining during plant development, leaves were also collected for accelerated solvent extraction (ASE). Five leaf samples were collected from five individual plants for each species for each developmental time extracted.

Tissue preparation

To prepare tissue for ASE, the tissue was dried in a freeze dryer (Freezemobile 12EL, VirTis, Gardiner, NY) . Dried tissue was ground with a coffee grinder (Hamilton Beach Custom Grind, Hamilton Beach Brands Inc., Washington, NC) until sample particles were finer than 2 to 3 mm. A weight of up to 1.5 g of ground sample was measured for each extraction sample.

Sample loading and lipid collection

Once the tissue was prepared, an 11-mL extraction cell with a 19.8-mm glass fiber filter (Dionex, Sunnyvale, CA) at the end of the cell was loaded with the sample mixed with the dispersing agent diatomaceous earth, or hydromatrix (Chem Tube Bond Elut Hydromatrix, Varian Inc., Palo Alto, CA). The remaining volume of the extraction cell was then filled with clean sand (Sand, washed and dried, Mallinckrodt Chemicals, Hazelwood, MO). Sealed extraction cells and collection vials were placed in the ASE.

Solvent choice and amount

Three solvents were used in this study: ethanol (Texas A&M University West Campus Stockroom, College Station, TX), hexane (Hexanes, Mallinckrodt Chemicals, Hazelwood, MO), and polar chloroform (Chloroform, Mallinckrodt Chemicals, Hazelwood, MO) with polar methanol (Methanol, EMD Chemicals Inc., Gibbstown, NJ) (C/M) (2/1, v/v). The amount of solvent used for ASE was 12 to 16 mL for every 10 g of solid. This translated to a solid-to-solvent ratio (g:mL) of 5:8 (0.625:1) or 5:6 (0.883:1) (Luthria 2004; Dionex 2004; Dionex 2006).

ASE conditions

Previous methodologies have been written by Dionex (2004, 2006) for ASE extraction of oil from oil seeds and phenolic acids from plant tissue. From these methodologies, the following conditions for performing ASE were selected: 1000 PSI (6.67 MPa) at 105 ºC for 5 min with a 100% flush volume and a 60 sec purge time for 1 static cycle. The ASE results showed that more than one static cycle extracted minimal extra lipids. From the collections vials, the resultant solvent-lipid mixture was pipetted into ten pre-weighed 1800- μL vials. Into each of these vials, 1500- μL was pipetted. The vials were placed in a Savant Automatic Environmental SpeedVac with Vapornet AES 2000 (GMI Inc., Ramsey, MN) to evaporate solvent off. The vials were re-weighed with just the lipids inside, and the total lipids extracted in the extraction cell was estimated by comparing the total weight of lipids from the 1500-μL samples of the solution to the total solution extracted from the cell which was normally 17 to 24 mL. Therefore, the average total lipids per a given dry weight of leaves from each species was calculated.

Determination of leaf lipid composition with TLC

A method adapted from Christiansen et al. (2007) was used for running thin-layer chromatography (TLC) on extracted lipid samples from moments of high oil body production to determine the different classes of leaf lipids present. Lipid samples were dissolved in chloroform and spotted onto 20 x 20-cm analytical normal phase silica gel TLC plates (Sigma Chemicals, St. Louis, MO). Lipid standards (Sigma Chemicals, St. Louis, MO) of monoglycerides, diglycerides, triglycerides, cholesterol, free fatty acids, alcohols, aldehydes, and tocopherol were also spotted on TLC plates. Plates were developed in a solvent system of hexane (Hexanes, Mallinckrodt Chemicals, Hazelwood, MO), diethyl ether (Sigma Chemicals, St. Louis, MO, and acetic acid (Acetic acid, glacial, Mallinckrodt Chemicals, Hazelwood, MO) (85:15:2) for approximately 1 h, when the solvent moved four cm below the top of the plate. After plates were dry, bands were stained with copper sulfate (II) (20 g of cupric sulfate crystals [Fisher Scientific

Company, Fair Lawn, NJ] in 200 mL of 8% phosphoric acid [EMD Chemicals Inc., Gibbstown, NJ]) followed by charring of the bands at 165 °C for 60 min in 1370 FM drying oven (VWR International, West Chester, PA). Charring made the cupric sulfate stained bands darker and readily visible on the plate.

CHAPTER IV

RESULTS

Species survey

First, numerous species (Table 3) from the study of Lersten et al. (2006) were grown in the greenhouses of Texas A&M University. From these plants, leaves were collected and sectioned. All these species tested positively for oil body production just as the research of Lersten et al. (2006) had reported. Sectioning these species also helped to refine sectioning and microscopy practices for this research. Figure 3 shows pictures of leaves sectioned from four of these species.

Table 3 The species surveyed for oil body production that were also tested by Lersten et al. (2006). All species tested positive for oil body production.

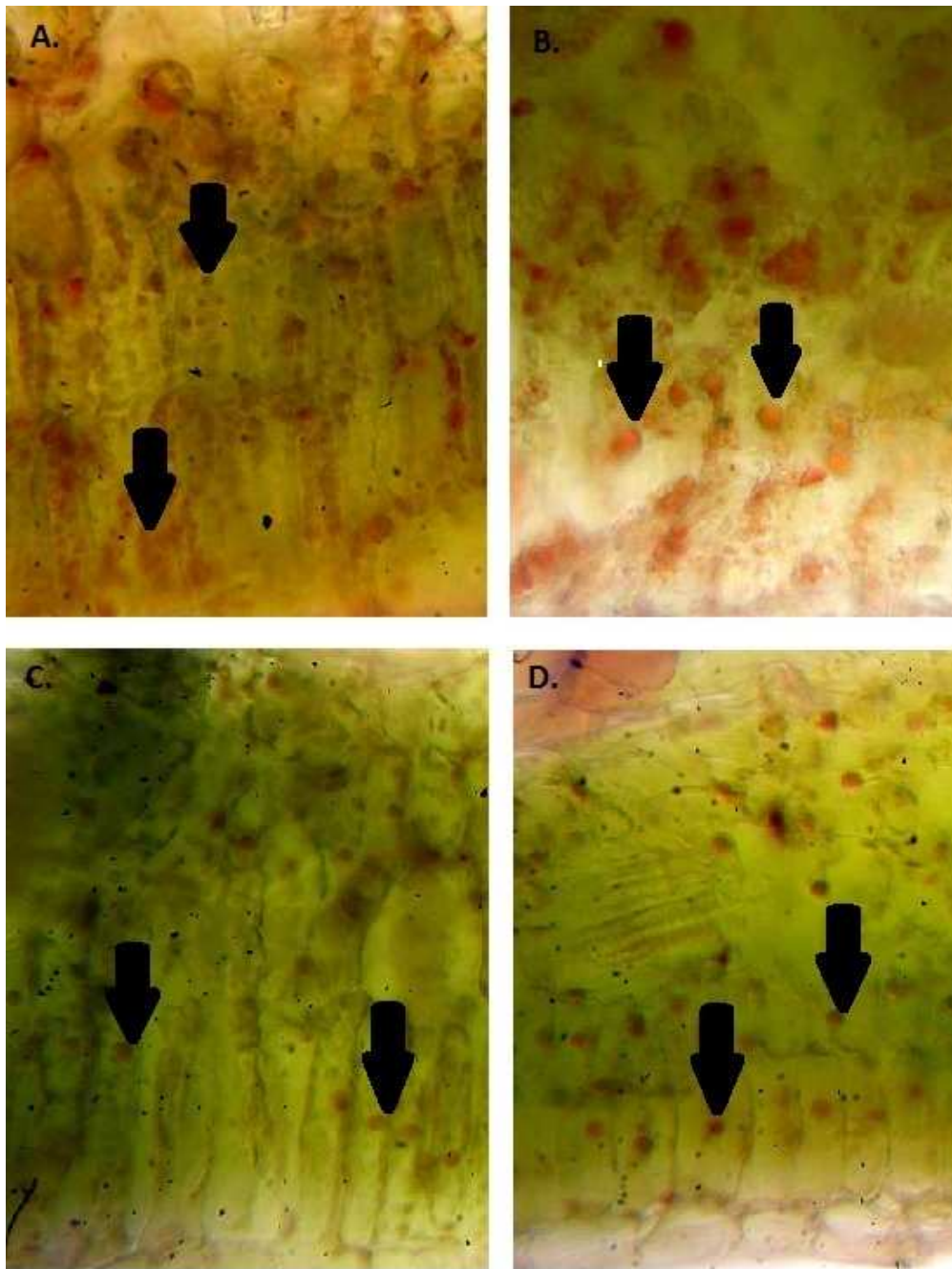| Order	Family	Genus and species
Asterales	Asteraceae	*Ambrosia trifida* **L. (Giant ragweed)***; Artemisia ludoviciana* **Nutt. (White sagebrush)***; Coreopsis lanceolata* **L. (Tickseed)***; Helianthus annuus L.* **(Common sunflower)***; Helianthus tuberosus* **L.(Jerusalem artichoke)***; Liatris ligulistylis* **(A. Nelson) K. Schum. (Meadow Blazing Star)***; Silphium perfoliatum* **L. (Cup plant)***; Solidago rugsoa* **Mill. (Rough goldenrod)***; Zinnia elegans* **Jacq. (Zinnia)**
Celastrales	Celastraceae	*Euonymus atropurpureus* **Jacq. (Eastern wahoo)**
Celastrales	Valerianaceae	*Weigela florida***(Bunge) A. DC. (Old fashioned weigela)**
Fabales	Fabaceae	*Baptisia alba* **(L.) Vent. (White wild indigo)***; Baptisia tinctoria* **(L.) R. Br. (yellow false indigo)**
Gentianales	Asclepiadaceae	*Asclepias incarnata* **L. (Swamp milkweed)***; Asclepias tuberosa* **L. (Butterfly milkweed)**
Malpighiales	Euphorbiaceae	*Euphorbia amygdaloides* **(L.) x characias (L.) (Cushion spurge)***; Euphorbia niciana* x *nicaeensis* **All. (Blue haze)***; Euphorbia stygiana* **H. Schäf (Honey spurge)**
Rosales	Rosaceae	*Malus* **Mill. hybrids (Crabapple)**

Fig. 3 Sudan IV stained leaf sections of species reported positive for oil body production by Lersten et al. (2006). Leaves were hand-sectioned. Magnification 250X. The four species shown are: (A) *Baptisia alba* (B) *Euonymus atropurpureus* (C) *Helianthus annuus* (D) *Zinnia elegans*

Several additional species were selected to be surveyed. Most species were ordered as seed or whole plants to be grown in the greenhouses at Texas A&M University. A few additional wild species were sampled from the College Station, TX, area. The additional species surveyed are listed in Table 4 with species positive for oil bodies bolded. Figure 4 shows pictures of leaves sectioned from four of these species.

Table 4 Additional selected species surveyed. Some species were predicted to grow well in the stress environments of native Texas land. Species positive for oil body production are listed in bold print.

