TERPENE CHEMISTRY OF LEMON VERBENA (*ALOYSIA CITRIODORA*): NATURAL VARIATION AND RESPONSE TO ECOLOGICAL AND AGRICULTURAL VARIABLES

by

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(Under the Direction of James M. Affolter)

ABSTRACT

Lemon verbena (*Aloysia citriodora* Paláu), Verbenaceae, is an aromatic, perennial shrub native to South America. The essential oil extracts and leaf material are valued medicinally, in tincture or tea form, and for their aromatic properties in culinary and cosmetic industries. Mono- and sesquiterpenoids are the primary constituents of essential oils, and their volatility is responsible for the characteristic odors of this and many other aromatic plant species. In addition to being valued economically, the terpenes play important ecological roles including plant defense, attracting pollinators, deterring microbial infections, and even ameliorating the effects of abiotic stresses for the plants that produce them.

The dominant monoterpenes of a common U.S. cultivar of lemon verbena were the *cis*- and *trans*-isomers of citral (neral and geranial), followed by δ-limonene, β-ocimene, and δ-+-3-carene; while the dominant sesquiterpenes were germacrene-D, *trans*-caryophyllene, bicyclogermacrene, and α-curcumene. Qualitative terpene composition did not change significantly throughout the harvest period (July – August). In agricultural study plots, mono- and sesquiterpene concentrations increased until peak flowering and then declined throughout the remainder of the growing season. Plant biomass was inversely related to plant density,

though density did not significantly affect terpene yield per plant or per kilogram of plant material. Water stress significantly increased terpene concentration for multiple mono- and sesquiterpenes (e.g., α-pinene, β-phellandrene, α-cubebene and caryophyllene) and significantly reduced foliar biomass yield per plant. Exclusion of ambient UVB radiation increased foliar concentrations of geranial (*trans*-citral) and β-bourbonene but did not significantly affect other terpenes. Mono- and sesquiterpene emissions were not affected by the exclusion of UVB radiation or water stress.

International trade in aromatic plants, such as lemon verbena, is increasing in both volume and value. These species, and the essential oils they contain, have been identified as a renewable resource and source of economic stimulus in many rural areas and developing nations. Implementing good agricultural and collection practices is critical to creation of a sustainable market while preserving and protecting native species of plants. Thin layer chromatography (TLC) was tested as a low-cost, rapid way to assess the monoterpenes of wild populations of aromatic species of plants using *A. citriodora* as a test species. It was possible to accurately identify the majority of the monoterpenes present with TLC and to distinguish chemotypes that clustered according to population in the different provinces sampled (Salta, Catamarca and La Rioja). Chemotypes containing deleterious compounds (i.e. thujone) could be avoided and those containing positive markers (i.e. citral) could be selected for collection and cultivar development through the use of TLC as a prescreening tool.

INDEX WORDS: *Aloysia citriodora*; *Aloysia triphylla*; lemon verbena; terpenoids; plant density; seasonal variation; phytochemistry; abiotic stress; UV; water stress; emissions; plant volatiles; monoterpene; sesquiterpene; TLC

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B.S. Emory University, 1996

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2009

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August 2009

DEDICATION

To Heriot Allan Chapman, my mother, who taught me to love science from the moment I could talk and to wonder at its beauty and study it until I had learned all that I could. To Stephen F. Allan, my granddaddy, who taught me to grow peas when I was three and saved a labeled pressing of every wildflower that bloomed while I was in college once he learned I loved botany. You gave me a sense of place, love of nature, and love of learning I will strive to pass on to others. To Grace Heriot Bailey, Raph Bailey Jr., and Chloé Jane Chapman, may you carry on the family tradition of a love of nature and knowledge in equal measure.

ACKNOWLEDGEMENTS

Many people shared their knowledge and support with me throughout my career as a graduate student. To those that I do not name, thank you very much for all you did. To those that are mentioned below, here is my attempt at a thank you that cannot ever be fully captured in words.

Jim Affolter, thank you for being my advisor and my mentor. For these many years you have guided me through academic decisions, helped me find funding, and helped me keep a focus despite many of life's challenges. You were always a patient guide who demonstrated how to be a mentor through your example. Thank you for sitting up with me that first night in Argentina when I thought I was dying from anaphylactic shock, and all the other drama you put up with. That first field season in Argentina was memorable and great fun. Fortunately my Spanish has improved! You have developed excellent projects for conservation and I hope to continue some of your efforts in my classes and future work.

Stan Kays, thank you for always encouraging me to choose the "right way" to do something and for opening your lab and your office to enable me to do so. I would not be here today without your expertise and assistance. Thank you for continuing to support my efforts and encouraging me to be the best researcher I can be. I strive to live up to your example.

David Giannasi, you have been a great teacher from my very first day. Your knowledge of natural products chemistry is amazing and your willingness to take time to teach students botanical systematics as well as analytical techniques is appreciated more than you could know.

Ron Carroll, you have been the heart of Ecology for me. From directing me to all the people associated with plant chemistry when I came for an informational visit to improving my research questions and approach you have brought your ecological insight to my projects. Thank you for always having time to discuss ideas, teaching, new developments in ecology and for always being a student advocate and passionate teacher and researcher.

Carl Jordan, thank you for serving on my committee, for offering space at Spring Valley Ecofarms when I tried to conduct a second experiment there. Thank you for understanding when I had undertaken more than I could handle. Also, thank you for all you do with teaching and outreach about agro-ecology and sustainable, community farming; you are helping put research into action in the academic and local community.

Brian, you have been here through the roughest parts of my Ph.D. and your dedication to help me through it has meant the world to me. I love you with all my heart. Laurel has passed the gauntlet to Pudge as the dissertation support dog, but both provided a lot of sanity during its writing and throughout my life. Thank you, Brian, for making sure our family worked when I couldn't and for being such a wonderful and supportive partner.

To my family, thank you, for all your support, for only asking once a year if I might finally finish and graduate at some point. Thank you for putting up with my rarely coming to visit because I was always working. And thank you for encouraging me to work hard and follow my dreams. Eliza, Christian, Poppa, and Lucile you have supported me through so much, and you believed in me and encouraged me to do this, even when it required sacrifice on your part. Ralph and Claire, thank you for being my siblings and support as well! Lukie, Jane and Jerry, Tony and Danielle, thank you for encouraging me to broaden my horizons and reach for this.

To the following organizations for financial and personal support: Jardín Botanico Dr. Miguel Culaciati, especially Paula Culaciati; The State Botanical Garden of Georgia; Instituto Nacional de Tecnologia y Agropecuaria (INTA); Center for Latin American and Caribbean Studies; IDEAS program (Office of International Development); the Tinker Foundation; and the Stanley Smith Horticultural Trust. Additionally I would like to thank Marta Lagrotteria; Fernando Guzmán, Dante Carabajal and INTA representatives in many provinces; Alejanda Gil, Arnoldo Bandoni, Carlos Balleré, and Ana Scopel for discussing research ideas. Roz Ayers, Heather Alley, and William Brooks for their help with collection and analysis. The staff at the UGA Horticulture farm over the years, especially Herb, Carl and Ryan, for keeping my plants alive and helping me fix nearly any problem I could create.

A special thanks to Betty Schroeder. There is no way this would be finished without you. Thank you for helping me whenever you could, guiding me when I needed it, staying late countless days and weekends, and in general being a great friend and exceptional person. Jenny Buley (and family) you are too wonderful for words. Thanks for helping me learn to grow things, or at least not kill all my plants. You taught me better ways to do so much, thank you for sharing all your knowledge with me. Dong Sik, thank you for helping me so much in the lab. For figuring out so many of the mechanics of the chemical collection and analysis and letting me follow behind you and learn. It was a pleasure to work with you.

Wonderful people, friends and alums of the Ecology Institute. You helped me, guided me, made me laugh, made me a better teacher, researcher, student and friend – for those I name, there are an equal number I do not: Patsy Pittman, Misha Boyd, Mary Freeman, Cathy Pringle, Thelma Richardson, Jeremy Sanderlin, Jim Richardson, Erin Lindquist, Marcelo Ardon, Chris Anderson, Beth Anderson, Jessica Seares, Sue Dye, John Davis, James Norman, Gretchen

Peltier, Lindsay Stallcup, Angela Romito, Krista Jacobsen, Scott Connelly, Lindsay Stallcup, Andrew Mehring, Hugo Collantes, and so many more. Thank you!

To everyone at Oxford College, thank you! You believed in, supported and encouraged me so much! Thank you for all that you have taught me, as a student and then as a new teacher!

Jennifer C. M. Reynolds, thank you for handling the panicked e-mails, early and late calls and for being my "Atlanta lab group" and friend. To Sonia Hernandez-Divers, Alex Reynolds, Liza Comita, and Rachel Spigler thank you for all the time you listened and gave advice and believed in me! To my dear friends near and far, Mandy, Mollie, Jill, Zoe, Adrienne, Rhett, Missy, Sarah, Diane, Billy and all of those crews, you have put up with my constant over commitment and inability to be there for baby christenings and even weddings, but you are the best of friends and dear to my heart. Thank you for being there for me through all this time. For listening to me when none of us were sure what I was doing, and for being supportive by taking care of me (and/or Laurel) when we most needed it. You are the best a person could wish for!

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

International trade in aromatic plants, such as lemon verbena (*Aloysia citriodora* Paláu), is increasing in both volume and value. These species, and the essential oils they contain, have been identified as a renewable resource and source of economic stimulus in many rural areas and developing nations. Mono- and sesquiterpenoids are the primary constituents of essential oils, and their volatility is responsible for the characteristic odors of many aromatic plants. In addition to being valued economically, the terpenes play important ecological roles including plant defense, attracting pollinators, deterring microbial infections, and even ameliorating the effects of abiotic stresses for the plants that produce them. It is this last characteristic that makes many aromatic plants of special interest for sustainable wild-harvest and/or possible cultivation. Aromatic species are often found in marginal habitats with frequent drought and low soil fertility. The ecological adaptations that have prepared them for those stresses make them excellent candidates for cultivation in similar habitats. As global climate change alters water availability and temperature ranges in traditional agricultural areas, species adapted to these stresses may become viable alternative crops. Aromatic plants are excellent candidates for an agroecological approach to sustainable development.

Understanding the biology, ecology and agronomy of aromatic species in general as well as each species in particular is critical to developing best-use agricultural practices and appropriate conservation strategies. As stated by Gershenzon and Dudareva (2007), “the word ‘biodiversity’ is on nearly everyone’s lips these days, but ‘chemodiversity’ is just as much a characteristic of life on Earth as biodiversity.” The studies in this dissertation focus on one

species of aromatic plant, lemon verbena (*Aloysia citriodora* Paláu). We chose this species because: 1) it is valued for its medicinal and aromatic properties in countries of origin (e.g., Argentina and Chile) and it shows economic growth in domestic and foreign markets; 2) it has been naturalized in many countries so we could conduct experiments in the U.S. without removing plant material from Argentina; and 3) the results would be applicable to many other species of aromatic plants for conservation or domestication efforts.

Medicinal and Aromatic Plants

Plants have been utilized by humans as a source of medicines for thousands of years. Today more than 80% of the world's population relies on traditional systems of medicine, the majority of which are plant-based, for their primary healthcare (Phillipson 1994). A review of prescription drugs sold in the USA between 1958 and 1980, revealed that a consistent percentage (25%) of medical prescriptions contained plant-derived compounds (Farnsworth and Soejarto 1985). The percentages of plant-based drugs commonly prescribed in many other developed nations are even higher - e.g., 60% in Eastern Europe and 80% in China (Husain 1991). The raw materials for medicinal plant products are often harvested from the wild with only a small percentage coming from cultivated stock. Due to growing populations in developing countries and an increased interest in herbal medicine in many developed countries, the demand for raw materials for medicinal plant products continues to rise. The total sale of herbal medicines in the European market in 2003 was 3.7 billion Euros (Zhang 2009). The size of the global medicinal and aromatic plant trade raises concerns about product safety and quality as well as source population sustainability and conservation. There is also growing interest in many developing nations in utilizing these medicinal and aromatic plants (MAPs) as renewable resources. Many of

the essential oil MAPs grow in marginal habitats with little agricultural input where they are adapted to low soil moisture as well as limited fertility. This makes them ideal candidates for rural economic development in many countries particularly in light of current climate change models that would preclude many forms of traditional agriculture.

Current Market, Trade, and Sources of Medicinal and Aromatic Plants

Trade in Medicinal Plants (FAO 2005) highlights the expanding trade in medicinal and aromatic plants (MAP) worldwide. The report provides an overview of the markets for MAPs and economic opportunities for improving the medicinal and aromatic plant trade through responsible collection/production practices (FAO 2005). In a single-country case study, Lange (1997) reported that 70-90% of the plant species traded in Germany are primarily harvested from the wild from a diverse array of countries including Europe, Africa, Asia, and the Americas (Lange 1997b). This high level of consumption of wild harvested species is true for most countries whether in the import or domestic market, and is evidenced by the fact that some of the most widely consumed drug plants have only been brought into wide-spread cultivation in the past few decades (Schumacher 1991, Lewington 1993, Cole 1996, Lange 1997a, Lagrotteria and Affolter 1999, Affolter and Pengelly 2007).

Sustainable Collection and Production: Wild-harvest vs. Cultivation

The wild-collection of medicinal plants can threaten: 1) the species of interest due to over-collection, 2) other associated species as a result of accidental collection or habitat modification, and 3) human health through improper identification and/or contamination of samples. Habitat loss due to land conversion (i.e. clearing land for grazing, burning to encourage

the growth of desired species, and construction of roads in developing areas) compounds the effects of over-collection. Many species of medicinal plants are becoming locally rare while some are listed as nationally and/or internationally endangered species (Akerele et al. 1991, Hamilton 2004). This crisis has led to an international call for increased efforts in research to better understand the biology of natural populations of species of medicinal and aromatic plants as well further efforts in the arena of conservation (Akerele et al. 1991, Caffini et al. 1999, Affolter and Pengelly 2007). The need for conservation initiatives and aspects of the global perspective on the conservation and use of medicinal and aromatic plants are reviewed by Affolter and Pengelley (2007) and Hamilton (2004).

Cultivation has been proposed as a viable method of conservation for many species of medicinal and aromatic plants (Bonati 1991, Palevitch 1991, Bernáth 1997, WHO 2003). Coupled with detailed study of natural populations, horticultural production of many medicinal plant species could serve to: 1) reduce the pressure on wild populations, 2) promote the standardization of desirable chemical constituents, 3) enhance plants' productivity, and 4) reduce contamination of plant samples due to indiscriminate collection and poor post-harvest processing. In combination with other forms of ex situ conservation (e.g., gene banks and collections in botanical gardens) cultivation could function to preserve the large degree of biodiversity present in wild populations of medicinal and aromatic plants.

In recent years, however, researchers have raised concerns about the "cry for cultivation" as a wide-spread solution to wild harvest (Schippmann et al. 2002). Cultivation is not always the clear answer to plant conservation and often can disenfranchise the human populations who rely on wild-harvest as a primary source of income while doing little to conserve the species (Schippmann et al. 2006). Schippmann et al. (2002) summarize from Homma's (1992) model of

extraction that the monetary incentive for cultivation does not typically appear until after a supply of raw materials has become limited. Furthermore, not all species are affected in the same way by harvesting pressures. Schippman (2002) outlines a chart based on data presented by Rabinowitz (1981) that combines geographic distribution, habitat specificity, and local population sizes to determine an index of rarity that can be translated to an index of susceptibility to over-harvest. If plants have a wide geographic distribution, can survive in a variety of habitats, and/or have sufficient local populations to support wild-harvesting practices there is not an immediate need for the development of cultivation practices from a conservation perspective.

Medicinal and aromatic plants incorporate a broad range of interrelated and complexly interwoven subjects, including agriculture, commerce, ecology, pharmacology, and social issues. In response to concerns about plant conservation, resource allocation, and sustainable use the World Health Organization published their Guidelines on the Conservation of Medicinal Plants (WHO 1993) addressing ecological and socioeconomic issues related to wild harvest of medicinal plants. This was followed by the WHO Guidelines on Good Agricultural and Collection Practices (GACP) in 2003 which provides general guidelines for sustainable development and quality control of medicinal and aromatic plant collection and production (WHO 2003). Continuing in an effort to improve the current standards of best-use practices, Bogers et al. (2006) present the summary of an international panel of specialists convened to develop the dialogue about the complex issues associated with medicinal plants' utilization and conservation. Their work highlights research developments and deficits in addition to examining current standards of good practices for continued use of medicinal plants. They also consider the prospect of growing, collecting, and processing these plants as a source of economic stimulus in rural and developing communities. Included in this summary is a proposed update of the 2003

GACP guidelines that include greater incorporation of biological diversity conservation, benefits sharing, and fair business practice in a sustainable development model as a combined effort of the World Health Organization (WHO), International Union for Conservation of Nature (IUCN), World Wildlife Fund (WWF), and TRAFFIC International (Kathe 2006, Leaman 2006).

Economic Development and Quality Control

Beyond the issues of conservation and sustainable use is the potentially large source of revenue available to many developing countries and rural communities through the production of end-products from medicinal and aromatic plants. The United Nations Industrial Development Organization (UNIDO) promotes essential oils from medicinal and aromatic plants as a renewable resource that could be industrially utilized for economic benefit (Silva 2006). According to the United Nations Commodity Trade Statistics Database (COMTRADE 2009) the world import market of essential oils and aromatic plants in the perfumery and cosmetic industry has grown from 11.6 billion US dollars in 2003 to 17.5 billion US dollars in 2007 (summary by country and region can be found at: http://comtrade.un.org/pb/CommodityPages.aspx?y=2007). In UNIDO's *Manual on the Essential Oil Industry*, Silva (2006) highlights the need for technological access and knowledge transfer to developing countries and rural areas so that they may reap the financial benefit of producing the value added products (e.g., essential oils, tinctures, and herbal mixtures) rather than simply the raw materials of MAPs. Included in all recommendations for sustainable use of MAPs is the need to develop management practices for collection and/or cultivation, research expertise in determining the effects of agronomic and environmental variables on medicinal and aromatic plants in their native habitats, as well as

research related to conservation issues and strategies for sustainable utilization of those resources (WHO 2003, Bogers et al. 2006, Silva 2006, Affolter and Pengelly 2007).

Essential Oils/Terpene Biochemistry

Aromatic plants, many of which are medicinal plants, are valued for their essential oils. Mono- and sesquiterpenoids are considered to be the primary constituents of essential oils, their volatility being responsible for the characteristic odors of many aromatic plants (Robinson 1993, Little and Croteau 1999). Monoterpenes are typically characterized as colorless, lipophilic, volatile substances and have been reported from nearly 50 plant families (Banthorpe and Charlwood 1980, Gershenzon and Croteau 1990). Terpenoids are usually cyclic, unsaturated hydrocarbons with substituent groups with varying degrees of oxygenation. Variability within this chemical class is increased by the existence of sterioisomers for many of the compounds, often present simultaneously in different mixtures (Harborne 1991).

Some terpenoid compounds have a role in the primary metabolic functions of growth and development (e.g., photosynthetic pigments, growth regulating steroids), but the majority of the mono- and sesquiterpenes are considered secondary metabolites. Though precise ecological roles have not been identified for all of these compounds, terpenes are thought to mediate a variety of above and below ground ecosystem processes including: plant defense, plant-pollinator interactions, plant-fungal interactions, allelopathy, and even tri-trophic interactions where insects sequester monoterpenes from plants and use them for defense and/or attraction (Gershenzon and Croteau 1991a, Robinson 1993, Langenheim 1994, Croteau and al. 2000, Gershenzon and Dudareva 2007).

These functions (i.e. defense and pollination) have dominated the development of theories regarding the production of terpenes as well as most other secondary metabolites (Fraenkel 1959, Harborne 1991, Gershenzon 1994, Langenheim 1994, Gershenzon and Dudareva 2007). It has recently been proposed, however, that abiotic stresses such as drought, photodamage, nutrient deficit and climate change acting individually as well as synergistically may be the important drivers in the evolutionary development and contemporary production of many types of secondary metabolites including terpenes (Close and McArthur 2002, Penuelas and Llusia 2003, Holopainen 2004, Peñuelas and Munné-Bosch 2005, Vickers et al. 2009).

Mono- and Sesquiterpene Biosynthesis

Terpenoids comprise the largest and most diverse group of plant chemicals, approximately 25,000 have been characterized (Banthorpe and Charlwood 1980, Croteau 1980, Gershenzon and Croteau 1991b, Robinson 1993, Bohlmann et al. 1998, Grayson 2000). Terpenes are synthesized by the condensation of isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Croteau and al. 2000, Lange et al. 2000, Dewick 2002). These 5-carbon isoprene units are combined to form a variety of classes of terpenes: 1) hemi- (C_5), 2) mono- (C_{10}), 3) sesqui- (C_{15}), 4) di- (C_{20}), 5) tri- (C_{30}), 6) tetra- (C_{40}), and 7) poly- (>C_{40}) terpenoids. For many years, it was believed that all terpenoids were products of the common mevalonate pathway, which was considered the universal route for the production of IPP from acetyl-CoA (Bohlmann et al. 1998, Mahmoud and Croteau 2002). It has now been established that plants produce IPP via two spatially separate, independent pathways (McConkey et al. 2000, Dewick 2002). IPP precursors for sesqui- and triterpene biosynthesis are synthesized in the cytosol via the mevalonate pathway (Newman and Chappell 1999) while hemi-, mono-, di-, and tetraterpene

precursors are synthesized in plastids via the methyl-erythritol-phosphate (MEP) pathway (Lange and Croteau 1999, Lichtenthaler and al. 1999, Rohmer 1999, Trapp and Croteau 2001, Rodriguez-Concepcion and Boronat 2002). This physical compartmentalization allows the pathways to operate independently, but considerable cross-talk has also been demonstrated (Hemmerlin et al. 2003, Laule et al. 2003, Dudareva et al. 2005). Isoprene is synthesized in the plastids from dimethylallyl pyrophosphate (DMAPP) produced via the MEP pathway by isoprene synthases with high specificity for the five-carbon substrate (Sharkey et al. 2005). The monoterpene precursor, geranyl pyrophosphate (GPP) is formed by the head to tail condensation of one molecule of isopentyl diphosphate (IPP) and one molecule of DMAPP in the plastid; GPP is then transformed by a number of monoterpene synthases with varying numbers of steps into the great diversity of monoterpenes the plants produce. In the cytosol, two molecules of IPP and one molecule of DMAPP are combined to form farnesyl pyrophosphate (FPP), the precursor for sesquiterpenes (Dewick 2002, Dudareva et al. 2004, Dudareva et al. 2006).

Peppermint (*Mentha* x *piperita* L.) is arguably the most important monoterpene-producing species from an economic standpoint and has been developed as a model system for the study of terpene metabolism. This is largely due to the ease with which it can be propagated and because its oil is chemically complex, with a biosynthetic pathway that encompasses nearly all the representative reaction types of monoterpenoid metabolism (Croteau and Gershenzon 1994, Gershenzon et al. 2000). Terpene biosynthesis and accumulation in mints is localized in the glandular trichomes on the leaf surface (McCaskill et al. 1992, McConkey et al. 2000). Gershenzon et al. (2000), using $^{14}CO_2$ pulse labeling, determined that the rate of monoterpene biosynthesis is restricted to a brief period early in leaf development. This corresponds to a similar increase in monoterpene content of these leaves, which stabilizes as the leaves continue

to expand. Mint, as well as many other species of plants, release volatile terpenes into the atmosphere (Lerdau et al. 1997), discussed in detail below. For mint and other terpene-storing species, the pattern of monoterpene accumulation seems to depend chiefly on the rate of biosynthesis (Gershenzon 2000). McConkey et al. (2000) suggest that regulation of monoterpene biosynthesis in mints occurs at the level of gene expression and this is supported by similar evidence regarding inducible monoterpene biosynthesis (Steele et al. 1998).