Order	Family	Genus and species
Asterales	Asteraceae	***Cosmos bipinnatus* (Garden cosmos)*; Cynara Cardunculus* (Cardoon)*; Echinacea angustifolia* (Narrow-leaf coneflower)*; Euphorbia amygdaloides* (L.) x *characias* (L.) (White wood aster); *Helianthus debilis* Nutt. ssp. *cucumerifolius* (Torr. & A. Gray) Heiser (Cucumberleaf sunflower)*; Helianthus maximiliana* Schrad. (Maximilian sunflower)*; Rudbeckia hirta* L. (Black-eyed susan)*; Rudbeckia amplexicaulis* Vahl (Clasping coneflower)*; Tithonia rotundifolia* (Mill.) S.F. Blake (Mexican sunflower)**
Brassicales	Brassicaceae	*Brassica napus* L. X *B. campestris* L. (Canola)
Celastrales	Celastraceae	***Euonymus alatus* (Thunb.) Siebold (Burning bush)*; Euonymus americanus* L. (Strawberry bush)**
Cornales	Hydrangeaceae	***Hydrangea macrophylla* (Thunb.) Ser. (Bigleaf hydrangea)**
Dipsacales	Caprifoliaceae	***Sambucus nigra* L. (Elderberry)*; Viburnum cinnamomifolium* Rehder (Cinnamon leaf viburnum)**; *Viburnum propinquum* Hemsl (Taiwanese viburnum)
Fabales	Fabaceae	***Desmanthus illinoensis* (Michx.) MacMill. ex B. L. Rob. & Fernald (Illinois bundleflower)**; *Indigofera heterantha* Wall. ex Brandis(Himalayan indigo)
Gentianales	Apocynaceae	***Nerium oleander* L. (Oleander)**

Table 4 continued

Lamiales	Lamiaceae	***Agastache foeniculum* (Pursh) Kuntze (Anise hyssop)*; Agastache rugosa* (Fisch. & C.A. Mey. Kuntze) (Purple giant hyssop);** *Mentha longifolia* **(L.) Huds. (Horse mint);***Mentha piperita* **L. (Peppermint)*; Mentha pulegium* **L. (PennyRoyal)*;** *Mentha spicata* L. (Spearmint)*;* ***Monarda didyma* L. (Scarlet beebalm**)*;* *Pycnanthemum virginianum* (L.) T. Dur. & B.D. Jacks. ex B.L. Rob. & Fernald (American mountain mint)
Poales	Poaceae	*Sorghum halepense* (L.) Pers. (Johnsongrass)
Ranunculales	Papaveraceae	***Argemone albiflora* Hornem. (Bluestem pricklypoppy)**
Rosales	Rosaceae	*Spirea vanhouttei* (Briot) Zabel. (Vanhoutte spirea)
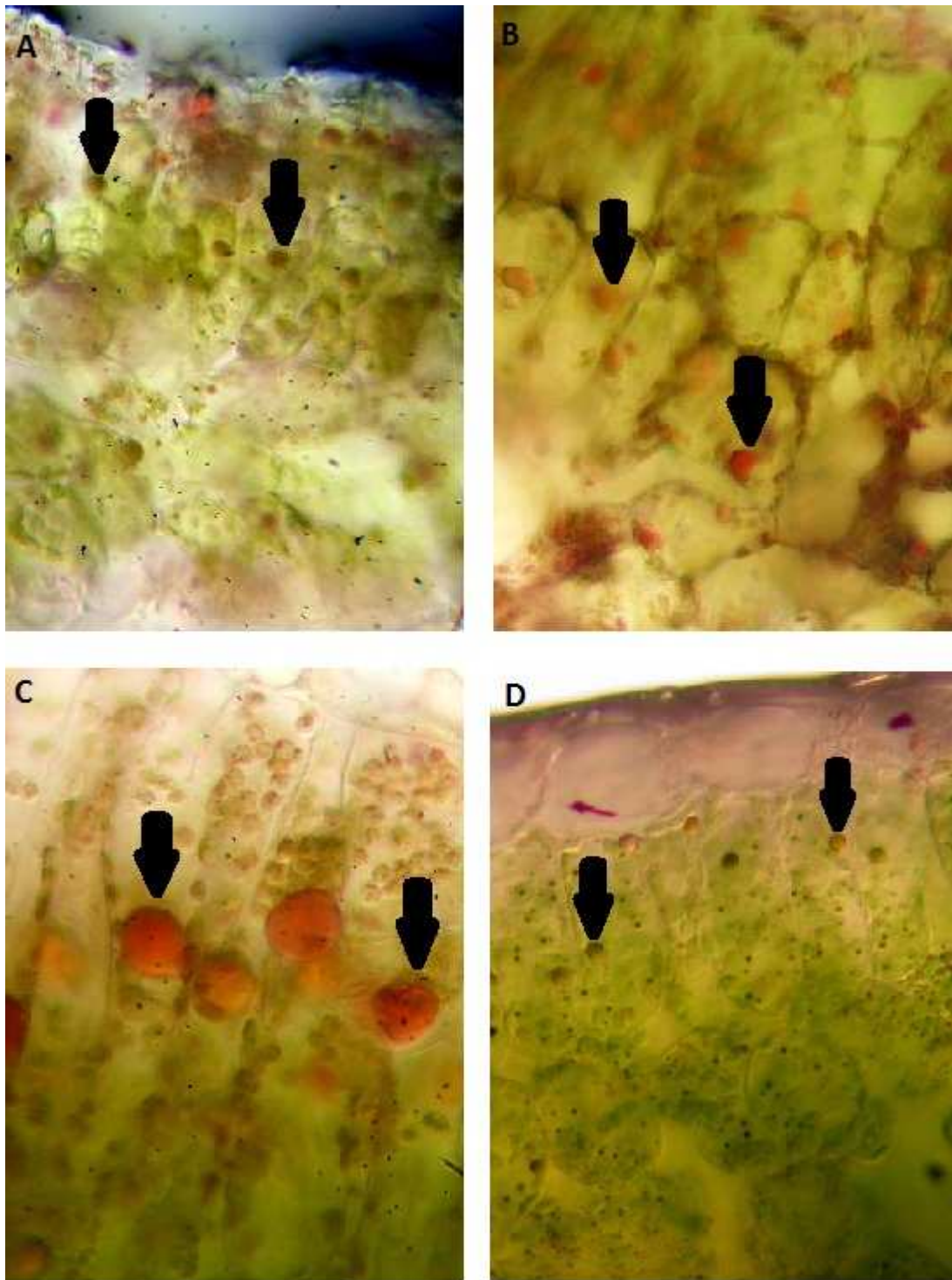
Fig. 4 Sudan IV stained leaf sections of additional selected species positive for oil body production. Leaves were hand-sectioned. Magnification 250X. The four species shown are: (A) *Euphorbia amygdaloides* (B) *Helianthus debilis* ssp. *cucumerifolius* (C) *Monarda didyma* (D) *Rudbeckia amplexicaulis*

Table 5 Additional species surveyed from a collection trip to the Kingsville-Corpus Christi areas. Species positive for oil body production are listed in bold print.

<u>Order</u>	<u>Family</u>	<u>Genus and species</u>
Asterales	Asteraceae	***Engelmannia peristenia* (Raf.) Goodman & C.A. Lawson (Engelmann's daisy)***; *Iva frutescens* L. (Jesuit's bark)**; *Ratibida columnaris* (Nutt.) Wooton & Standl. (Mexican Hat)***; *Verbesina microptera* DC. (Crownbeard)**
Fabales	Fabaceae	***Acacia angustissima* (Mill.) Kuntze (Prairie acacia);** *Dalea scandens* (Mill.) R.T. Clausen (Low prairie clover)
Fagales	Myricaceae	***Myrica pusilla* Raf. (Dwarf wax myrtle)**
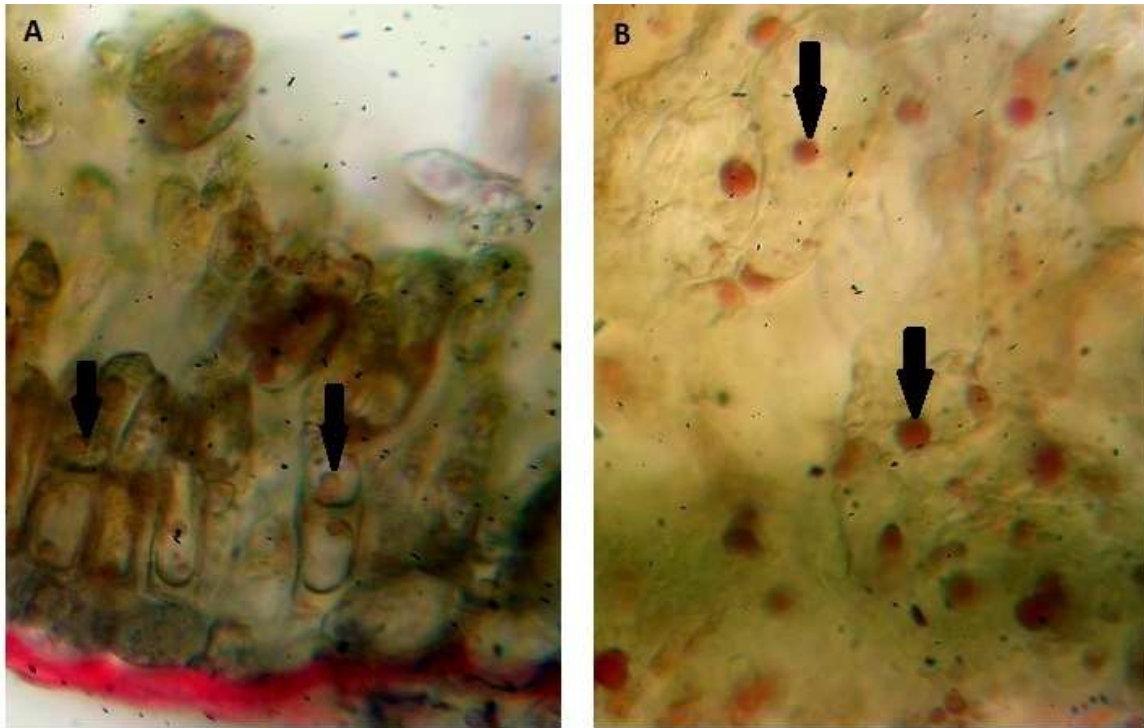
Fig. 5 Sudan IV stained leaf sections of two of the species positive for oil body production surveyed from a collection trip to the Kingsville and Corpus Christi areas of Texas. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 250X. The two species shown are: (A) *Myrica pusilla* (B) *Engelmannia peristenia*

Additional species were also sectioned from a collection trip to the Kingsville-Corpus Christi areas. The species sectioned from the collection trip are listed in Table 5 with species positive for oil bodies bolded. Figure 5 shows pictures of leaves sectioned from two of these species.

Species selection

From species surveyed, four species were selected for further study of their foliar oil bodies. The species were selected based on their growth patterns, foliage production, and ready adaptability to stressed conditions. Also species were chosen that from visual estimation of images were predicted to have significantly different oil body production from each other.

The four species chosen were *Agastache foeniculum*, *Asclepias incarnata*, *Cynara cardunculus*, and *Helianthus maximiliani*. The species are shown in Figure 6. These species grow readily from seed within two to three months from planting in a greenhouse environment and produce significant foliage. Each is adaptable to varying climates of Texas. These select species were studied to determine the variation in their leaf oil body production and extractable lipids during the course of their development.

Fig. 6 The four species selected for further study of their foliar oil bodies. Selection criteria included growth patterns, foliage production, and ready adaptability to land in an environment of stressful conditions. (A) *Agastache foeniculum* (B) *Asclepias incarnata* (C) *Cynara cardunculus* (D) *Helianthus maximiliani*.

Leaf oil body sectioning

Figures 7 and 8 show the results for leaf sectioning of each species. For each collection date Figure 7 shows the average percent oil body surface area measured in a 5000-μm^2 leaf surface area of ten to fifteen images each from a different whole plant. Also the standard deviation for each mean is given. Figure 8 shows the average percent oil body surface area measured in a 5000-μm^2 leaf surface area of images by acropetal, intermediate, and basipetal leaves. Acropetal leaves are younger than the basipetal leaves.