Cost of Synthesis. The accumulation of mono- and sesquiterpenes in plants is thought to be constrained by the metabolic cost of these compounds (Gershenzon 1994). Though initially more costly than other primary and secondary metabolites due to: 1) their extensive reduction, 2) the unique set of enzymes necessary for their biosynthesis, and 3) the need to sequester them in complex secretory structures, once synthesized they remain in a metabolically stable pool in these secretory structures (Mihaliak and Lincoln 1989, Mihaliak et al. 1991a, Mihaliak et al. 1991b, Gershenzon 1994) with a relatively small percentage of that pool being lost to emission. According to the method employed by Gershenzon (1994) for terpenes whose biosynthetic pathway was well known, the average monoterpene "cost" can be estimated based on glucose as a unit of cellular currency. Considering all enzymes, substrates, and cofactors the cost of monoterpenes ranges from 3.12 grams of glucose per gram monoterpene for linalool ($C_{10}H_{18}O$) to 3.54 g glucose/g α-pinene ($C_{10}H_{16}$). A similar range exists for the sesquiterpenes with cost varying inversely with the degree of oxygenation (Gershenzon 1994). While they are synthesized early in leaf development and are present in stable pools throughout the life of the plant, monoterpenes are either transported to other organs or are catabolized to primary metabolites prior to leaf senescence (Croteau 1988). Lerdau et al. (1997) highlight that emission into the atmosphere is the most likely route of loss of monoterpenes from plants; however, Gershenzon et

al. (1993) found that in many diverse species of monoterpene-producing plants, there was an absence of terpenoid turnover. Those terpenes that are volatized from the plants are thus considered to represent a small portion of the overall terpene pool in the secretory structures (Gershenzon et al. 2000). These facts have important implications for current theories of plant defense, especially the resource availability hypothesis (Coley et al. 1985) which maintains that there are two classes of defense compounds (mobile and immobile) defined by their ability to be recovered from leaves prior to senescence. Mobile defenses, such as monoterpenes, are expected to be restricted to those plants whose leaves have short life spans because the energy required to replenish stores of defense compounds would exceed the cost of immobile defenses. Due to their lack of rapid turnover, however, monoterpenes may entail less metabolic costs than immobile defenses, especially since many of their constitutive metabolites may be able to be recovered prior to senescence (Croteau 1987, 1988, Mihaliak et al. 1991b, Gershenzon 1994).

Plant Volatile Compounds: Focus on Mono- and Sesquiterpene Emissions

Plants synthesize and emit a variety of biogenic volatile organic compounds, the majority of which are lipophilic molecules with high vapor pressures. Over 1700 volatile compounds have been isolated for more than 90 plant families (Knudsen et al. 2006). These compounds are released from leaves, flowers, and fruits into the atmosphere and from roots into the rhizosphere and are mainly represented by the following chemical classes: 1) terpenoids, 2) benzoids or phenylpropanoids, 3) fatty acid derivatives, and 4) amino acid derivatives (Kesselmeier and Staudt 1999, Dudareva et al. 2004, Dudareva et al. 2006, Sharkey et al. 2008). Terpenoids are the primary volatiles emitted from essential oil producing plants and will be the focus of the remainder of the review. Emitted terpenes are synthesized in the tissues from which they are

emitted, and their production is spatially and temporally regulated (Dudareva et al. 2004, Dudareva et al. 2006).

Isoprene. Isoprene is a significant biogenic source of hydrocarbons in the atmosphere and has been related to ozone formation in the atmosphere (Guenther et al. 2006). Isoprene is emitted by a broad range of taxa from mosses (Hanson et al. 1999) and ferns (Tingey et al. 1987) to gymnosperms and angiosperms (for comprehensive list see http://www.es.lancs.ac.uk/cnhgroup/iso-emissions.pdf). Isoprene emission has been linked to thermotolerance (Sharkey and Singsaas 1995) and reducing damage due to reactive oxygen species, ozone, and other forms of oxidative stress (Loreto et al. 2001, Loreto and Velikova 2001, Velikova et al. 2005, Velikova et al. 2008). Additional theories regarding the regulation and stimulus for isoprene biosynthesis and emission are reviewed by Sharkey et al. (2008).

Mono-and sesquiterpenes. Mono- and sesquiterpene emissions have predominately been investigated in relation to defense against herbivory and pathogens and as attractants for pollinators and seed dispersers (Pichersky and Gershenzon 2002). They have also been implicated in roles in tritrophic interactions, below ground defenses, and intra and inter-plant interactions (Dudareva et al. 2006, Duhl et al. 2008) as well as in response to abiotic factors (addressed in later sections).

Ecology of Mono- and Sesquiterpenes

Mono- and sesquiterpenes play significant roles in multiple ecological interactions including: 1) direct plant defense (vs. herbivores and pathogens), 2) allelopathy, 3) indirect plant defense (entomophages), 4) plant pollination, 5) allelopathy, and 6) intra- and interplant communication (Harborne 1991, Langenheim 1994, Theis and Lerdau 2003, Gershenzon and

Dudareva 2007, Heil 2008, Dicke 2009). Many of the same terpenes have been indicated in multiple roles, and it is postulated that these diverse roles of terpenes may defray their "opportunity costs" as defense compounds (Gershenzon et al. 1993, Gershenzon 1994, Langenheim 1994, Gershenzon and Dudareva 2007, Frost et al. 2008).

Since Fraenkel (1959) described the "raison d'être" for plant secondary metabolites as being defense compounds against plant herbivores and Erlich and Raven (1964) hypothesized the coevolutionary relationships between insects and the plant groups on which they feed, theories regarding plant secondary metabolites (e.g., terpenes) have been dominated by their link to insect herbivory and the factors that affect trade-offs between growth and defense. The ultimate goal of the various hypotheses is to explain and predict phenotypic, genetic, and geographic variation in plant defenses and explain why most plants seem to be so well defended. The current dominant hypotheses are: Optimal Defense (Feeny 1975, 1976, Cates and Rhoades 1977), Carbon: Nutrient Balance (Bryant et al. 1983, Tuomi et al. 1988), Growth Rate or Resource Availability (Coley et al. 1985), and Growth-Differentiation Balance (Herms and Mattson 1992, 1994). These defense hypotheses and surrounding debates are reviewed by Stamp (2003) and Tuomi (1992). The volatile terpenoids have been of particular interest due to their roles as both constitutive and inducible defenses as well as being involved in multiple incidences of plant-pollinator and multi-trophic level interactions (Langenheim 1994).

Studies over the past decade have indicated that, in addition to isoprene, and especially in those species that do not emit isoprene, the low molecular weight terpenes (mono- and sesquiterpenes) may also act to ameliorate abiotic stress responses by scavenging and neutralizing the reactive oxygen species (ROS) that are commonly produced when a plant undergoes various abiotic stresses (Sharkey and Loreto 1993, Velikova and Loreto 2005, Vickers

et al. 2009). Recent research has suggested that many secondary compounds, though effective against or correlated with herbivory, may be acting in a photoprotective role (Close and McArthur 2002) and that this, in concert with other ecological factors, has been a driving selective force for these compounds. Photoprotective roles have been demonstrated for many phenolics, and are proposed for other classes of secondary compounds including the terpenoids. Vickers et al. (2009) proposed a new model for evaluating the role of volatile isoprenoids in their "single biochemical mechanism for multiple physiological stressors" in which abiotic selective pressures (e.g., high light, temperature, and water stress) receive prominence. This model is built on decades of research with isoprene and recent studies of other low molecular weight terpenoids. They highlight the need for a greater body of research that investigates mono- and sesquiterpene accumulation and emission in response to these abiotic stresses.

Genetic and Environmental Variation in Wild Populations

Genetic, biochemical, and ecological factors have been shown to have a role in determining the occurrence of mono- and sesquiterpenes in the essential oils of aromatic plants. Chemical profiles have long been used in chemotaxonomy to distinguish between species and varieties of plants, and there is a growing body of evidence for infraspecific geographic variation in monoterpene composition for many species – e.g., *Eucalyptus spp., Thuja plicata, Juniperus spp., Satureja douglasii, Artemisia herba-alba, Cymbopogon distans, Coriandrum sativum, Pinus sylvestris,* and *Aloysia citriodora* among others (Lincoln and Langenheim 1976, Rhoades et al. 1976, Comer et al. 1982, Segal et al. 1987, Simmons and Parsons 1987, Mathela et al. 1988, Von Rudloff et al. 1988, Nerg et al. 1994, Cavaleiro et al. 2002, Gil et al. 2002, Gil et al.

2007). The existence of chemical "races" or chemotypes[1] within species of plants is supported by current research regarding the biosynthetic pathways of terpenes which indicate that while many biotic and abiotic factors affect the expression of certain genes regulating biosynthesis, thereby affecting the concentrations of many terpenes, the ability to produce certain compounds is under tight genetic control (Gershenzon et al. 2000, Dewick 2002, Mahmoud and Croteau 2002). Biodiversity at the species level is well known, but it is equally important to understand the "chemodiversity" within and among populations of plants throughout their distribution (Gershenzon and Dudareva 2007).

Bearing this in mind, there is also the potential for a great deal of variation in chemical constituents of medicinal and aromatic plants, particularly in geographically distinct populations. While many medicinal and aromatic plants are sold as bulk plant material (leaves, roots, stems), the majority of quality control has focused on reducing contamination with other species of plants rather than infraspecific qualitative variation, which is often overlooked. Improving the efficacy of a plant medicine and the market-value of an end-product (e.g., extracted essential oils) is based on both chemical composition and concentration, quality control should be implemented at the level of qualitative chemical constituents and their relative concentrations (Silva 2006).

Abiotic Factors

Water Stress. Terpene responses to water stress have been investigated in light of prevalent theories regarding defense hypotheses. Increased levels of terpenes have often been attributed to a simple decrease in leaf expansion and thus an increase in concentration in terpene-

[1] A chemotype is a chemical phenotype, based on the presence of certain complements of compounds (e.g., terpenes)

storing species such as pine and mint. Based on defense theories the increase in terpenes is due simply to reduced growth/leaf expansion (Gershenzon et al. 1978, Gershenzon 1984, Gershenzon and Croteau 1991b, Gershenzon et al. 2000). Many studies have found that plants respond contrary to that simple model and the responses vary greatly dependent on species and environment as well as native geographic range. Water deficits have been shown to increase concentration in the essential oils of several species (e.g., *Marjorana hortensis, Mentha piperita*, and *Satureja douglasii*) (Gershenzon et al. 1978, El-Keltawi and Croteau 1987, Charles et al. 1990), yet Penuelas and Llusiá (1997) found no significant effects of water stress on the accumulation of essential oils in *Rosmarinus officinalis*. They postulated that this was due to this species' adaptation to semi-arid environments. Simon et al. (1987) found that while total plant biomass and leaf area decreased with increasing levels of stress, the essential oil content of *Ocimum basilicum* tended to increase; furthermore, percent composition of specific oils was affected with some oils (i.e. chavicol) increasing and others (i.e. linalool) decreasing in response to water stress. Singh (2000), however, found that the essential oil concentration and composition of *Pelargonium graveolens* was not significantly influenced by different irrigation regimes or nitrogen addition, but the herbage yield was affected by these parameters serving to increase overall yield of essential oils per plant. Similar results were found for *Cymbopogon* spp. under mild and moderate water-stress regimes (Singhsangwan et al. 1994).

Llusiá and Peñuelas (1998, 2000) demonstrated decreased emission rates in a variety of species (*Pinus halepensis, Pistacia lentiscus, Cistus albidus, C.monspeliensis, Quercus ilex, Phyllyrea latifolia, P. angustifolia,* and *Arbutus unedo*) studied under severe drought. *Rosmarinus officinalis, Pinus halepensis, Cistus albidus,* and *Quercus coccifera* studied under drought stress (simulated by multiple days of water withholding) had a range of monoterpene

responses, from no change to a moderate increase in emissions,; sesquiterpenes, however, had decreased emission rates over time (Ormeno et al. 2007). Most essential oil work related to drought stress has been conducted with an eye toward agricultural production rather than the possible ecological roles/drivers of abiotic pressures. Given predicted patterns of water deficit due to consumption and global climate change, predicting a plant species' chemical and physiological responses to drought stress will be important in both ecological and agricultural models.

UVB radiation. Varying models predict increased levels of incident ultraviolet radiation (particularly UVB radiation) relative to photosynthetic active radiation to continue for the next 50 years, with an expected leveling off thereafter. Many plants are native to regions with historically high UVB levels and some new theories postulate that UV radiation may be as important a selective pressure for many plant secondary metabolites as herbivory (Close and McArthur 2002, Peñuelas and Munné-Bosch 2005, Vickers et al. 2009). The magnitude of increased UVB due to current and projected ozone loss does not exceed the geographical differences in UVB from the equator to the poles or from sea level to high mountains, thus understanding the high altitude/high latitude species' responses to UV stress may be a key to predicting plant response patterns to these changes in the environment (Rozema et al. 1997a, Rozema et al. 1997b, Ballaré et al. 2001).

Terrestrial plants often exhibit photomorphogenic effects when exposed to elevated ultraviolet radiation, particularly in the UV-B range (280-320 nm) (Day and Neale 2002, Flint et al. 2003). Plants have a wide array of stress responses to UVB including: decreased height and leaf area; increased leaf thickness and axillary branching; altered leaf angle, plant architecture

and canopy structure; as well as modified emergence of new leaves, senescence and seed production (Caldwell 1968, Ballaré et al. 1996, Allen et al. 1998, Jansen et al. 1998, Ballaré 2003). The impact of UV-B on plant biomass is less clear as results are mixed (Jansen et al. 1998, Day and Neale 2002, Kakani et al. 2003). Increased UV-B exposure also has been shown to cause DNA, cell membrane, photosystem II and phytohormone damage in plants (Searles et al. 1995, Allen et al. 1998, Jansen et al. 1998, Searles et al. 2001, Day and Neale 2002).

Many terrestrial plant secondary metabolites (e.g., flavonoids, lignin, and tannins) filter UV-B radiation and reduce its damaging effects (Mazza et al. 2000, Close and McArthur 2002, Kakani et al. 2003, Gobbo-Neto and Lopes 2007, Winter and Rostas 2008). The terpenoids are an additional class of secondary metabolites that have been proposed as an evolutionary base for photoprotection (Karousou et al. 1998, Zavala and Ravetta 2002, Peñuelas and Munné-Bosch 2005). Certain terpenoids (e.g., carotenoids and tocopherols) have well-known roles in photoprotection. There is strong evolutionary evidence of these compounds' conservation in that all photosynthetic organisms contain carotenoids such as the carotenoid-xanthophyll complexes that assist in dissipating excess light energy as heat or β-carotene, an oxygen-free radical scavenging species and membrane stabilizer (Havaux 1998, Peñuelas and Munné-Bosch 2005). Many terpenoids increase in response to elevated photosynthetic active radiation (PAR, 400-700 nm), but their response to UV-B has not been studied extensively (Bohlmann et al. 1998, Zavala and Ravetta 2002, Turtola et al. 2006).

Isoprene has been researched for its role in heat dissipation and allowing for increased thermo- and photo-tolerance in some species of plants as well as reducing oxidative stress (Loreto et al. 2001, Loreto and Velikova 2001, Affek and Yakir 2002, Cinege et al. 2009). It has been hypothesized that, due to the high number of double bonds relative to molecule size, the

low molecular weight terpenoids are well positioned to quench oxygen free radicals by allowing energy transfer and dissipation (Kainulainen et al. 2000, Penuelas and Llusia 2003, Velikova et al. 2004, Delfine et al. 2005, Grassmann 2005, Peñuelas and Munné-Bosch 2005, Vickers et al. 2009). Few studies have investigated the mono- and sesquiterpenes that comprise the majority of the essential oils of many terpene-storing species in response to this particular form of light stress. The overall levels of essential oils have been shown to respond to UV-B exposure in the field as well as in growth chamber and greenhouse experiments in *Grindelia chiloensis*, *Ocimum basilicum* and *Mentha spicata* (Karousou et al. 1998, Johnson et al. 1999, Zavala and Ravetta 2001, Ioannidis et al. 2002, Zavala and Ravetta 2002).

Due to the multiple ecological roles of many plant terpenoids, the effects of UVB radiation on plant chemistry could in turn affect pollinators, phytophagous insects and mammals, and other species of plants. These effects may be direct, such as changes in feeding preference based on alteration in compounds or perception of compounds (Mazza et al. 1999, Thines et al. 2007) or indirect based on multi-trophic level effects and interactions (Langenheim 1994, Holopainen 2004, Izaguirre et al. 2007).

Agronomic and Wild- Collection Variables

Seasonal variation. Seasonal variation of foliar concentration has been demonstrated in nearly all investigations regarding essential oil plants. Rao et al. (1996, 2001) demonstrated significant seasonal effects on both the yield and percent composition of essential oils in *Pelargonium spp.* with inverse relationships between certain monoterpenes throughout the growing season – e.g., low levels of geraniol and high levels of citronellol during summer months followed by high levels of geraniol and low levels of citronellol during winter months.

Work with *Eucalyptus* spp., *Mentha piperita,* and *Mentha spicata* indicates that the response of monoterpene composition and concentration is heavily dependent on genotype, with some chemotypes exhibiting seasonal variation while others did not (Gasic et al. 1987, Simmons and Parsons 1987, Kofidis et al. 2004). In *Lavandula stoechas* the leaf essential oil (volume per dry weight) remained the same throughout the year, while the essential oil concentration in the flowers decreased from the beginning to the end of the flowering stage; these results indicate that plant part sampled can determine if there is a seasonal response (Angioni et al. 2006). Jordán et al. (2006) demonstrated different patterns of seasonal variation for *Thymus hyemalis* and *Thymus vulgaris* with different vegetative cycles being recommended for the maximization of particular components of the essential oils of both species. Sampling to optimize thymol concentration (the character impact compound for thyme) would need to be conducted at full bloom/beginning of fruit maturation. Studies of seasonal variations in the chemical compositions of essential oils of wild populations of native aromatic plants in Turkey (*Thymbra spicata* var. *spicata*, *Satureja thymbra*, *Salvia fruticosa*, *Mentha pulegium*, *Laurus nobilis*, and *Inula viscose*) showed that the harvest time with the greatest essential oil yield would be July for the majority of the species, but September for *Laurus nobilis* (Muller-Riebau et al. 1997). An extensive study with four dominant species in situ in a Mediterranean shrubland (*Pinus halepensis*, *Pistacia lentiscus*, *Rosmarinus officinalis*, and *Globularia alypum*) showed variable seasonal responses when submitted to experimental drought (Joan et al. 2006). Owens (1998) linked the seasonal variation in monoterpene content in *Juniperus ashei* to seasonal patterns of flammability. A positive correlation between the level of limonene and flammability indicates that there could be chemotypic seasonal effects on fire management of ecosystems. In addition to variation in monoterpene accumulation, it has been found that some shrubs in wild populations exhibit

seasonal variation in terpene emission. The emission rates were correlated to temperature variation with higher emissions in warmer periods of the year (Penuelas and Llusia 1999, Llusia and Penuelas 2000, Staudt et al. 2002, Llusia et al. 2008). Finally, seasonal variation in essential oil concentration and composition was shown to affect insect antifeedant effects against aphids and antifungal and alleopathic effects in *Laurus novacanariensis* (Rodilla et al. 2008), demonstrating the ecological ramifications of some agronomic variables and their potential agroecological applications. The variability in species' responses to time of harvest and concurrent agricultural factors indicates that individual species biology must be investigated before implementing blanket conservation or collection practices.

Plant Density. Planting density is an important consideration in any cultivation regime. Spacing experiments with *Thymus vulgaris* have indicated that while wider spacing led to the highest level of biomass and essential oil yield per plant, dense cultivation significantly increased the essential oil yield per unit area (Clark and Menary 1979, Shalaby and Razin 1992). An inverse relationship between planting density and biomass yield without a significant change in terpene production, resulting in oil yield becoming a simple product of biomass yield, has been found for lavender, thyme, Australian mint, Hungarian & Tasmanian peppermint, fennel and the newly cultivated Tasmanian dusty daisy bush (Clark and Menary 1979, Shalaby and Razin 1992, Dragar and Menary 1996, Zheljazkov and Topalov 1996, Rissanen et al. 2002, Falzari et al. 2006, Arabaci et al. 2007). Density was shown to have an overall effect on *Mentha piperita* essential oil yield per hectare, giving highest yields in the mid-range 10 and 30 cm planting densities, due simply to increased biomass, as essential oil concentration did not differ significantly among spacings (Rissanen et al. 2002). In a different approach to plant density, Gil

(2002) investigated the response of cultivars of *Coriandrum sativum* to weed density and found that while essential oil yield was affected by other environmental variables (location and nitrogen addition) it was not significantly affected by weed density in the plots. The majority of the reports on plant density and essential oil production indicate that planting density should be determined to optimize biomass yield per hectare, as essential oil yield per plant is not significantly affected by plant density.

Aloysia citriodora Natural History and Essential Oil Composition

Lemon verbena, *Aloysia citriodora* Paláu (=*Aloysia triphylla* (L'Hér.) Britton; synonyms include: *Lippia citriodora* Kunth, *Lippia triphylla* (L'Hér.) Kuntze), Verbenaceae, is native to Argentina, Chile and Peru and is grown throughout Latin America as well as North Africa (Morocco), southern Europe, and parts of Asia (Botta 1979, Carnat et al. 1999, Rotman and Mulgura de Romero 1999). Lemon verbena is an aromatic, perennial shrub approximately 2.5 m tall. Leaves typically occur in whorls of 3 (though 4 and 5 are possible), are lanceolate and entire, and approximately 3-7 cm long by 1-2 cm wide. The leaves have trichome hairs on the underside. Flowers are white. The plant is easily propagated vegetatively (Botta 1979, Carnat et al. 1999, Rotman and Mulgura de Romero 1999).

Aloysia citriodora is valued for its medicinal use as a digestive aid, antimicrobial, antispasmodic, analgesic, and diuretic as well as being used as a treatment for colds, insomnia and anxiety (Botta 1979, Martinez and Pochettino 1992, Carnat et al. 1999, Rotman and Mulgura de Romero 1999). Infusions are typically made from dried or fresh leaves and it is consumed as a tincture, tea, or added to maté (a traditional drink made by steeping the leaves of *Ilex paraguariensis*). Antibacterial and antioxidant activity of *A. citriodora* have been demonstrated

for the essential oils, tea, and tinctures (Cowan 1999, Valentao et al. 2002, Ohno et al. 2003, Sartoratto et al. 2004, Pereira and Meireles 2007, Bilia et al. 2008). The leaves are also used as a flavoring agent in beverages, jellies and deserts. Essential oil extracts of the leaves are used extensively in the perfumery and cosmetic industries.

The major compounds responsible for *A. citriodora's* aroma and flavor are the mono- and sesquiterpenes found in its leaves and flowers. Reports on the essential oil composition of *A. citriodora* growing in its native range in South America indicate the presence of multiple chemotypes of this species (Gil et al. 2007). The commonly cultivated chemotype of *A. citriodora* worldwide is known as lemon verbena due to the dominance of lemon-scented monoterpenes, especially citral; however, even within this type there is a great deal of variation in the proportion of essential oil components. The oil is composed primarily of isomers of citral (i.e. neral and geranial, 10-40% depending on chemotype), accompanied by sabinene, limonene, cineole, geraniol, caryophyllene, linalool and spathulenol in varying proportions (Carnat et al. 1999, Santos-Gomes et al. 2005, Argyropoulou et al. 2007, Gil et al. 2007, Pereira and Meireles 2007, Bandoni et al. 2008).