Figures 9 through 17 show images from the sectioning results. Two to three figures have been shown for each plant species, with figures separated by leaf position – acropetal, intermediate, and basipetal. Each figure shows an image from each collection date. These images show differences among species and among collection dates in foliar oil body production.
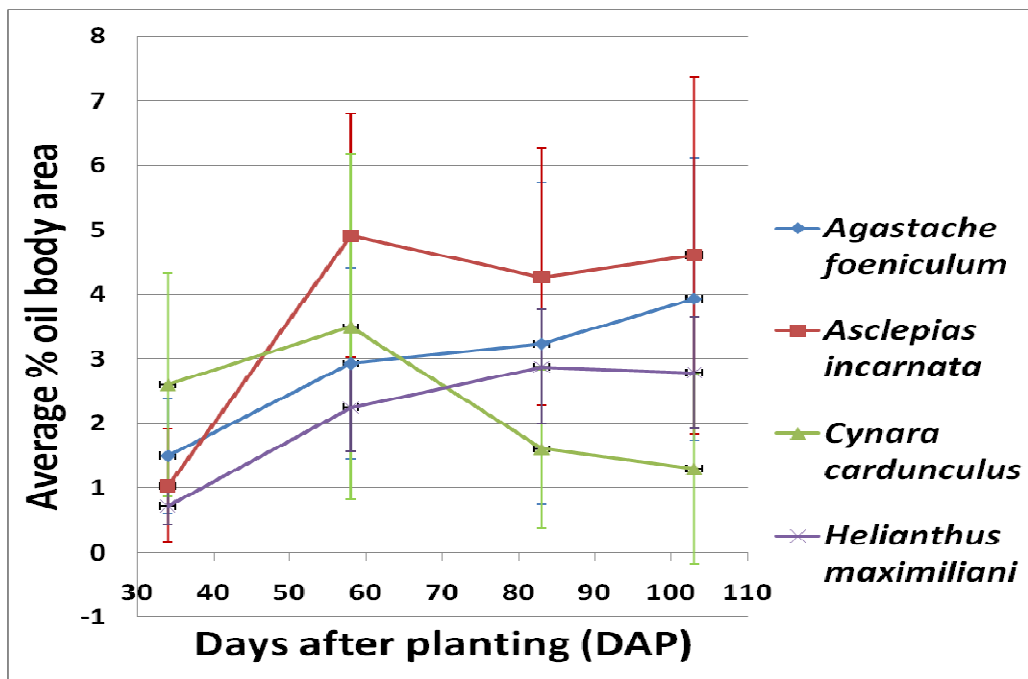
Fig. 7 Leaf sectioning results for plant species over developmental time. "Average % oil body area" is the percent surface area of oil bodies per 5000-µm^2 leaf surface area in an image, calculated using ImageJ (National Institute of Health). Each value is the average of ten to fifteen images, from a mixture of acropetal, intermediate, and basipetal leaves. Error bars are one standard deviation.
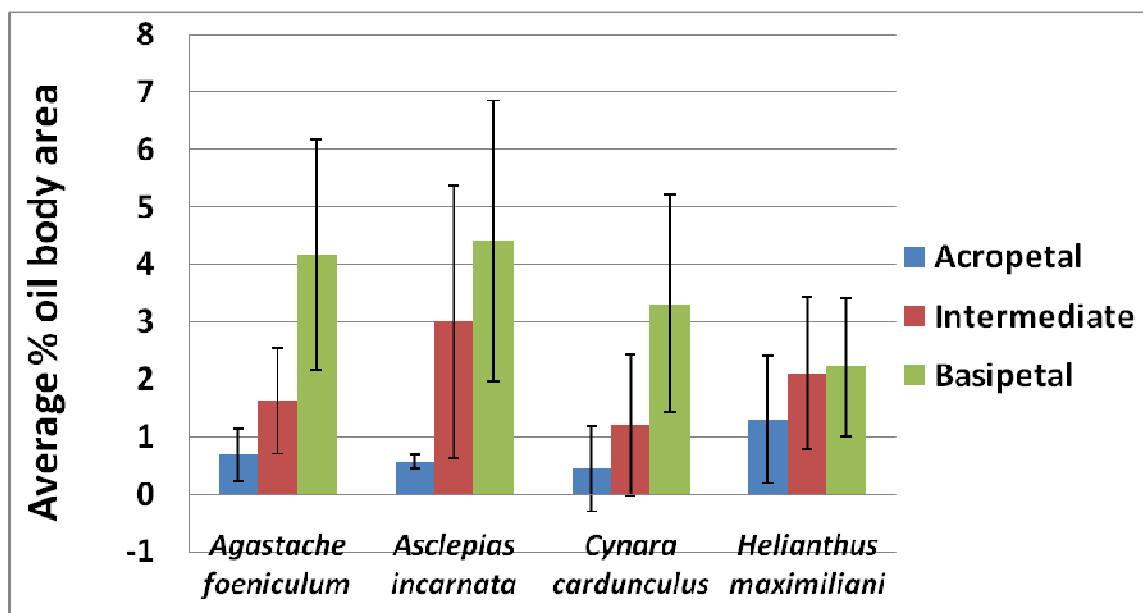
Fig. 8 Leaf sectioning results by leaf location on the whole plant for plant species. "Average oil body area" is the percent surface area of oil bodies per 5000-µm^2 leaf surface area in an image, calculated using ImageJ (National Institute of Health). Each value is the average of five images taken from each of the four collection dates, for a total of twenty images. Exceptions are *Agastache foeniculum* and *Asclepias* incarnata and *Cynara cardunculus* "acropetal" values which are each from normally only one image for each of the collection dates, for a total of four images. Error bars are one standard deviation.