The quality of the essential oil of lemon verbena is largely determined by the concentration of the two isomers of citral (neral and geranial) and limonene. Studies have shown that these compounds are present in all chemotypes of *A. citriodora*, but their concentrations vary considerably based on source of origin, plant part sampled, and plant developmental stage (Argyropoulou et al. 2007, Gil et al. 2007). Lemon verbena is easily propagated and grows well in cultivation under a broad range of climatic conditions. Lemon verbena is increasingly used in cosmetic and aromatherapy products as well as being grown for tea production and in home gardens for medicinal and culinary use. It is therefore important to determine the variability in

the production of various components of the essential oils, specifically the mono- and sesquiterpenes that contribute to its characteristic aroma. These attributes contribute to growing interest in developing cultivars of lemon verbena that allow growers to maximize the citral concentration (Gil et al. 2007). In addition to the lemon scented variety, other chemotypes have been indicated and documenting this chemodiversity could be used to develop protocols for good use practices with similar and/or related species of medicinal and aromatic plants. Though it exhibits the greatest chemotypic variation in its natural range, *Aloysia citriodora* is grown throughout the world. This allows experiments to be conducted without transfer of germplasm from countries of origin, thus we avoid conflict regarding genetic or intellectual property rights in conducting these studies. Finally, while *A. citriodora* is not threatened or endangered, other closely related aromatic species are. This study can be carried out without the pressures and constraints of working with an endangered species, but the results could be applied to developing management strategies and good agricultural and collection practices for many aromatic species.

Research Objectives

The development of good collection and cultivation practices in medicinal and aromatic plants requires research into each species' biology including: 1) natural variation of the chemicals of interest within and among populations, 2) the response of the plant physiologically and chemically to environmental variables that may act as stressors in its natural environment (e.g., water, nutrients, or light intensity) and 3) the horticultural practices that will affect chemistry and biomass yield. The objectives of this dissertation research are: 1) to study the effects of time of harvest and planting density on the composition and concentration of the mono- and sesquiterpenes of lemon verbena grown in a temperate climate (Chapter 2); 2) to investigate the

effects of water stress and UV-B solar radiation on the foliar concentrations and emissions of mono- and sesquiterpenes in lemon verbena(Chapter 3); and 3) to develop and analyze a rapid means of assessing terpene variation within and among wild populations of *Aloysia citriodora* in its native range in Argentina (Chapter 4).

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CHAPTER 2

CONCENTRATION OF MONO- AND SESQITERPENES FROM *ALOYSIA CITRIODORA* IN RESPONSE TO TIME OF HARVEST AND PLANT SPACING[2]

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ABSTRACT

Lemon verbena (*Aloysia citriodora* Paláu) is an aromatic, perennial shrub that is valued for its medicinal use (in tincture or tea form) as well as for its aromatic properties in culinary and cosmetic industries. We conducted a study to examine the effects of agronomic practices such as time of harvest and planting density on the composition and concentration of the mono- and sesquiterpenes of lemon verbena grown in a temperate climate. The dominant monoterpenes were the *cis*- and *trans*-isomers of citral (neral and geranial), followed by δ-limonene, β-ocimene, and δ-+-3-carene; while the dominant sesquiterpenes were germacrene-D, *trans*-caryophyllene, bicyclogermacrene, and α-curcumene. Terpene composition did not change significantly throughout the harvest period. Mono- and sesquiterpene concentration increased until peak flowering and then declined throughout the remainder of the growing season. Plant biomass was inversely related to plant density (grams/plant) but overall yield per hectare was highest in the highest density. Planting density did not significantly affect terpene concentration per gram leaf material or total yield per plant. These results indicate that there are optimal harvest times (near peak flowering) to increase essential oil yield per gram of leaf material while planting densities should be determined considering optimal leaf biomass yield for a given area. If being used fresh or in teas, adjustments for seasonal variation should be made when determining the amount of leaf material used.

KEYWORDS: *Aloysia citriodora*; lemon verbena; terpenoids; monoterpene; sesquiterpene; citral; plant density; seasonal variation; phytochemistry

INTRODUCTION

Aloysia citriodora Paláu (= *Aloysia triphylla* (L'Hér.) Britton; synonyms include: *Lippia citriodora* Kunth, *Lippia triphylla* (L'Hér.) Kuntze), Verbenaceae, is native to Argentina, Chile and Peru and is grown throughout Latin America as well as North Africa (Morocco), southern Europe, and parts of Asia (Botta 1979, Carnat et al. 1999, Rotman and Mulgura de Romero 1999). *Aloysia citriodora* is an aromatic, perennial shrub which is valued for its medicinal use (in tincture or tea form) as a digestive aid, antimicrobial, antispasmodic, analgesic, and diuretic as well as being used as a treatment for colds, insomnia and anxiety (Botta 1979, Carnat et al. 1999, Rotman and Mulgura de Romero 1999). Antibacterial and antioxidant activity of lemon verbena have been demonstrated for the essential oils, tea, and tinctures (Cowan 1999, Valentao et al. 2002, Ohno et al. 2003, Sartoratto et al. 2004, Pereira and Meireles 2007, Bilia et al. 2008). Essential oil extracts of the leaves are also used extensively in the flavoring and cosmetic industries.

The major compounds responsible for *A. citriodora's* aroma and flavor are the mono- and sesquiterpenes found in its leaves and flowers. Reports on the essential oil composition of *A. citriodora* growing in its native range in South America indicate the presence of multiple chemotypes[3] of this species (Gil et al. 2007, Bandoni et al. 2008). The commonly cultivated chemotype of *A. citriodora* worldwide is known as lemon verbena due to the dominance of lemon-scented monoterpenes, especially citral; however, even within this type there is a great deal of variation in the proportion of essential oil components. The oil is composed primarily of isomers of citral (i.e. neral and geranial, 10-40% depending on chemotype), accompanied by sabinene, limonene, cineole, geraniol, caryophyllene, linalool and spathulenol in varying

[3] A chemotype is a true-breeding chemical phenotype, based on the presence of certain complements of compounds (e.g., terpenes)

proportions (Carnat et al. 1999, Argyropoulou et al. 2007, Gil et al. 2007, Pereira and Meireles 2007, Bandoni et al. 2008).

The quality of the essential oil of lemon verbena is largely determined by the concentration of the two isomers of citral (neral and geranial) and limonene. Studies have shown that these compounds are present in all chemotypes of *A. citriodora*, but their concentrations vary considerably based on source of origin, plant part sampled, and plant developmental stage (Argyropoulou et al. 2007, Gil et al. 2007). Lemon verbena is easily propagated and grows well in cultivation under a broad range of climatic conditions; however no studies have reported the response of the essential oil composition to the parameters typically associated with agricultural production. Lemon verbena is increasingly used in cosmetic and aromatherapy products as well as being grown for tea production and in home gardens for medicinal and culinary use. It is therefore important to determine the variability in the production of various components of the essential oils, specifically the mono- and sesquiterpenes that contribute to its characteristic aroma.

The goal of the present work was to study the effects of agronomic practices such as time of harvest and planting density on the composition and concentration of the mono- and sesquiterpenes of lemon verbena grown in a temperate climate.

MATERIALS AND METHODS

Plant Material. Lemon verbena plants were obtained from a local nursery in Athens, Georgia (Wolfskin Growers). These plants were all of the lemon-scented variety commonly sold in US markets. Clones were made by propagating cuttings at the University of Georgia (UGA) Botany greenhouses. Clones were planted in a 1:1 mixture of Fafard #2 growing medium and

perlite, and were left for two weeks under mist, after which they were planted into 4 in pots in Fafard #2 growing mix. Plants were grown in the greenhouse for three weeks after rooting, acclimated for one week at the field site in pots and two weeks in the ground prior to being used in any experiments.

Time of Harvest. One hundred twenty *A. citriodora* clones were planted in a field at the UGA Horticulture Farm, with a spacing of 1 m between plants in mid-June 2001. Plants were watered as needed and were fertilized every two weeks throughout the experiment with soluble 20N-8P-12K. Once the plants were established, leaves were harvested at the beginning of each month (July-November). Two leaves (A and B) were collected from the middle node of five stems from two plants in each replicate (20 leaves total). The middle position was experimentally determined to be representative of all fully expanded, non-senescing leaves along any given stem (R.E. Chapman, unpublished data). Collections were made in the early morning of sunny days during the first week of each month of the harvest dates. The A and B leaves were placed in aluminum foil packets and stored in a cooler for transport to the lab for analysis. The ten A and B leaves were weighed for fresh leaf weight. The A leaves were used for terpene extraction and analysis. The B leaves were used to measure surface area and were dried for two days at 60 $^{\circ}$C to obtain dry weight data.

Plant Spacing. Clones were planted at the University of Georgia Horticulture Farm at three plant densities (0.75 m, 1.0 m and 1.25 m apart; 17,777, 10,000 and 6,400 plants per hectare). There were three replicate plots along the course of a single field separated by fallow areas. Plants were watered as needed and were fertilized every two weeks throughout the experiment with soluble 20N-8P-12K. Leaves were collected at the end of the growing season (early November) and were transported, processed, and analyzed as described above.

Terpene Extraction and Analysis. The A leaves were extracted two times in 30 mL of redistilled hexane (HPLC grade, Fischer Scientific) that contained 4 μL of trans-2-hexenyl butyrate as an internal standard. This yielded an extract of 60 mL, which was condensed to 6 mL by slowly flushing with purified nitrogen gas. Extracts were stored at -20 °C until they were analyzed by gas chromatography.

Chemical Standards. Analytical standards used were: Citral mixture of isomers (61 % cis, 36 % trans), nerol, geraniol α-humulene/ α-caryophyllene, R-(+)-limonene (Sigma, St. Louis, MO): β-caryophyllene, linalool oxide (TCI America, Portland, OR); α-pinene, myrcene, α-copaene, alloaromadendrene, germacrene-D, Bicyclogermacrene (Fluka Chem. Co., Milwaukee, WI); α-cubebene, S-(-)-limonene, α-terpineol, δ-(+)-3-Carene, δ-Cadinene, *trans*-nerolidol (Aldrich Chem. Co., Milwaukee, WI), caryophyllene oxide (ACROS, Morris Plains, NJ); β-pinene, piperitone (ICN, Plainview, NY). Trans-2-hexenyl butyrate was purchased from Sigma Chemical Co. (Milwaukee, WI) and used as an internal standard to quantify the concentration of all other compounds.

GC. Gas chromatography analysis was performed on a 1 μL aliquot of each sample using a Hewlett Packard 5890 (Hewlett-Packard, San Fernando, CA) gas chromatograph with a split-splitless injection port temperature of 225 °C and a flame ionization detector (FID) temperature of 280 °C. Separations were made on a 15 m x .53 mm i.d. fused silica column coated with DB-1 (Alltech Associates, Deerfield, IL). The samples were auto injected in the splitless mode (50 °C) with a purge time of 0.5 min and held at that temperature for 2 min, after which the oven temperature was increased to 130 °C at a rate of 2 °C /min followed by another increase in temperature of 10 °C /min to a final temperature of 280 °C, holding the column at 280 °C for 15 min. A Hewlett Packard 3396 Series III integrator was used to compute peak areas.

The relative concentrations were calculated from peak area ratios to the internal standard and expressed as µg/g dried leaf material. When available, analytical standards were used to determine absolute concentrations.

GC-MS. A Hewlett Packard model 5985B GC-MS system was used to identify the compounds. Mass spectrometer conditions were: ion source 200 °C; electron energy 70 eV; multiplier voltage 220 V; GC/MS interface zone 300 °C scan range 40-400 atomic mass units. Mass-spectral identification was done using the Wiley MS database. When available, analytical standards were used as reference compounds to compare retention times.

Statistical Analysis. Analysis of variance (ANOVA) was performed for all experiments, with multivariate analysis of variance (MANOVA) being used for the dominant terpenes when correlation coefficients were greater than 0.80. Means were compared with a Tukey-Kramer HSD test at $p < 0.05$ and Pearson Correlation Coefficients were calculated to determine correlation among dependent variables. Statistics were calculated using JMP v.7.0 and SAS v. 9.1 statistical packages.

RESULTS

The mono- and sesquiterpenes found in foliar extracts of lemon verbena throughout the growing season are listed in Table 2.1. The dominant monoterpenes were the *cis*- and *trans*-isomers of citral (neral and geranial), followed by δ-limonene, β-ocimene, and δ-+-3-carene. The dominant sesquiterpenes were germacrene-D, *trans*-caryophyllene, bicyclogermacrene, and α-curcumene with relative dominance varying with harvest date (Figure 2.1). The composition (presence of a certain complement of compounds) of these dominant terpenes was not affected

by harvest time or plant density; however the concentration of the compounds and in some cases the plant biomass were significantly affected by each of these variables.

Seasonal variation. Time of harvest had a significant effect on the concentration of the mono- and sesquiterpenes in lemon verbena (Table 2.1, n=20, $F<0.0001$ unless otherwise indicated, Figure 2.1). Composition of the main essential oil constituents was not affected by season, and geranial consistently had the highest concentration followed by neral then δ-limonene. The concentrations of neral, geranial, δ-limonene and all other monoterpenes were much higher In August (Table 2.1). The majority of sesquiterpenes were significantly higher in August, following a similar pattern as shown by the monoterpenes. A few sesquiterpenes, however, were present in trace amounts through much of the growing season, then increased at the November (α-caryophyllene, δ-cadinene) while others showed equally high levels in August and October (*trans*-nerolidol, caryophyllene oxide) (Table 2.1).

Plant spacing. There was a significant difference in biomass yield per plant at the three densities with plants at the highest density (0.75 m between plants) having significantly lower biomass yield per plant (54 g/plant, $F=0.0022$, n=9, Tukey-Kramer HSD, $p=0.05$). Plants grown at medium and high densities had an average plant biomass of 84.4 g and 108.3 g, respectively. When extrapolated to yield for a hectare plot, biomass yield was significantly effected by planting density, distinguishing the higher yield per hectare of the high density plots (976.96 kg/ha, $F=.0109$, n=9, Tukey-Kramer HSD, $p<.05$) from the low-density plots (692.82 kg/ha). The medium-density plots were not significantly different (844.33 kg/ha) from the other densities. Plant spacing did not significantly effect the foliar concentration of any mono- or sesquiterpenes in *A. citriodora* in terms of micrograms per gram dried leaf material or oil yield per hectare (Table 2.2).

DISCUSSION

The profile for the dominant terpenes in the leaf extracts of lemon verbena grown in this experiment is generally consistent with other reports of the essential oil composition of lemon verbena (Carnat et al. 1999, Santos-Gomes et al. 2005, Argyropoulou et al. 2007, Gil et al. 2007, Pereira and Meireles 2007). The general trend of an increase in terpenoid compounds from early in the growing season with a peak during the flowering stage (Table 2.1, July & August respectively) is in line with the results found by Argyropoulou et al.(2007), but we did not observe the shift in relative percentages of citral isomers and δ-limonene found in their study. Their sampling ended at peak flowering although the leaves continue to persist in temperate climates until frost (November in our study). It is important to note from the results of this study that after flowering and throughout the remainder of the growing season there is a dramatic drop in terpene concentration. The concentration of the major terpenes declined to a quarter, and up to a tenth, of the concentrations present in August (Table 2.1). This is commercially significant as this late developmental period represents approximately three months during which leaf samples are likely to be commercially harvested and sold as bulk material even though they have a substantially reduced essential oil yield per gram. The initial drop in concentration following flowering is much more dramatic than one would expect from dilution of the compounds due to leaf expansion. Terpenes are mobile carbon resources within the plant and while the leaves are not senescing, this decline in terpene concentration likely indicates a shift in the allocation of carbon resources mediated through the catabolism of the mono- and sesquiterpenes as a carbon source (Croteau 1988, Mihaliak et al. 1991, Gershenzon 1994). A portion of the decline may be due to continued release of the volatile mono- and sesquiterpenes from the leaf trichomes without compensation via synthesis but these levels are often low unless induced by herbivory or

some other means (McGarvey and Croteau 1995, Baldwin and Preston 1999, Gershenzon et al. 2000, Sangwan et al. 2001, Figueiredo et al. 2008, Frost et al. 2008).

The inverse relationship between plant density and foliar biomass indicates there is an optimal planting density, above which one will not receive a significant increase in yield per hectare. The oil yield becomes a product of biomass yield as there were no significant differences in oil yield related to the planting densities in this study. This does not comply with many conventional ecological theories regarding carbon-based secondary metabolites such as the mono- and sesquiterpenes. These results do, however, coincide with data collected for other species of medicinal and aromatic plants. Similar results have been shown for leaf biomass and terpene/essential oil production for lavender, thyme, Australian mint, and Hungarian & Tasmanian perppermint (Clark and Menary 1979, Shalaby and Razin 1992, Zheljazkov and Topalov 1996, Rissanen et al. 2002, Arabaci et al. 2007).

Based on the results of our study lemon verbena should be planted in stands of high to intermediate density and harvested during peak flowering time (i.e. August in temperate climates of northern latitudes) to optimize the yield of essential oils from the leaves. If being used fresh or in teas, adjustments for seasonal variation should be made when determining the amount of leaf material used.

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Table 2.1: Foliar mono- and sesquiterpenes extracted from lemon verbena throughout the growing season[4].

Compound[5]	ID method[6]	July[7]	August	September	October	November
α-Pinene	MS, std	294.74 b[8]	739.38 a	146.73 bc	18.10 c	94.80 bc
δ-(+)-3-Carene	MS, std	482.50 b	1378.30 a	193.64 bc	26.57 c	125.91 c
β-Myrcene	MS, std	198.06 b	398.28 a	45.90 c	tr[9] c	3.95 c
δ-Limonene	MS, std	2318.77 b	4808.04 a	1143.14 bc	330.38 c	955.67 c
β-Ocimene	MS	829.67 b	1524.71 a	79.55 c	tr c	8.39 c
γ-Terpinene	MS	196.02 b	471.95 a	95.66 bc	31.47 bc	111.97 c
α-Terpineol	MS, std	169.16 b	432.65 a	45.23 c	tr c	41.21 c
***trans*-Chrysanthemal**	MS,	308.47 b	785.63 a	81.71 c	tr c	68.01c
β-Fenchyl alcohol	MS	365.40 b	1030.45 a	137.48 c	36.80 c	159.91 c
Neral (*cis*-Citral)	MS, std	2764.64 b	11911.71 a	1280.08 b	1466.80 b	1162.74 b
Geranial (*trans*-Citral)	MS, std	4785.72 b	16950.77 a	1304.13 c	1318.38 c	980.39 c
Bicycloelemene	MS	9.12 b	214.09 a	56.53 ab	tr b	9.43 b
α-Copaene	MS, std	154.10 bc	795.22 a	131.63 bc	tr c	200.98 b
β-Bourbonene	MS	43.75 bc	317.45 a	80.03 bc	tr c	119.60 b
Nerol (Geranyl proprionate)	MS, std	129.32 b	593.94 a	47.15 b	tr b	63.57 b
***trans*-Caryophyllene**	MS, std	555.06 b	1832.85 a	147.93 bc	80.37 c	118.09 c
α-Caryophyllene	MS, std	tr b	tr b	tr b	tr b	98.38 a
Germacrene-D	MS, std	1224.01 b	3068.24 a	148.62 bc	tr c	tr c
α-Curcumene	MS	454.15 bc	2538.12 a	532.66 b	845.23 b	tr c
Bicyclogermacrene	MS, std	990.91 ab	1710.17 a	66.86 b	tr b	347.75 b
1-H-3a, 7-Methanoazulene	MS	489.93 b	1395.11 a	128.09 bc	tr c	tr c
δ-Cadinene	MS, std	24.61 b	tr b	tr b	tr b	137.05 a
***trans*-Nerolidol**	MS, std	111.12 b	432.48 a	61.00 b	592.16 a	125.47 b
(-)-Spathulenol	MS	371.82 b	1283.65 a	481.78 b	tr c	583.93 b
Caryophyllene oxide	MS, std	235.28 b	1452.10 a	797.98 ab	1497.58 a	878.12 ab

[4] Compounds presented (µg per gram dried leaf material) are averages of 4 replicates at each sample date.
[5] Mono- and sesquiterpenes are presented in order of their elution from a DB-5 column.
[6] Identification method: MS: by comparison with spectral libraries; std: identification by injection of standard.
[7] Leaves were harvested at the beginning of each month.
[8] Compounds with different letters are significantly different according to Tukey-Kramer HSD at alpha = 0.05
[9] These compounds were present but in trace amounts (below the threshold of the GC integrator)
[i]F<.0097, [ii]F<,0020m, [iii]F<.0008

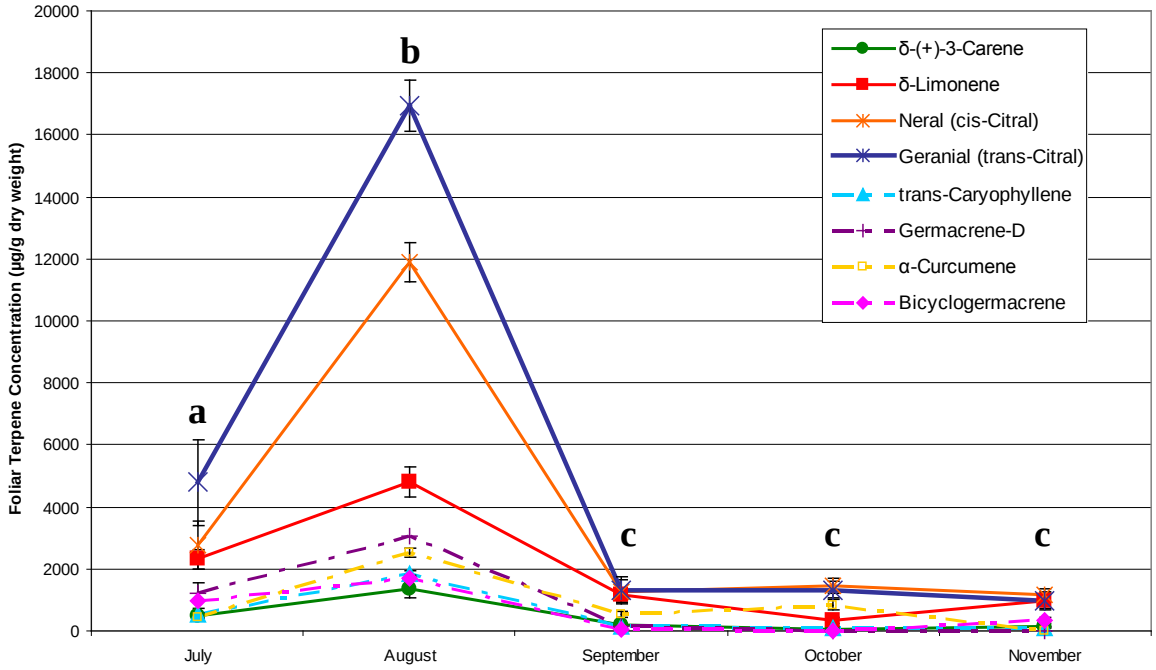

Figure 2.1: The dominant monoterpenes (δ-+3-carene, δ -limonene, neral, and geranial) and sesquiterpenes (germacrene-D, α-curcumene, trans-caryophyllene, and bicyclogermacrene) were significantly affected by time of harvest and illustrate the trend for all terpenes in relation to time of harvest (n=20, F<0.001, MANOVA).

Table 2.2: Foliar mono- and sesquiterpenes extracted from lemon verbena in different plant densities[10].

Compound[11]	ID method[12]	Plant Spacing 0.75 m µg/g dry wt	0.75 m g/hectare	1.0 m µg/g dry wt	1.0 m g/hectare	1.25 m µg/g dry wt	1.25 m g/hectare
α-Pinene	MS, std	193.9	196.1	52.2	43.1	342.6	242.3
δ-(+)-3-Carene	MS, std	725.4	739.4	244.5	202.3	649.5	456.4
β-Myrcene	MS, std	31.8	32.7	1.9	1.7	23.1	16.8
δ-Limonene	MS, std	2516.9	2581.8	1044.9	866.2	1553.5	1106.1
γ-Terpinene	MS	136.6	140.4	58.8	49.0	77.5	54.8
α-Terpineol	MS, std	13.3	13.2	44.8	38.1	113.1	79.1
***trans*-Chrysanthemal**	MS	0.1	0.1	0.0	0.0	0.1	0.1
β-Fenchyl alcohol	MS	193.7	189.4	94.8	79.8	120.1	85.3
Neral (*cis*-Citral)	MS, std	2140.0	2139.7	1382.1	1167.6	1569.8	1122.3
Geranial (*trans*-Citral)	MS, std	1485.6	1471.4	1076.8	906.0	2054.0	1480.3
α-Copaene	MS, std	119.3	122.2	112.6	94.4	136.6	95.9
β-Bourbonene	MS	45.1	44.6	66.6	55.9	78.2	54.9
Nerol	MS, std	7.8	7.7	35.4	30.1	45.9	32.7
***trans*-Caryophyllene**	MS, std	97.3	99.5	129.2	108.9	225.1	156.2
α-Caryophyllene	MS, std	40.1	39.7	53.6	45.2	54.0	37.9
Bicyclogermacrene	MS, std	600.8	577.5	184.9	154.5	417.4	292.6
δ-Cadinene	MS, std	16.7	16.5	76.7	64.6	88.6	62.6
***trans*-Nerolidol**	MS, std	14.7	14.5	72.2	61.8	97.7	69.3
(-)-Spathulenol	MS	643.1	618.4	366.5	309.1	400.4	281.3
Caryophyllene oxide	MS, std	1087.6	1037.1	529.6	444.2	879.4	600.7

[10] Compounds presented are averages of 3 replicates at each plant density.
[11] Mono- and sesquiterpenes are presented in order of their elution from a DB-5 column.
[12] Identification method: MS, by comparison with spectral libraries; std, identification by injection of standard.