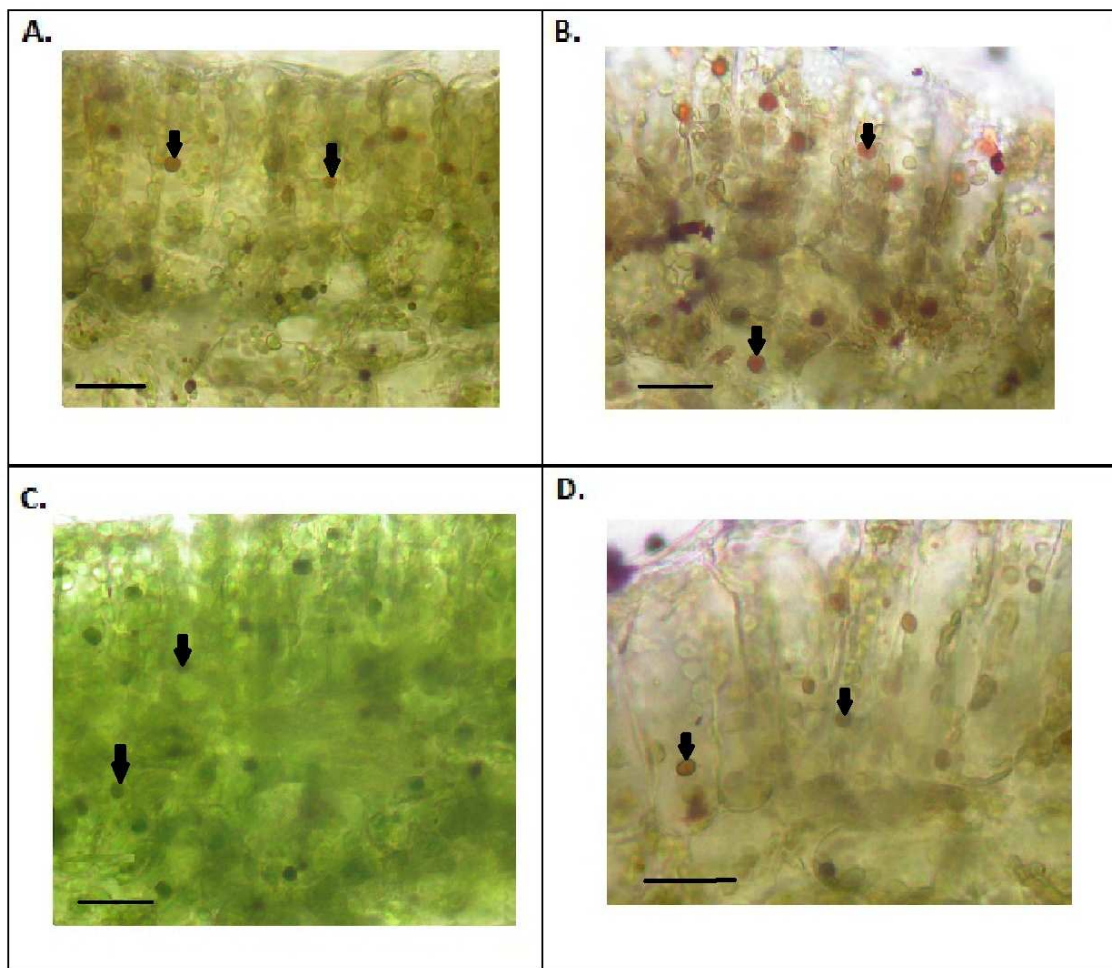
Fig. 9 Sectioned intermediate leaves stained with the lipid stain Sudan IV for oil bodies from *Agastache foeniculum*. A leaf from each collection time is given: (A) 36 DAP (B) 63 DAP (C) 84 DAP (D) 105 DAP. Scale shown is 20 μm.
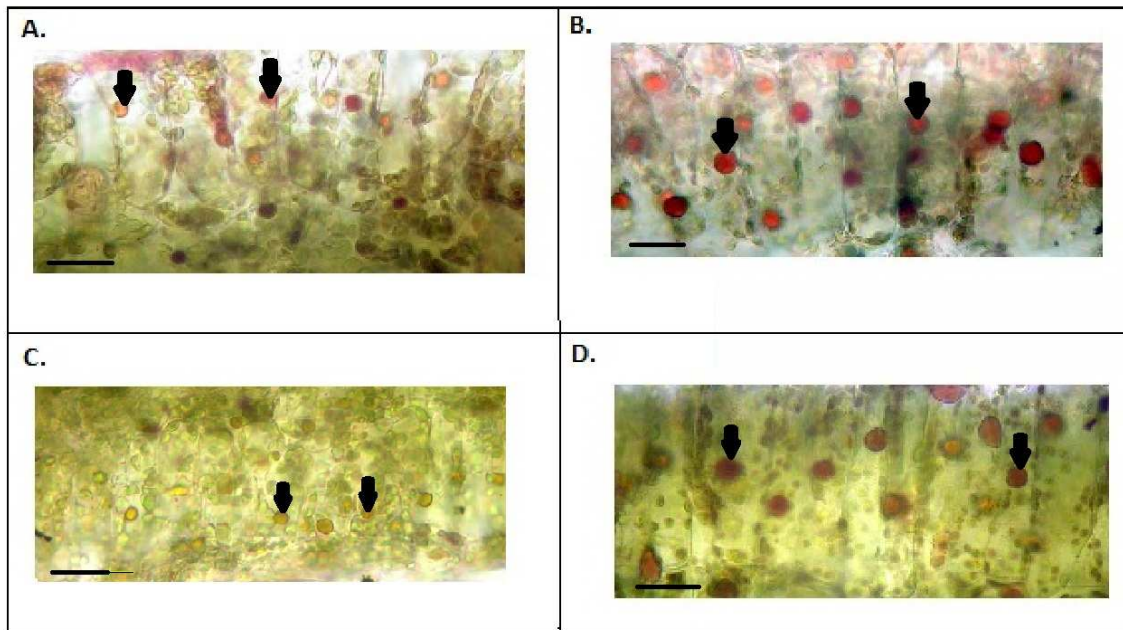
Fig. 10 Sectioned basipetal leaves stained with the lipid stain Sudan IV for oil bodies from *Agastache foeniculum*. A leaf from each collection time is given: (A) 36 DAP (B) 63 DAP (C) 84 DAP (D) 105 DAP. Scale shown is 20 μm.
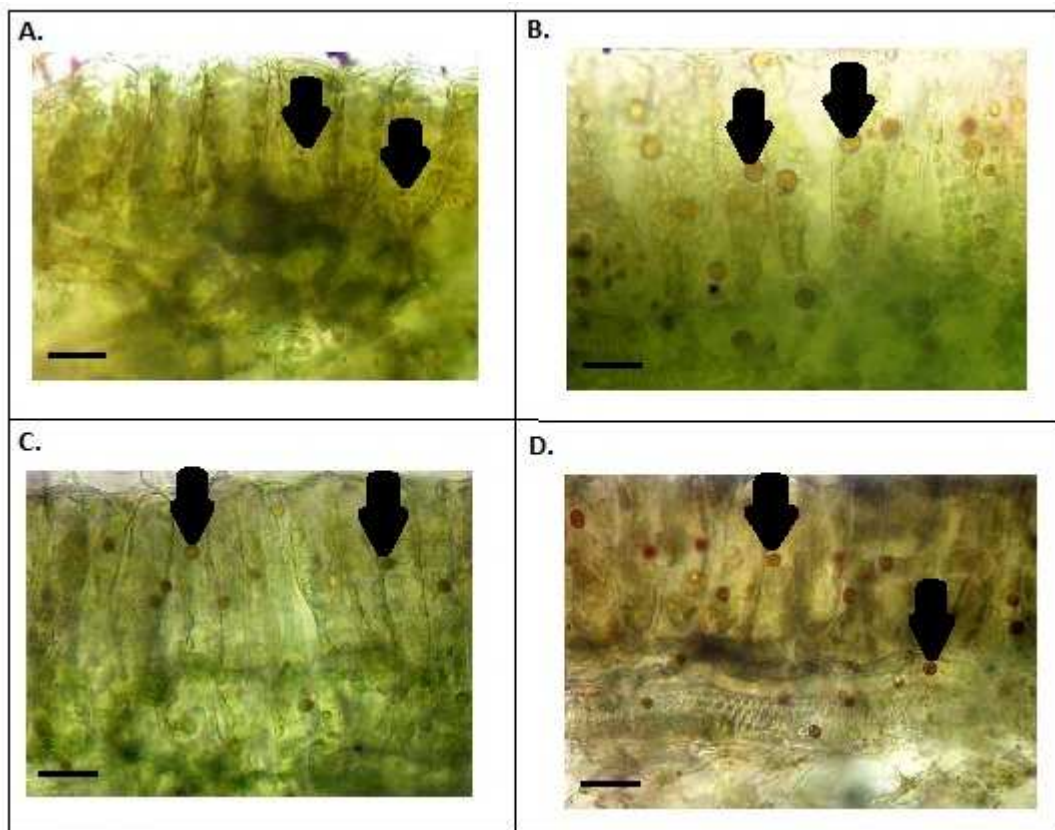
Fig. 11 Sectioned intermediate leaves stained with the lipid stain Sudan IV for oil bodies from *Asclepias incarnata*. A leaf from each collection time is given: (A) 36 DAP (B) 63 DAP (C) 84 DAP (D) 105 DAP. Scale shown is 20 μm.
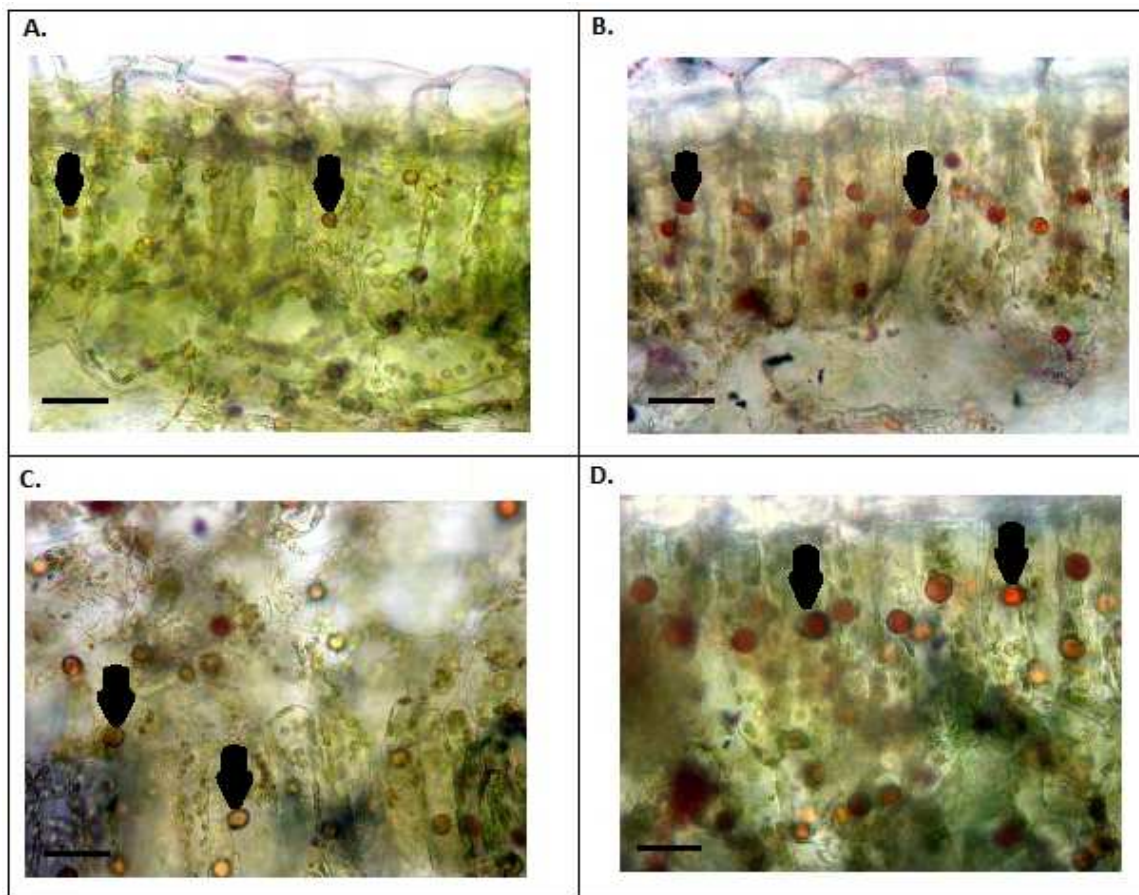
Fig. 12 Sectioned basipetal leaves stained with the lipid stain Sudan IV for oil bodies from *Asclepias incarnata*. A leaf from each collection time is given: (A) 36 DAP (B) 63 DAP (C) 84 DAP (D) 105 DAP. Scale shown is 20 µm.
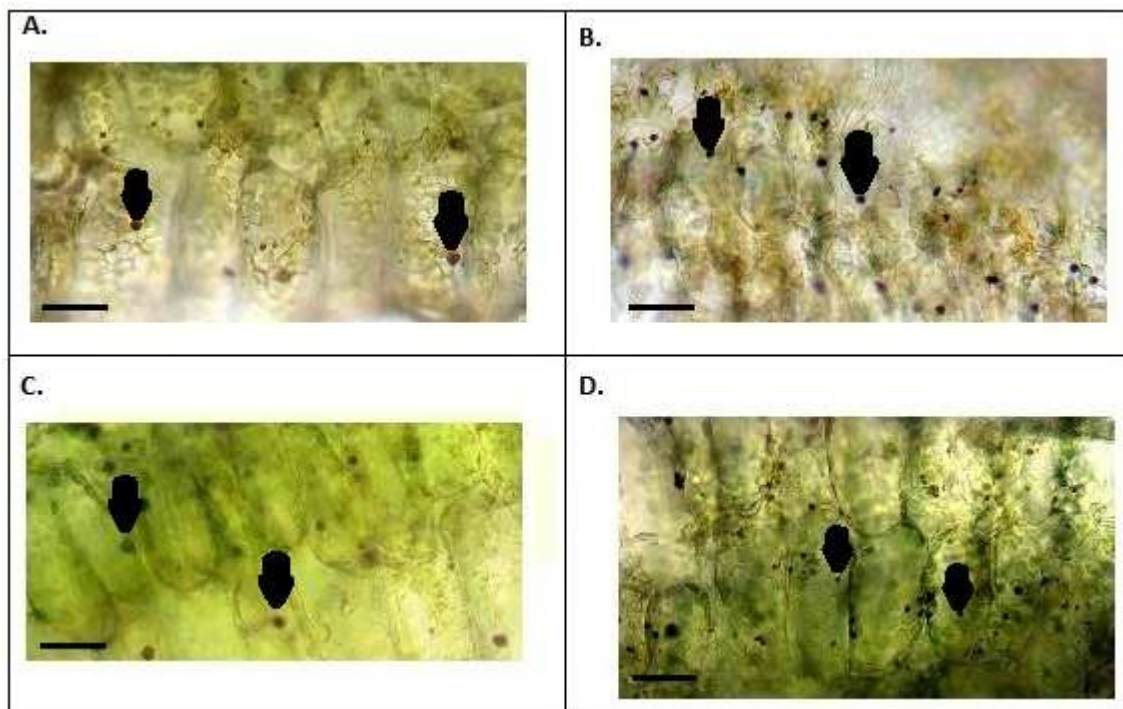
Fig. 13 Sectioned intermediate leaves stained with the lipid stain Sudan IV for oil bodies from *Cynara cardunculus*. A leaf from each collection time is given: (A) 36 DAP (B) 63 DAP (C) 84 DAP (D) 105 DAP. Scale shown is 20 μm.
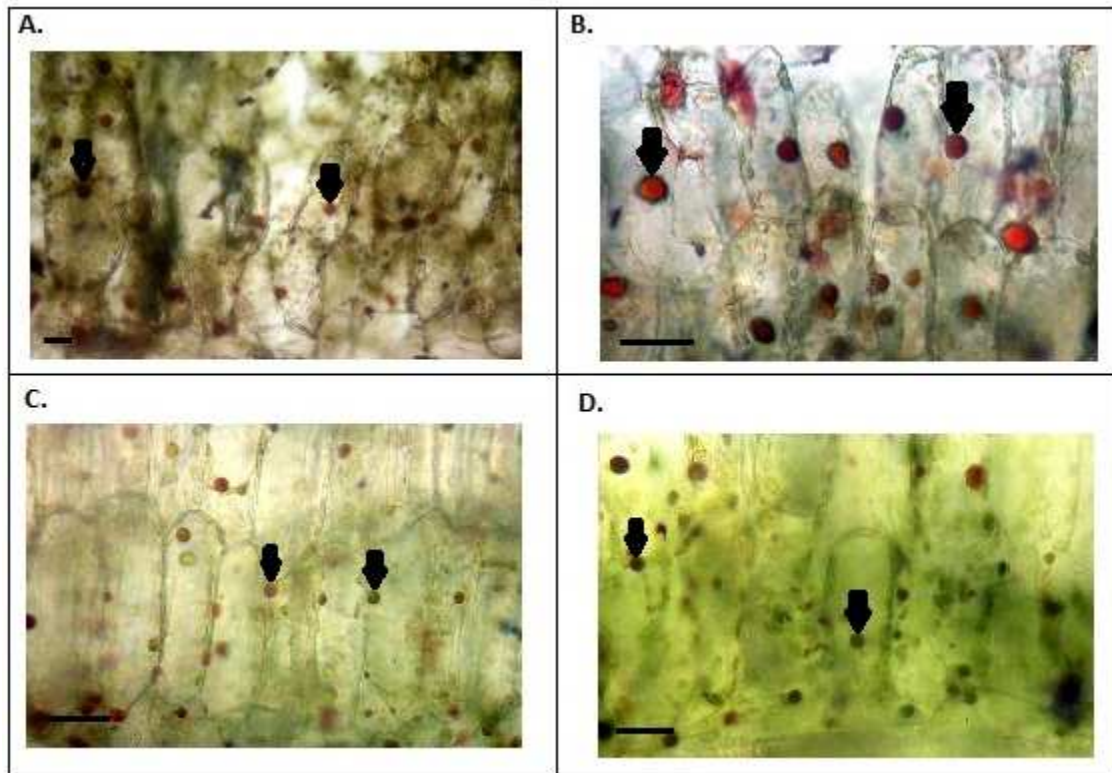
Fig. 14 Sectioned basipetal leaves stained with the lipid stain Sudan IV for oil bodies from *Cynara cardunculus*. A leaf from each collection time is given: (A) 36 DAP (B) 63 DAP (C) 84 DAP (D) 105 DAP. Scale shown is 20 µm.
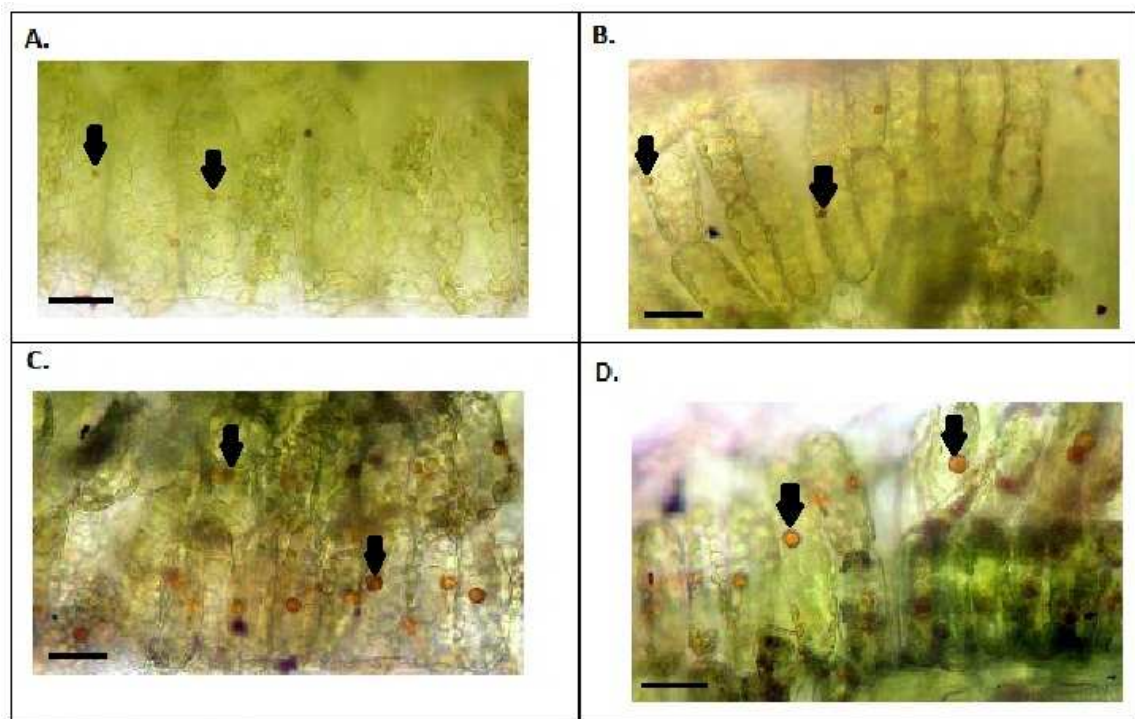
Fig. 15 Sectioned acropetal leaves stained with the lipid stain Sudan IV for oil bodies from *Helianthus maximiliani*. A leaf from each collection time is given: (A) 36 DAP (B) 63 DAP (C) 84 DAP (D) 105 DAP. Scale shown is 20 µm.
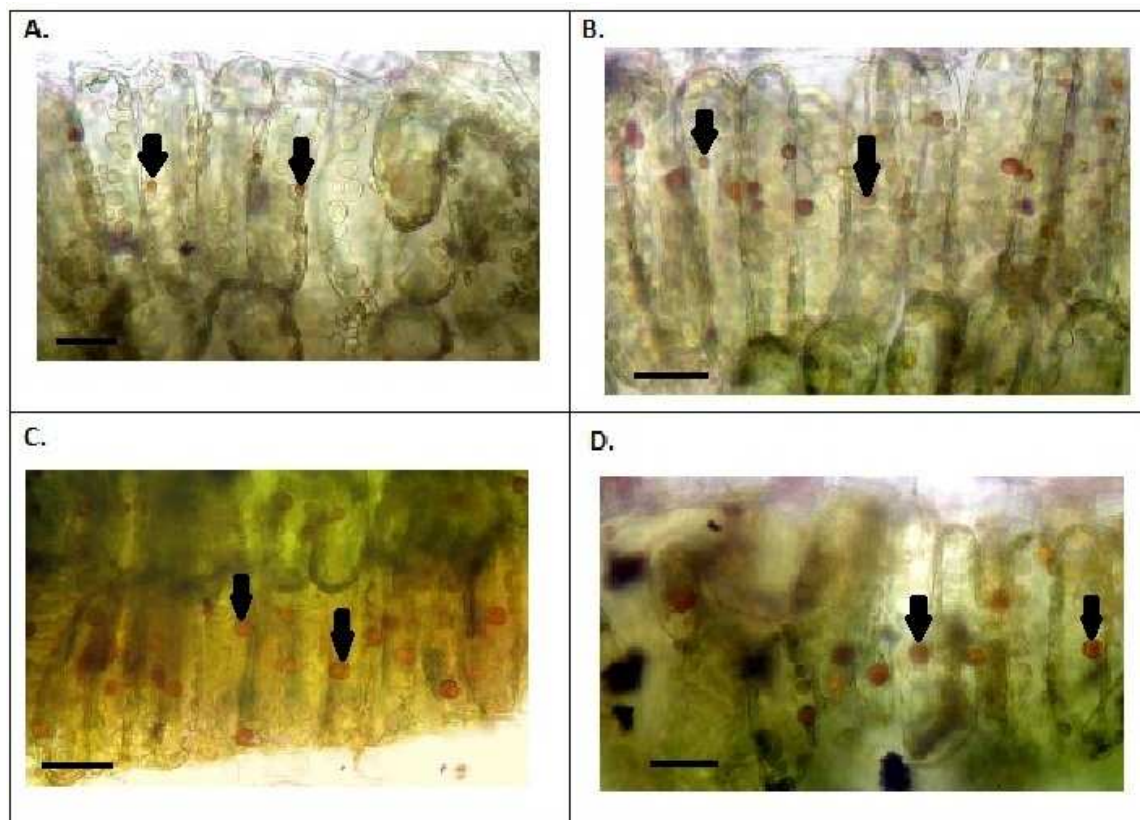
Fig. 16 Sectioned intermediate leaves stained with the lipid stain Sudan IV for oil bodies from *Helianthus maximiliani*. A leaf from each collection time is given: (A) 36 DAP (B) 63 DAP (C) 84 DAP (D) 105 DAP. Scale shown is 20 μm.
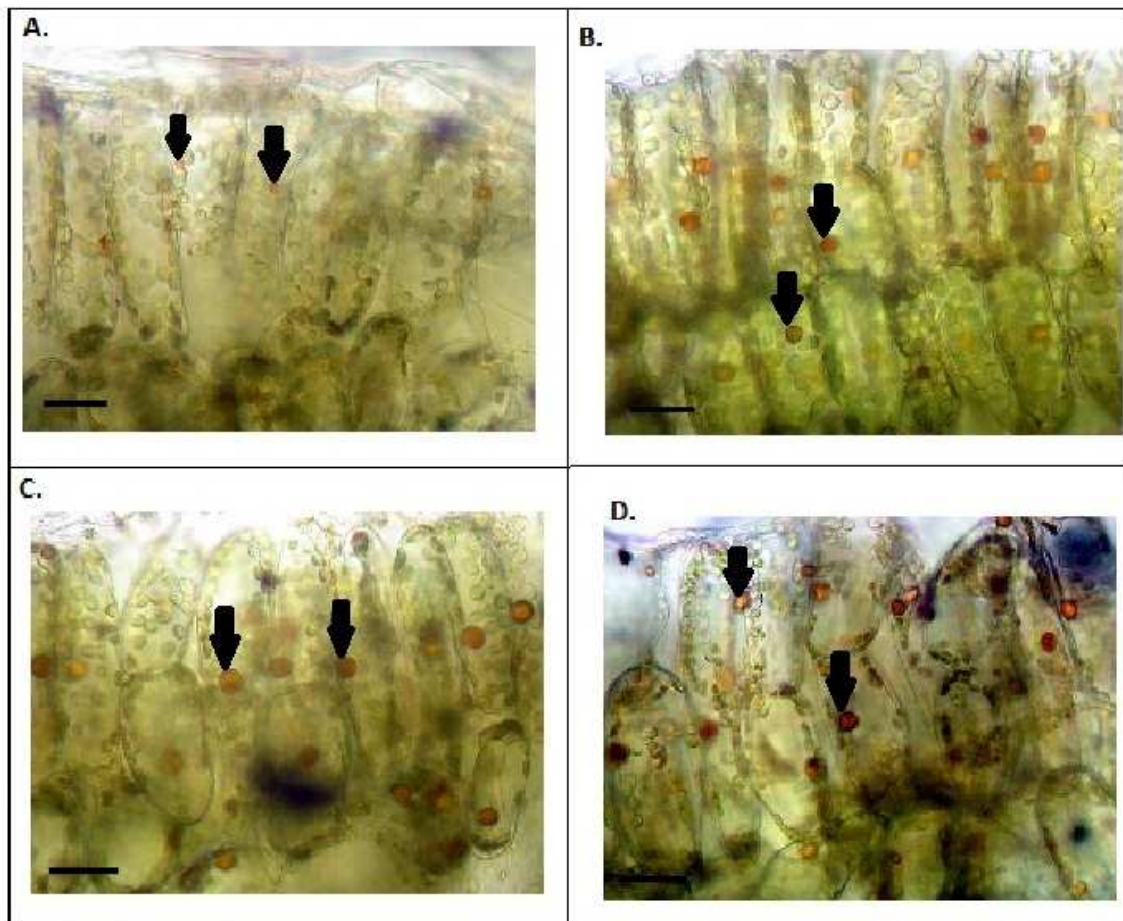
Fig. 17 Sectioned basipetal leaves stained with the lipid stain Sudan IV for oil bodies from *Helianthus maximiliani*. A leaf from each collection time is given: (A) 36 DAP (B) 63 DAP (C) 84 DAP (D) 105 DAP. Scale shown is 20 μm.