CHAPTER 3

EFFECTS OF DROUGHT STRESS AND SOLAR UV-B RADIATION ON THE FOLIAR CONCENTRATION AND EMISSIONS OF MONO- AND SESQUITERPENES IN LEMON VERBENA (*ALOYSIA CITRIODORA*)[13]

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ABSTRACT

We investigated the effects of UV-B radiation and water stress on the mono- and sesquiterpenes produced by lemon verbena (*Aloysia citriodora*) which is native to semi-arid, mountainous regions of Argentina, Chile and Peru. While these compounds are recognized for their activity in a variety of ecological roles such as feeding deterrents, antimicrobial agents, pollination attractants, and as allelopathic chemicals, it has also been proposed that they play a role in reducing oxidative stress caused by abiotic factors in the environment (e.g., light and water stress). The objective of our study was to elucidate the effects of UV-B radiation and drought stress on the foliar concentration and emission of terpenes in lemon verbena. Plants were grown in pots outside for both the water stress and UV-B exclusion experiments. Foliar concentrations of terpenes were analyzed using gas chromatography-mass spectrometry (GC-MS) and emissions were collected from intact leaves via dynamic headspace sampling and analyzed with a thermal desorption-GC-MS system. Water stress significantly increased terpene concentration for multiple mono- and sesquiterpenes (e.g., α-pinene, β-phellandrene, α-cubebene and caryophyllene) and significantly reduced foliar biomass yield per plant. Exclusion of ambient UV-B radiation increased foliar concentrations of geranial (*trans*-citral) and β-bourbonene but did not significantly affect other terpenes. Mono- and sesquiterpene emissions were not affected by the exclusion of UV-B radiation or water stress. Terpenes are important in plant physiology and ecological interactions at multiple trophic levels. The role of UV-B- or water stress-induced alteration of terpene synthesis and storage should be incorporated in theoretical models of plant defense, response to abiotic stresses, and carbon allocation.

KEYWORDS: *Aloysia citriodora*; lemon verbena; terpenoids; monoterpene; sesquiterpene; abiotic stress; UV; water stress; phytochemistry; emissions; plant volatiles

INTRODUCTION

The terpenoids are recognized for their activity in a variety of ecological roles such as feeding deterrents, antimicrobial agents, pollination attractants, as well as being allelopathic and plant communication chemicals. These functions (i.e. defense and pollination) have dominated the development of theories regarding the production of terpenes as well as most other secondary metabolites (Fraenkel 1959, Harborne 1991, Gershenzon 1994, Langenheim 1994). It has recently been proposed, however, that abiotic stresses such as drought, photodamage, nutrient deficit and climate change acting individually as well as synergistically may be the important driving forces in the evolutionary development and contemporary production of many types of secondary metabolites including terpenes (Close and McArthur 2002, Penuelas and Llusia 2003, Holopainen 2004, Peñuelas and Munné-Bosch 2005).

Current models for climate change predict a continued decline in annual precipitation over landmasses in temperate climates (10^{o} N to 30^{o} N latitude) with greater extremes expected in precipitation and temperature in the future (IPCC 2007). These changes in climate could exacerbate the extremes and change the current patterns of two common environmental plant stressors, low moisture and high light. This is particularly true in the semi-arid mountainous regions and/or Mediterranean climates from which many aromatic plants originate. An increased understanding of the relationship between the abiotic stressors and plants' chemical responses is critical to developing better descriptive and predictive models of plant chemical responses to changing environments.

Carbon-based plant secondary chemicals such as the terpenoids have been researched extensively in terms of competing defense hypotheses: Optimal Defense (OD), Carbon: Nutrient Balance (CNB), Growth Rate (GR), and Growth-Differentiation Balance (GDB) (Stamp 2003).

The volatile terpenoids have been of particular interest due to their roles as both constitutive and inducible defenses as well as being involved in multiple incidences of plant-pollinator and multi-trophic level interactions (Langenheim 1994). Studies over the past decade have indicated that, in addition to isoprene, and especially in those species that do not emit isoprene, the low molecular weight terpenes (mono- and sesquiterpenes) may also act to ameliorate abiotic stress responses by scavenging and neutralizing the reactive oxygen species (ROS) that are commonly produced when a plant undergoes various abiotic stresses (Sharkey and Loreto 1993, Velikova and Loreto 2005, Vickers et al. 2009a). Vickers et al. (2009) proposed a new model for evaluating the role of volatile isoprenoids in their "single biochemical mechanism for multiple physiological stressors" in which abiotic selective pressures receive prominence. This model is built on decades of research with isoprene and recent studies of other low molecular weight isoprenoids, and they highlight the need for a greater body of research that investigates mono- and sesquiterpene accumulation and emission in response to dominant abiotic stresses.

While terpene responses to water stress have been investigated in light of prevalent theories regarding defense hypotheses, the increased levels of terpenes have often been attributed to a simple decrease in leaf expansion and thus an increase in concentration in terpene-storing species such as pine and mint; based on those theories the increase in terpenes is due simply to reduced growth/leaf expansion (Gershenzon et al. 1978, Gershenzon 1984, Gershenzon and Croteau 1991, Gershenzon et al. 2000). Many studies have found that plants respond contrary to that simple model and that the responses vary greatly dependent on species and environment as well as native geographic range. Water deficits have been shown to increase concentration in the essential oils of several species (e.g., *Marjorana hortensis, Mentha piperita*, and *Satureja douglasii*), while Penuelas and Llusiá (1997) found no significant effects of water stress on the

accumulation of essential oils in *Rosmarinus officinalis* and postulated that this was due to this species' adaptation to semi-arid environments with thickened, narrow leaves (Gershenzon et al. 1978, Clark and Menary 1980). Most essential oil work related to drought stress has been conducted with an eye toward agricultural production rather than ecological roles/drivers and little has been done concerning ecological models that explore plant terpenoids and their emissions related to abiotic rather than biotic pressures.

In addition to water stress, varying models predict increased levels of incident ultraviolet radiation (particularly UV-B radiation) relative to photosynthetic active radiation to continue for the next 50 years, with an expected leveling off thereafter. Many plants are native to regions with historically high UV-B levels and some new theories postulate that UV radiation may be as important a selective pressure for many plant secondary metabolites as herbivory (Close and McArthur 2002, Peñuelas and Munné-Bosch 2005, Vickers et al. 2009a). The magnitude of increased UV-B due to current and projected ozone loss does not exceed the geographical differences in UV-B from the equator to the poles or from sea level to high mountains, thus understanding the high altitude/high latitude species' responses to UV stress may be a key to predicting plant response patterns to these changes in the environment (Rozema et al. 1997a, Rozema et al. 1997b). Many terrestrial plant secondary metabolites (e.g., flavonoids, lignin, and tannins) filter UV-B radiation and reduce its damaging effects. The terpenoids are an additional class of secondary metabolites that have been proposed as an evolutionary base for photoprotection (Karousou et al. 1998, Zavala and Ravetta 2002, Peñuelas and Munné-Bosch 2005). Certain terpenoids such as carotenoids and tocopherols have well-known roles in photoprotection. Furthermore, there is strong evolutionary evidence of conservation of these compounds, in that all photosynthetic organisms contain carotenoids such as the carotenoid-

xanthophyll complexes that assist in dissipating excess light energy as heat or β-carotene, an oxygen-free radical scavenging species and membrane stabilizer (Havaux 1998, Peñuelas and Munné-Bosch 2005). Many terpenoids increase in response to elevated photosynthetic active radiation (PAR, 400-700 nm), but their response to UV-B is less understood (Bohlmann et al. 1998).

Few studies have investigated the mono- and sesquiterpenes that comprise the majority of the essential oils of many terpene storing species in response to this particular form of light stress. The overall level of essential oils have been shown to respond to UV-B exposure in the field as well as in growth chamber and greenhouse experiments in *Grindelia chiloensis*, *Ocimum basilicum* and *Mentha spicata* (Karousou et al. 1998, Johnson et al. 1999, Zavala and Ravetta 2001, Zavala and Ravetta 2002). The responses of individual terpenoids, however, were either not investigated or were not shown to differ significantly between plants exposed to ambient/elevated UV-B and those that were not.

In light of new theories on the roles of abiotic stresses as drivers for plant terpenoid production we designed a study to investigate the effects of UV-B radiation and water stress on the mono- and sesquiterpenes produced by lemon verbena, which is native to semi-arid, mountainous regions of Argentina, Chile and Peru, and is grown in cultivation throughout the world. We developed an experiment to elucidate the effect of the environmental stresses of UV-B and water stress on the foliar concentration and emission of specific terpenes in lemon verbena grown in a temperate climate. Our objectives were to: 1) determine the effects of ambient UV-B radiation on leaf biomass, foliar concentration and emission of mono- and sesquiterpenes and 2) study the effects of prolonged water stress on growth (leaf biomass), photosynthetic efficiency

(chlorophyll fluorescence) and mono and sesquiterpene foliar concentration and emissions in lemon verbena grown in a temperate climate.

MATERIALS AND METHODS

Plant Material. Lemon verbena plants were obtained from a local nursery in Athens, Georgia (Wolfskin Growers). These plants were all of the lemon-scented variety commonly sold in US markets. Clones were made from one original plant by propagating cuttings at the University of Georgia (UGA) Botany greenhouses. Clones were planted in a 1:1 mixture of Fafard #2 growing medium and perlite, and were left for two weeks under mist, after which they were acclimated outside of mist for one week. The plugs were then planted into four-inch pots in Fafard #2 growing mix. Plants were grown in the greenhouse for three weeks after rooting, and then transferred to white one-gallon pots.

UV-B-Radiation Exclusion Experiment. Eighty lemon verbena clones were placed in a randomized block design. Ten plants were placed in each of four blocked replicates per treatment in an outside area at the University of Georgia's Horticulture Farm in Watkinsville, GA. Mylar plastic was used as a filter to exclude UV-B radiation (wavelength $\leq$ 330 nm) without significantly reducing photosynthetic active radiation. Stretch film was used as the full spectrum control and did not reduce UV-B or PAR. There were no significant reductions in transmittance of the plastic over the two month course of the experiment. The plastic was secured to adjustable PVC frames and was adjusted regularly to keep the plastic 5 – 10 cm above the tops of the plants throughout the experiment. Frames were positioned and angled according to the sun's path for May-July and thus filtered incident radiation. Plants were watered as needed and were fertilized on a bi-weekly basis. Soil and air temperature did not differ between treatments. Plants were

grown under experimental conditions for two months. Volatile collection and foliar terpene extraction methods are outlined below. The volatile emissions results were expressed as an average of two collections per plant and two plants per replicate; these were averaged and compared for four replicates of each of the two treatments. The foliar terpene concentrations are expressed as an average of three, 1 μL injections from each sample (plant). Three plants from the center of each blocked replicate were analyzed for four blocked replicates.