Statistical analysis

Sectioning results show upwards of 8 to 9% oil body surface area in images for some plant species. A three-way ANOVA test on time by plant species by leaf location did not show any significant interactions among all three of these factors in affecting oil body presence (P=0.2111) (Table 6). Two-way ANOVA tests were analyzed on leaf location by plant species, time by plant species, and time by leaf location to determine if interactions of these factors affected oil body presence. There were significant interactions between leaf location by plant species (P=0.0001) and time by plant species

(*P*=0.027) in affecting oil body presence. Time by leaf location was an insignificant interaction in affecting oil body presence (*P*=0.384).

One-way ANOVA tests were analyzed with time, plant species, and leaf location each as factors affecting oil body presence. All factors were considered fixed. The one-way ANOVA tests showed that time and leaf location were statistically significant factors for affecting oil body presence (*P*=0.003 and *P*=0.000, respectively) but did not show plant species as a statistically significant factor for affecting oil body presence (*P*=0.068).

Table 6 ANOVA for the factors and their interactions that would affect leaf oil body production. The dependent variable is the leaf oil body production that had been quantitatively identified through image analysis of leaf sections of each species.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	503.48[a]	47	10.71	5.76	0.0001
Intercept	501.40	1	501.40	269.53	0.0001
time	35.26	3	11.75	6.32	0.0001
leaf location	176.88	2	88.43	47.54	0.0001
species	13.52	3	4.51	2.42	0.068
time * leaf location	11.94	6	1.99	1.07	0.384
time * species	36.36	9	4.04	2.17	0.027
leaf location * species	53.24	6	8.87	4.77	0.0001
time * leaf location * species	42.78	18	2.377	1.28	0.211
Error	260.44	140	0.72		
Total	1934.08	188			
Corrected Total	763.92	187			

[a] R^2 =0.659 (Adjusted R^2 = 0.545)
LSD=0.5699 for plant species, LSD=0.5456 for time, and LSD=0.5733 for leaf location

Lipid extraction

The results of the leaf lipid variation in content for each plant species revealed common trends on a dry weight basis. The lipid percentage ranged from 1% to 9% oil when using hexane to extract, 7% to 25% oil when using ethanol to extract, and 12% to 23% oil when using chloroform/methanol (2:1) to extract (Figure 18). The average lipids extracted by ethanol from greatest to least among plant species was: *Cynara cardunculus* (24%), *Asclepias incarnata* (19%), *Agastache foeniculum* (16%), and *Helianthus maximiliani* (15%) (Figure 18). Then for hexane it was: *Asclepias incarnata* (7%), *Agastache foeniculum* (6%), *Cynara cardunculus* (6%), and *Helianthus maximiliani* (6%) (Figure 18). For chloroform/methanol (2:1, v/v), lipid percentage from greatest to least was: *Asclepias incarnata* (20%), *Cynara cardunculus* (17%), *Agastache foeniculum* (16%), and *Helianthus maximiliani* (14%) (Figure 18). Figure 19 shows these lipid extraction amounts for all solvents over time for each of the four plant species.
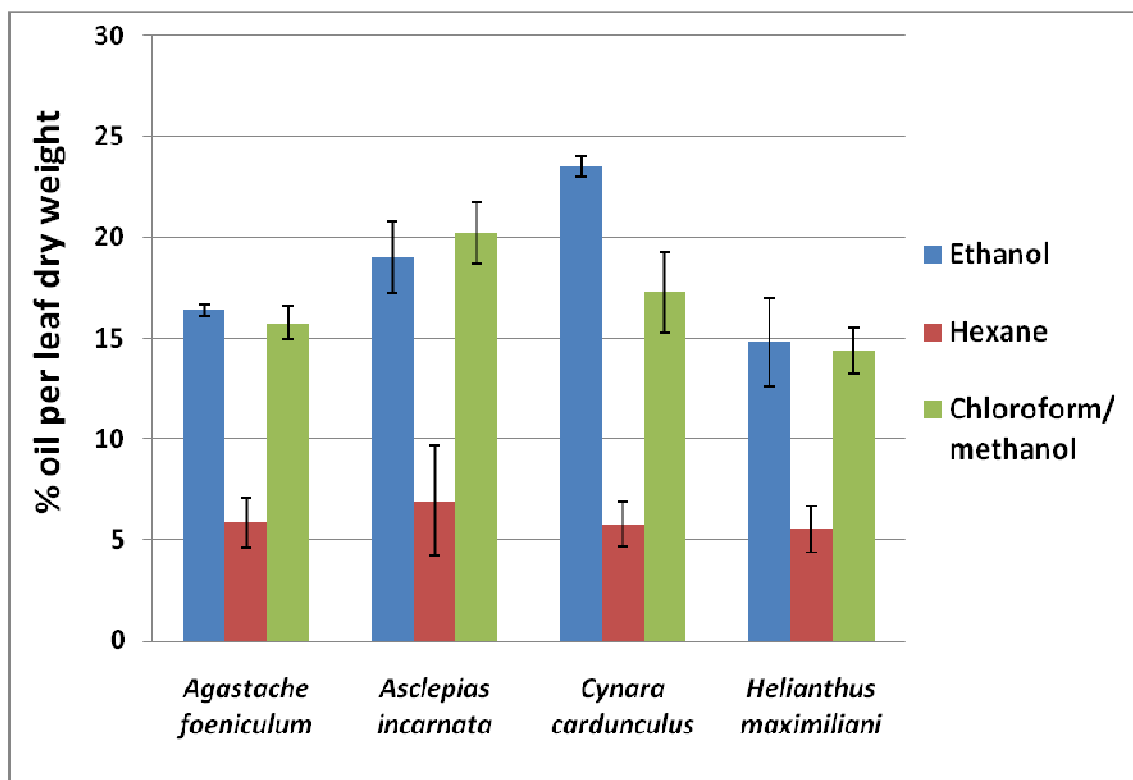
Fig. 18 Leaf extraction results by solvent for plant species. Extraction amounts are reported as the percent oil extracted from a dry weight of foliage of each plant species. Each value represents the average of five different extraction samples made from leaves collected from several different whole plants for each of three to four collection dates, for a total of 15 to 20 samples. Error bars are one standard deviation.
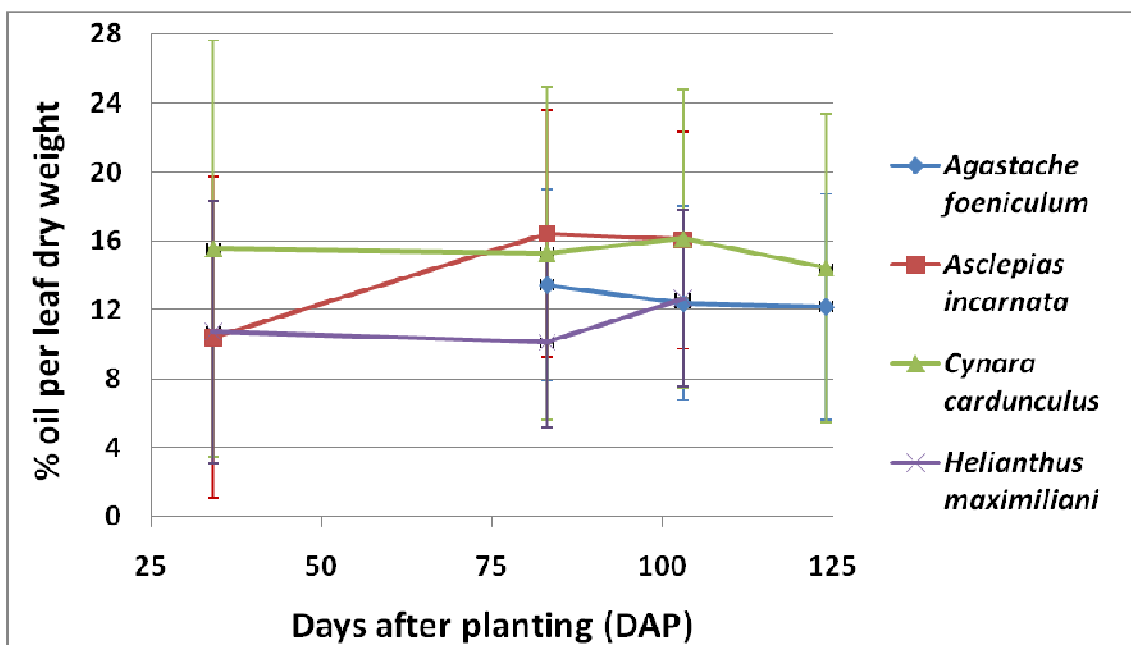
Fig. 19 Leaf extraction results for each plant species over developmental time. Extraction amounts are reported as the percent oil extracted from a dry weight of foliage of each plant species. Each value represents the average of five different extraction samples from each of three solvents, totaling fifteen samples. Error bars are one standard deviation. No data is given for *Agastache foeniculum* at 34 DAP because whole plants had too low foliage for making extraction samples. No data is given for all species at 58 DAP because collected samples had become damaged. *Agastache foeniculum* and *Helianthus maximiliani* were extracted again at 124 DAP because species had already flowered and neared the end of their first developmental life-cycle.

Statistical analysis

A three-way ANOVA test on time by plant species by extraction solvent did not show any significant interactions among all three of these factors in affecting lipid extraction (*P*=0.308) (Table 7). Two-way ANOVA tests were analyzed on extraction solvent by plant species, time by plant species, and time by extraction solvent to determine if any interactions of these factors affected lipid extraction. There were significant interactions between extraction solvent by plant species (*P*=0.0001) and time by plant species (*P*=0.006) in affecting lipid extraction. Time by extraction solvent was an insignificant interaction in affecting lipid extraction (*P*=0.432).

One-way ANOVA tests were analyzed on time, plant species, and extraction solvent each as factors affecting lipid extraction. All factors were considered fixed. The one-way ANOVA tests showed plant species and solvent as statistically significant factors in affecting lipid extraction (P=0.0001 for both). However, the results of these one-way ANOVA tests are not relevant since each of the factors were shown to have a significant interaction with another factor affecting it.

Table 7 ANOVA for the factors and their interactions that would affect total extractable lipids from leaves. The dependent variable is the percent oil extracted from a dry weight of foliage.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5966.03[a]	29	205.73	81.76	0.0001
Intercept	26140.97	1	26140.97	10388.88	0.0001
time	14.73	2	7.36	2.93	0.057
solvent	4663.40	2	2331.70	926.66	0.0001
species	467.70	3	155.90	61.96	0.0001
time * solvent	15.23	3	5.08	2.02	0.115
time * species	163.12	5	32.63	12.97	0.0001
solvent * species	302.33	6	50.39	20.03	0.0001
time * solvent * species	24.06	8	3.01	1.20	0.308
Error	301.95	120	2.52		
Total	34377.93	150			
Corrected Total	6267.99	149			

[a] R^2 = 0.952 (Adjusted R^2 = 0.940)
LSD=0.7585 for plant species, LSD=0.7023 for time, and LSD=0.6526 for solvent

Correlation analysis

Correlation analysis was run between the results for percent oil bodies in the sectioning data and the results for percent oil extracted in the extraction data. A few significant correlations were found in some of the data but not all (Table 8-10). There were significant correlations between sectioning data and both hexane and ethanol extraction data for *Asclepias incarnata* over developmental time ($P=0.01$ and $P=0.06$ respectively) (Table 8). There was also a significant correlation between sectioning data and hexane extraction data for *Agastache foeniculum* over developmental time ($P=0.01$) (Table 8). There were significant correlations between sectioning data and ethanol extraction data at 34 DAP and 103 DAP for each of the species ($P=0.01$ and $P=0.0001$ respectively) (Table 9). Also, there was significant correlation between sectioning data and chloroform/methanol extraction data at 103 DAP for each of the species ($P=0.03$) (Table 9). Overall, there were no significant correlations or variance between sectioning data and extraction data when correlations were analyzed for each of the individual species at each of the individual DAP (Tables 10).

Table 8 Correlation analysis of leaf sectioning results to lipid extraction results by separating data by plant species. These results show any correlation or variation between the abundance of oil bodies in a species leaves determined by image analysis and the abundance of extractable lipids from a leaf over developmental time for each of the species and each of the solvents. Lipid extraction results were separated by solvent because each solvent may extract different lipid amounts.

	Hexane	Ethanol	Chloroform/ Methanol (2:1, v:v)
Agastache foeniculum	0.74	0.05	0.186
	$P = 0.02$	$P = 0.89$	$P = 0.61$
Asclepias incarnata	0.76	0.50	-0.165
	$P = 0.01$	$P = 0.06$	$P = 0.65$

Table 8 continued

Cynara cardunculus	0.3	-0.03	0.385
	P = 0.28	*P = 0.90*	*P* = 0.27
Helianthus maximiliani	0.06	-0.07	0.258
	P = 0.84	*P* = 0.81	*P* = 0.47

Table 9 Correlation analysis of leaf sectioning results to lipid extraction results by separating data by DAP. These results show any correlation or variation between the abundance of oil bodies in a species leaves determined by image analysis and the abundance of extractable lipids from a leaf over developmental time among species for each of the collection dates and each of the solvents. Lipid extraction results were separated by solvent because each solvent may extract different lipid amounts. * values were not included because chloroform/methanol (2:1, v:v) was not yet being used as a solvent at 34 DAP.

	Hexane	Ethanol	Chloroform/ methanol (2:1, v:v)
34 DAP	0.45	0.63	*
	P = 0.08	*P* = 0.01	*
83 DAP	0.308	-0.29	0.04
	P = 0.187	*P* = 0.22	*P* = 0.86
103 DAP	0.116	0.77	0.48
	P = 0.626	*P* = 0.0001	*P* = 0.03

Table 10 Correlation analysis of leaf sectioning results to lipid extraction results by separating data by plant species and DAP. These results show any correlation or variation between the abundance of oil bodies in a species leaves determined by image analysis and the abundance of extractable lipids from a leaf for each of the collection dates, solvents, and plant species. Lipid extraction results were separated by solvent because each solvent may extract different lipid amounts. * values were not included because *Agastache foeniculum* had little foliage at 34 DAP. ** values were not included because chloroform/methanol (2:1, v:v) was not yet being used as a solvent at 34 DAP.

		Hexane	Ethanol	Chloroform/ methanol (2:1, v:v)
Agastache foeniculum	34 DAP	*	*	*
		*	*	*
	83 DAP	0.21	-0.43	-0.51
		$P = 0.73$	$P = 0.47$	$P = 0.38$
	103 DAP	0.34	-0.33	-0.75
		$P = 0.57$	$P = 0.58$	$P = 0.15$
Asclepias incarnata	34 DAP	-0.07	-0.81	**
		$P = 0.91$	$P = 0.10$	**
	83 DAP	0.49	-0.32	-0.35
		$P = 0.40$	$P = 0.60$	$P = 0.56$
	103 DAP	0.67	0.21	-0.04
		$P = 0.22$	$P = 0.73$	$P = 0.95$
Cynara cardunculus	34 DAP	0.35	-0.51	**
		$P = 0.56$	$P = 0.38$	**
	83 DAP	-0.03	0.71	0.29
		$P = 0.96$	$P = 0.18$	$P = 0.64$
	103 DAP	-0.67	0.86	0.56
		$P = 0.21$	$P = 0.06$	$P = 0.33$

Table 10 continued

Helianthus maximiliani	34 DAP	-0.13	0.23	**
		P = 0.84	P = 0.71	**
	83 DAP	-0.49	0.68	0.60
		P = 40	P = 0.20	P = 0.29
	103 DAP	0.55	0.51	-0.16
		P = 0.34	P = 0.38	P = 0.80

Thin-layer chromatography

The thin-layer chromatography (TLC) results for lipids from each of the four species are shown in Figure 20. For *Agastache foeniculum*, bands for monoglycerides, diglycerides, sterols, alcohols, and wax esters were identified. For *Helianthus maximiliani*, bands for monoglycerides, sterols, alcohols, and wax esters were identified. For *Cynara cardunculus*, bands for monoglycerides, sterols, and alcohols were identified. For *Asclepias incarnata*, bands for monoglycerides, sterols, alcohols, TAGs, and wax esters were identified.
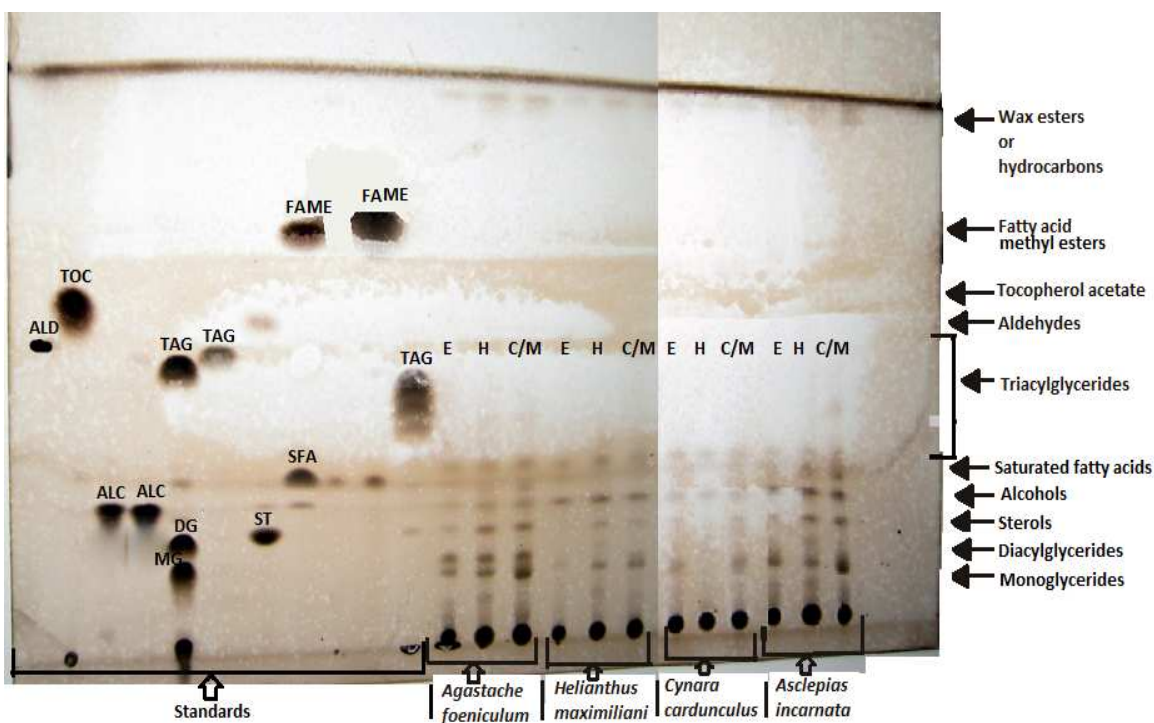
Fig. 20 Thin-layer chromatography (TLC) on standards and lipids from each of the four plant species. The three columns for each plant species represents lipids extracted from a different solvent, from left to right: ethanol [E], hexane [H], and chloroform/methanol (2:1, v/v) [C/M].

Comparing these results to lipid standards at different concentrations in TLC, showed that species seemed to have some differences in the concentrations of lipids present. The concentration of lipids in bands ranged from: 10-500 µg/mL for *Cynara cardunculus*, 150-4000 µg/mL for *Asclepias incarnata*, 75-250 µg/mL for *Agastache foeniculum*, and 100-900 µg/mL for *Helianthus maximiliani*.

CHAPTER V

DISCUSSION

Survey and the species chosen

Four species were chosen out of the survey for further study. These species were a good fit for this study because of their ready ability to grow to a significant size within a short time frame in greenhouse conditions and because of their potential to be a high biomass plant in various field stress conditions that might be found in different regions of Texas. Each species is suited for different types of stress conditions compared with the others. Also, ultimately these species were chosen because they all readily stained for oil bodies and because they varied in relative foliar oil body abundance.

Also, each of the species has its own advantage compared to the others, including adaptation to different stress environments in Texas. For example, *Asclepias incarnata* is better for moist or damp environments, which are more in South or East Texas and not the Texas Panhandle. *Asclepias incarnata* has a tall erect height and high foliage that makes it favorable as a biodiesel source. The plant is sometimes classified as a weed and grows in many regions of the U.S., including Texas. The potential for *Agastache foeniculum* as a biodiesel source is its ability to grow in dry to mesic conditions and soils with loam, clay-loam, or rocky material. Compared with other mint species, the plant reaches a sizable height with much branching and large leaves. *Agastache foeniculum* is better for cooler temperatures, which within Texas are mostly during the Fall and Winter in the Texas Panhandle. *Helianthus maximiliani* or other sunflower species are more readily cultivable for agriculture than the other species. *Cynara cardunculus* has very high biomass because its leaves reach a large size. *Helianthus maximiliani* and *Cynara cardunculus* are species whose seed oil has already been recognized as a potential source of biodiesel. If the foliage of these plants could also be used for biodiesel production, it would be of benefit. *Cynara cardunculus*, sometimes classified as a weed, is a cool season plant that can grow in dry climates and is a high biomass plant that can reach up to 5 feet tall and 4 feet wide. *Helianthus maximiliani* can grow in a variety of environments

from dry to moist and is found throughout the US. The plant grows many leaves and can reach a height upwards of three meters.

Sectioning results

ImageJ software (National Institute of Health) was used to quantify the abundance of oil bodies among species. To duplicate or compare to the results of this study, the surface area of oil bodies in a 5000-μm^2 area of a leaf cross-section image can be measured. Leaf cross-sections are to be taken perpendicular to the surface of the leaf.

From our results it still seems uncertain whether using imaging software to measure the surface area of oil bodies in sectioning images is an effective means to quantify and compare oil body abundance between leaves. Despite this uncertainty of effectiveness, results obtained through image analysis did show statistically significant differences and interactions between the factors of interest that may affect oil body abundance: time, plant species, and leaf location.

There were statistically significant interactions between time and plant species (*P*=0.027) and between leaf location and plant species (*P*=0.0001). These interactions seem to suggest that both time and leaf location affected leaf oil abundance but variation between species existed in this effect. Newly budded leaves seemed to have a lower abundance of oil bodies compared to more developed and senescing leaves. Therefore, there was variation in abundance of oil bodies in a species' leaves dependent on the plant age and leaf location. Oil body abundance seemed to depend on the development of the leaf. The significance of the age of the plant might be an artifact of the significance of the age of the leaf since a younger plant will have a low percentage of senescing leaves compared to an older one. From these results, the suggestion of the accumulation of these oil bodies relative to leaf senescence requires a review of previous literature discussing the origin of leaf cell oil globules and TAGs.

A study by Guiamét et al. (1999) suggested that plastogobuli (lipid-protein globules) on the chloroplasts of leaf cells accumulated lipids from chloroplast membrane lipid breakdown over senescence. This suggestion was further supported by mutants

against chloroplast breakdown that had low plastoglobuli formation (Guiamét et al.1999). Electron microscope images showed these plastoglobuli protruded through the chloroplast envelope into the cytosol (Guiamét et al.1999). In the cytosol, these globules sometimes acquired a coat, perhaps with clathrin, but then disintegrated (Guiamét et al.1999). Harwood 1984 had suggested that 80% of total lipids are in chloroplasts.

Another study by Kaup et al. (2002) examined these plastoglobuli more but also focused on the influence of diacylglycerol acyltransferase 1 (*DGAT1*) on the accumulation of TAGs during leaf senescence. First, the study showed that the activity of *DGAT1* increased in *Arabidopsis* rosette leaves over time (Kaup et al. 2002). Also, there was a change in the lipid composition of leaves over time that included an increase in TAGs and a change in the fatty acid (FA) composition of TAGs to more membrane-type FAs such as linolenic acid (18:3) and hexadecatrienoic acid (16:3) (Kaup et al. 2002). Kaup et al. (2002) correlated this change in TAG composition in leaves over time to the activity of *DGAT1* that was shown to be active in the chloroplasts of the leaf cells. Kaup et al. (2002) through electron microscopy also showed the formation of the plastoglobuli reported by Guiamét et al. (1999) on the chloroplasts, and the plastoglobuli accumulated TAGs from the breaking apart of the thylakoid membrane in chloroplasts. Therefore, Kaup et al. (2002) reported an increase in TAGs over leaf senescence with membrane-type FAs that they theorized came from the activity of *DGAT1* on chloroplast membranes leading to TAG accumulation in plastoglobuli.

Slocombe et al. (2009) did another study that looked at the accumulation of TAGs in leaves but also looked at the diurnal changes in leaf TAGs, particularly during dark periods. Slocombe et al. (2009) studied two mutants for fatty acid breakdown *cts-2* and *pxa-1* and looked at their changes during extended dark treatment and during senescence. Extended dark treatment in these mutants showed severe leaf wilting and a TAG leaf increase of at least 10-fold. In the *pxa-1*mutant there was a change in plastid structure correlating to wilting and an increase in visible cytosolic droplets. Senescence in these mutants showed an increase in TAG, particularly an eventual increase in 16:3 TAG that is associated with the breakdown of plastids with senescence. Despite the accumulation

of cytosolic droplets with senescence, there were actually no differences between the observed plastoglobuli from electron microscopy of the mutants compared to the wildtypes, suggesting the plastoglobuli were not responsible for the TAG accumulation. Interesting from these results was that the TAG accumulated from senescence was two to five times that from dark treatment. Also, Slocombe et al. (2009) reported that a *DGAT1* mutant SK353 accumulated less TAG when early flowering and senescence occurred. Slocombe et al. (2009) writes that plants may be partitioning fatty acids to TAG as a means to avoid the toxic effects of free fatty acids.

Another study by Dahlqvist et al. (2000) reported the activity of phospholipid:diacylglycerol acyltransferase (PDAT) in the production of TAGs from DAGs in an acyl-CoA independent pathway that transferred an acyl moiety from phosphatidylchline (PC). DGAT acts in an acyl-CoA dependent pathway. Dahlqvist et al. (2000) suggested that this activity of PDAT is a form of regulation of membrane lipid composition. PDAT was shown to be active in both yeast and plants. Therefore, as suggested for DGAT, TAGs from PDAT seem to arise from activity associated with plant cell membranes.

Lin and Oliver (2008) did another study in which they suggested that cytosolic TAGs were not associated with the TAGs of membrane breakdown that come from stress and senescence. In the study, they showed that TAGs in *Malus* leaves did increase over senescence and the FAs associated to these TAGs did change to more membrane associated FAs because of a decrease in oleic acid (18:1) and increase in linolenic acid (18:3). However, Lin and Oliver (2008) reported a second group of TAGs not associated with senescence that increased after a day of photosynthesis in plant leaves, and this TAG production was confirmed by the incorporation of ^{14}C-acetate into TAGs in leaves during the daytime. Lin and Oliver (2008) suggested that these TAGs are acting as an energy store, and the FA composition of these TAGs were 16:0, 18:1, and 18:0, different from the membrane TAGs. However, Lin and Oliver (2008) was unsure of the purpose of having this energy store since it represent only 6% energy of the total photosynthetic pool. Lin and Oliver suggested that the oil body TAGs were different from TAGs

associated with stress and senescence that come from membrane breakdown but instead may be a photosynthetic energy store.

Contrary to the suggestions of Lin and Oliver (2008), the results of our study suggest that oil bodies stained in leaf mesophyll cells according to the method of Lersten et al. (2006) are still possibly a byproduct of leaf development and senescence. These oil bodies may arise from breakdown of membrane lipids, particularly chloroplastic, that move out to the cytosol. Suggestions from previous papers that these TAGs may be a form of carbon energy storage in senescing leaves or a means to avoid the toxicity of these accumulating FAs (Slocombe et al. 2009) still seem like possible conclusions.

Extraction results

There were statistically significant interactions between time and plant species (P=0.0001) and between solvent and plant species (P=0.0001) in affecting the amount of extractable lipids from leaves. The interaction between plant species and solvent is explainable since each solvent extracts different lipids and lipid composition varies among species (Roughan and Batt 1969). The interaction between time and plant species suggests that the amount of extractable lipids changes over time in each of the plant species.

This change in lipid composition of leaves over time is supported by the findings of Kaup et al. (2002) that studied the composition of senescing rosette leaves of *Arabidopsis* over time. Their results showed that concentrations of DAGs, free fatty acids, TAGs, and steryl/wax esters over that of polar lipids was higher for senescing leaves. Also, of the neutral lipids, Kaup et al. (2002) wrote that steryl/wax esters and TAGs were higher in senescing leaves but DAGs and free fatty acids were higher in young leaves. Also, as already mentioned, there was a change in the FA composition of TAGs over time in leaves.

The change in lipid composition of leaves over time particularly in plant senescence was previously reported by Harwood and Russell (1984) and who indicated leaf senescence brought about a large decrease in chloroplast acyl lipids.

There was thought some correlation might occur between the sectioning and extraction results of this study. Some results did show correlation but not all. For both *Agastache foeniculum* and *Asclepias incarnata* there were some correlations of the sectioning results to the extraction results for changes in each over developmental time depending on which solvent was used. Notably, both experienced correlations with hexane that would have extracted only non-polar lipids that include TAGs. Also, at 34 DAP and 103 DAP, there were some correlations of the sectioning results to the extraction results for differences between species. This result showed that variation between species in the amount of lipids extracted at these time points can be correlated to variation between species in the amount of oil bodies identified in leaf sectioning.

Though some correlations did occur in the data, they did not occur in all data such that the results from sectioning data showing leaf oil body abundance do not broadly correspond to results from extraction data showing the amount of extractable lipids from a leaf. These results suggest that image analysis to try to quantify the amount of oil body accumulation is limited as an indication of the amount of lipids that may be extracted from a leaf. Rather, it seems that so many different lipids are extracted with each of these solvents that changes in lipid extracts associated with oil bodies were not always detectable. It seems lipid extracts may be largely composed of the membrane lipids present rather than oil body lipids present. These results could be confirmed by doing a fatty acid analysis of the lipids extracted to determine whether the lipids are composed of membrane-associated fatty acids such as linolenic acid.

TLC results

To further understand the sectioning and extraction results, TLC was performed. The first purpose of TLC was to confirm the presence of TAGs in each of these species that sectioned positively for oil bodies. Lin and Oliver (2008) had previously tested 24 species positive for oil bodies for the presence of TAGs. Surprising from these results was that only 13 of the 24 species had a detectable level of TAGs. Thus, confirmation of detectable TAG presence in each of these species needed to be determined. If these oil

bodies are not actually TAGs, it would be difficult to compare oil bodies to previous studies focusing on TAGs over leaf senescence or diurnal periods.

Also, TLC needed to be performed to understand the composition of the extractable lipids from leaves and not just the amount of lipids. TLC results showed lipids for monoglycerides, diglycerides, sterols, alcohols, and wax esters in plant leaves. However, *Asclepias incarnata* was the only species in which TAGs were detected by TLC. Thus, our results like Lin and Oliver's suggest that either the oil bodies are not TAGs or the lipids of the oil bodies are below detectable amounts.

CHAPTER VI

CONCLUSIONS

This study is one of many concerned with developing plants as a replenishable feedstock for biofuel production. This study also touched on several other areas of knowledge such as plant cell physiology, leaf lipid composition, leaf senescence, etc.

Considering the results of this study and the literature spoken of in the discussion section, important conclusions can be reached for each of the experiment sets. From the sectioning results, the main conclusion is that the amount of leaf cell oil bodies does increase with leaf senescence. The main conclusion from extraction results is that membrane lipids may be the dominant extractable lipids out of a leaf. Whether oil bodies can accumulate enough to become the dominant extractable lipid from a leaf is questionable. The main conclusion from TLC is that the oil bodies may not be TAGs, or if they are, the TAGs are below a detectible level, except for *Asclepias incarnata.*

Given these conclusions, there are several different directions research can go to help explain more of the unanswered questions. Given that so many previous studies had studied genetic activity on leaf TAGs, oil body level changes in response to these TAG-related genes could be studied. Another paper by Andrianov et al. (2010) had suggested tobacco to be an interesting crop to study the potential of plant biomass for biofuels, and certainly tobacco is one of several species that a large amount of research could be done with.

Also, for our results and those of other plant species, the FA composition of TAGs from TLC on the leaves would be of interest to determine whether the TAGs separated were from membrane lipids. Also, it would be of interest to look at leaves much farther into senescence in all species studied to see whether TAG or oil body levels increased, stayed the same, or decreased.

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APPENDIX A
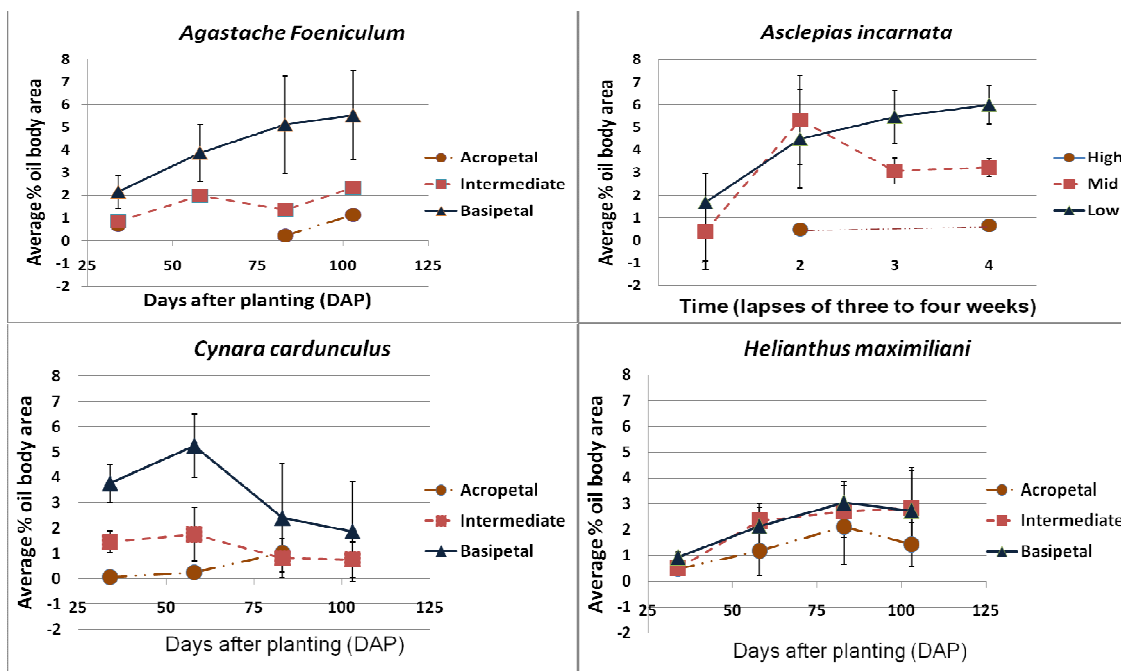
Leaf sectioning results for plant species over developmental time. "Acropetal," "Intermediate," and "Basipetal" leaves all come from their respective positions on the whole plant. "Average % oil body area" is the percent surface area of oil bodies per 5000-μm² leaf surface area in an image, calculated using ImageJ (National Institute of Health). Each value is the average of five images, each from a leaf of a different whole plant. Exceptions are *Agastache foeniculum* and *Asclepias* incarnata "Acropetal" values which are each from one image. Error bars are one standard deviation.

APPENDIX B
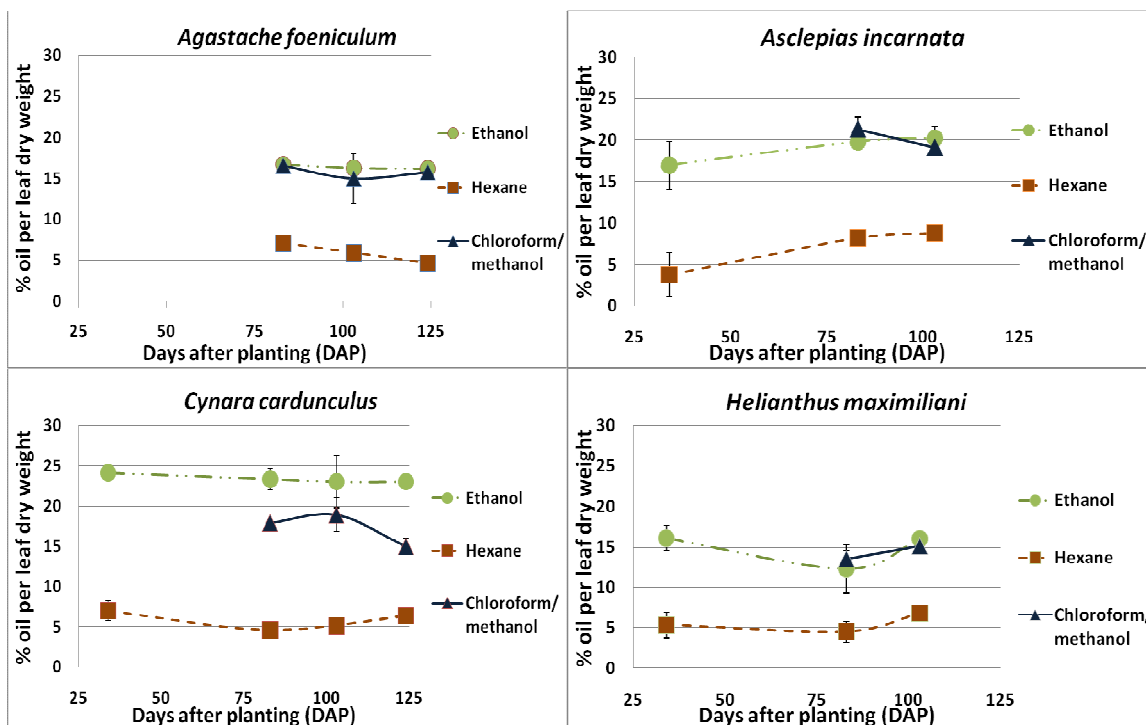
Leaf extraction results with three different solvents over developmental time. Extraction amounts are reported as the percent oil extracted from a dry weight of foliage of each plant species. Each value represents the average of five different extraction samples made from leaves collected from several different plants. Error bars are one standard deviation.

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