Water Stress. Twenty *A. citriodora* clones were placed in a randomized design and allowed to acclimate outside for two weeks before onset of water stress. Plants were watered to runoff every other day and plants were fertilized before and every two weeks throughout the experiment with soluble 20N-8P-12K. The water stress treatment was applied in a modified method outlined by Bianco et al. (2000). Ten plants from the well-watered group were weighed every other morning on a Mettler Toledo (Greifensee, Switzerland) 32000 balance to the nearest gram to determine the evapotranspiration rate ($ET/g^{-1}\ day^{-1}$). Water was then supplied every other day to the plants in the water-stressed (WS) group at a rate of 50% of ET. The well-watered plants were watered to run off every other day. In the water-stress groups, water was supplied to plastic containers under the plants to ensure water reached the roots and to allow dry-down to approximate what would happen in the field under progressive drought conditions. Reduction in stem elongation was used to approximate water stress based on previous experimental correlations (R.E. Chapman unpublished data). When significant water stress was indicated, the volatile analysis was conducted and leaves were harvested for foliar terpene analysis and biomass yield (see below). The results of the water stress volatiles are an average of two collections per plant, and four replicates of each treatment (water-stressed and well-watered). The foliar terpene concentrations are expressed as an average of three, 1 μL injections

from each sample (plant). Six replicates (plants) were analyzed per treatment for the water-stress study.

Chemical Standards. Analytical standards used were: citral mixture of isomers (61 % cis, 36 % trans), nerol, geraniol α-humulene/ α-caryophyllene, R-(+)-limonene (Sigma, St. Louis, MO): β-caryophyllene, linalool oxide (TCI America, Portland, OR); α-pinene, myrcene, α-copaene, alloaromadendrene (Fluka Chem. Co., Milwaukee, WI); α-cubebene, S-(-)-limonene (Aldrich Chem. Co., Milwaukee, WI), caryophyllene oxide (ACROS, Morris Plains, NJ); β-pinene, piperitone (ICN, Plainview, NY). Trans-2-hexenyl butyrate was purchased from Sigma Chem. Co. (Milwaukee, WI) and used as an internal standard to quantify concentration of all other compounds; (-)-menthone was purchased from Fluka Chem. Co., Milwaukee, WI and was used as in internal standard for emissions.

Dynamic Headspace Sampling. Intact *A. citriodora* stems, 16-18 cm in length, were fitted with a specially constructed collar that would form the cap of a 500 ml enclosure for dynamic throughput headspace sampling. At the time of volatile collection, plants were brought from the field experiment into a growth chamber and allowed to acclimate for 24 hours. In the UV-B experiment the growth chambers were fitted with appropriate filters to maintain UV-B exclusion and control treatments. During the collection process plants were maintained at standard air temperatures of approximately 30º C and PAR of 600 mol $m^{-2}s^{-1}$ Volatiles were collected by sealing the intact stems in a 500 mL jar using silicone gel approximately 5 days prior to trapping. Air was immediately passed through the jar using a vacuum that was moderated by a needle regulator at 500 mL min^{-1} for two hours Air entering the jar passed through carbon filter, then through 200 mg of 60/80 mesh Tenax-TA (Alltech Assoc. Inc., Deerfield, IL) packed in two cm of a modified pasture pipette and contained with silated glass

wool. After passing through the collection chamber, the volatiles were trapped in a 10 cm long, 6 mm o.d., 4 mm i.d. stainless steel sorbent trap (Scientific Instrument Services, Inc. Ringoes, NJ) which contained 100 mg of 60/80 Tenax contained with silated glass wool. The Tenax trap was preconditioned at 280 ºC for 2 hr with purified He at 20 mL min^{-1}. Connections in the trapping system were made with nalgene tubing. Menthone (5 mL collected from supersaturated air) was injected in the first minute of each 2 h collection as an internal standard.

Short Path Thermal Desorption. The samples were desorbed at an initial temperature of 200 ºC for 3 min, then the temperature was increased to 220 ºC for 2 min, and finally to 250 ºC where it was held for 2 min. Thermal desorption occurred with He at a flow rate of 10 mL min^{-1} onto the gas chromatograph column (GC/MS, model 6890N/5973, Agilent, Palo Alto, CA) using an automated short-path thermal desorption system (model TD-5, Scientific Instrument Services, NJ). Compounds were collected on the first 4 cm of the GC column using a CO_2 cooled cryofocus trap (-40 ºC) (SIS 2" Cryo-Trap, Scientific Instrument Services, NJ). After desorption the cryofocus trap was rapidly heated to 200 ºC to elute the compounds at which point the GC program started.

GC/MS Analysis of Volatiles. Identification and quantification of the volatile compounds were performed using a GC/MS equipped with a 30 m, 0.25 mm i.d., 0.25 μm fused silica capillary column (HP-5MS, Agilent, Palo Alto, CA). The injection port temperature was 225 ºC, with a split ratio of 5:1. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The initial temperature of the column was 32 ºC, the GC oven was held at that temperature for 5 min. The oven temperature was then increased at a rate of 1 ºC min^{-1} to 45 ºC and held for 5 min., increased again at ºC min^{-1} to 70 ºC and held for 5 min., then increased at a rate of ºC min^{-1} to 120 ºC at which point the oven temperature was increased at 30 ºC min^{-1} to 280 ºC and held

for 10 min. Mass spectrometry conditions were: ion source, 230 ºC; electron energy 70 eV; multiplier voltage 1247 V; GC/MS interface zone, 280 ºC; and scan range, 35 – 350 mass units with a threshold of 150 mass units.

Identification and Quantification of Volatile Compounds. Compounds were identified based on comparison of their mass spectra and relative abundances with NIST 02 and Wiley 7 spectral data bases. These identities were confirmed by comparison of the Kovats retention index (RI) and mass spectra with authentic standards when available. The levels of volatile compounds are expressed in ng g^{-1} when standards were available (e.g., α-pinene, limonene, *cis*- and *trans*-isomers of citral, *trans*-caryophyllene, and alloaromadendrene); others are expressed as menthone equivalents (ng g^{-1} assuming a response factor of 1). The concentration of the internal standard, menthone, which was injected into samples during collection, was determined by comparison to a standard curve for menthone generated by direct injection into the GC of a series of concentrations of menthone in hexane.

Terpene Extraction and Analysis. Plants were harvested at the end of each experiment as outlined above. The leaves were stripped from the plants, and fresh weights were recorded for leaf biomass. Two, 2.5 g samples of the leaves were taken (exact fresh weights were recorded) and labeled A and B leaves. The A leaves were extracted two times in 10 mL of redistilled hexane (HPLC grade-Fischer Scientific) that contained 4 μL of trans-2-hexenyl butyrate as an internal standard. This yielded an extract of approximately 10 mL, which was condensed to 7.5 mL by slowly flushing with purified nitrogen gas. Extracts were sealed in glass scintillation vials and stored at -20 °C until they were analyzed by gas chromatography. The B leaves were scanned and leaf areas were calculated using NIH-Image J. These leaves were dried at 60 ºC for

48 hours. The relationship of the B leaves fresh weight to dry weight ratio was used to approximate the dry weight of the A leaves from the known fresh weight.

Identification and Quantification of Foliar Compounds. Gas chromatography analysis was performed on 1 μL of each sample using a Hewlett Packard 5890 (Hewlett-Packard, San Fernando, CA) gas chromatograph with a split-splitless injection port temperature of 225 °C and a flame ionization detector (FID) temperature of 280 °C. Separations were made on a 15 m x .53 mm i.d. fused silica column coated with DB-1 (Alltech Associates, Deerfield, IL). The samples were auto injected in the splitless mode (32°C) with a purge time of 0.5 min. and held at that temperature for 5 min. The oven temperature was then increased at a rate of 1 ºC min^{-1} to 45 ºC and held for 5 min., increased again at ºC min^{-1} to 70 ºC and held for 5 min., then increased at a rate of 1 ºC min^{-1} to 120 ºC at which point the oven temperature was increased at 30 ºC min^{-1} to 280 ºC and held for 10 min.

A Hewlett Packard model 5985B GC-MS system was used to identify the compounds. Mass spectrometry conditions were: ion source, 230 ºC; electron energy 70 eV; multiplier voltage 1247 V; GC/MS interface zone, 280 ºC; and scan range, 35 – 350 atomic mass units. Mass-spectral identification was done using the NIST 02 and Wiley 7 mass spectral databases. When available, analytical standards were used as reference compounds to compare retention times.

A Hewlett Packard 3396 Series III integrator was used to compute peak areas. The concentrations were calculated from peak area ratios to standard peaks for α-pinene, β-phellandrene, δ-limonene, linalool, *cis*- and *trans*-citral, α-cubebene, and caryophyllene. All other compounds' concentrations are relative to the internal standard (trans-2-hexenyl butyrate)

and expressed as μg g^{-1} dried leaf material. When available, analytical standards were used to determine absolute concentrations as detailed above.

Statistical Analysis. Student's t-test and analysis of variance (ANOVA) were performed for experiments. Multivariate analysis of variance (MANOVA) was used for the dominant terpenes when correlation coefficients were greater than 0.80. In these cases means were compared with a Tukey-Kramer HSD test at $p < 0.05$ and Pearson Correlation Coefficients were calculated to determine correlation among dependent variables. Statistics were calculated using JMP v.7.0 and SAS v. 9.1 statistical packages.

RESULTS

Physiological parameters. Plants that were water stressed had a significantly lower leaf biomass (8.34g) than those that were not stressed (12.12 g; p=.017), as well as smaller leaf areas and slower stem elongation. Total stem length did not differ significantly between the two treatments (23.9cm in water stressed plants and 23.4cm in non-stressed plants). Mid-day quantum yield of chlorophyll fluorescence was significantly higher in water stressed plants (760 vs. 715, p=.008), though that difference is not considered biologically relevant indication of stress as neither measures are at levels known to indicate compromise of photosystem II (PSII). UV-B exposure did not have a significant effect on leaf biomass in the exclusion experiment. Plants under ambient UV-B had slightly longer stems versus those from which UV-B was excluded (26 cm vs. 24cm, p= .0039) and larger leaf areas in general. Chlorophyll fluorescence indicates that PSII was not significantly affected by UV-B treatment, as there were no significant differences between mid-day, dark-adapted quantum yields.

Phytochemistry. We identified 21 different mono- (10) and sesquiterpenes (11) from the foliar extracts of lemon verbena (Tables 3.1 & 3.2). Four of the ten monoterpenes and seven of the eleven sesquiterpenes had significantly higher foliar concentrations in water stressed plants (μg per g dry weight; Table 3.1, Figure 3.1). Plants exposed to ambient levels of UV-B radiation had significantly lower levels of the dominant citral isomer, geranial (a Character Impact Compound, CIC, for lemon verbena); the monoterpene, linalool, was only present in detectable amounts in those plants from which UV-B was excluded while the sesquiterpene, β-bourbonene, was only present in detectable amounts in the presence of ambient UV-B radiation (Table 3.2).

Over 100 compounds were collected as emissions from the leaves of *A. citriodora* from which 27 mono- and sesquiterpenes were identified (Tables 3.3 and 3.4). Many of the emitted terpenes were consistent with those found in foliar extracts in this experiment, however there were some differences in the constituents of the terpenes (e.g., α-pinene and sabinene are present in foliar extracts but absent from emissions; α-terpineol and piperitone are emitted but are not found stored in the leaf, Table 3.5). Emitted mono- and sesquiterpenes did not show significant differences in response to water stress or UV-B radiation and were not detected in large enough concentrations to have a significant effect on the overall terpene pool over the course of the growing season.

DISCUSSION

The qualitative profile for the dominant terpenes in the foliar extracts of *A. citriodora* in both the UV-B exclusion and water stress experiments are generally consistent with other reports of the essential oil composition of lemon-scented *A. citriodora*, lemon verbena (Carnat et al. 1999, Santos-Gomes et al. 2005, Argyropoulou et al. 2007, Gil et al. 2007, Pereira and Meireles

2007). Water stress, representing 30-50% reduced water over the course of two months of active growth, significantly increased foliar terpene concentrations per gram dry weight of many of the mono- and sesquiterpenes and significantly reduced leaf biomass yield per plant but did not have an effect on either mono- or sesquiterpene emissions (g^{-1} min^{-1} fresh or dry weight) for lemon verbena (Table 3.1, 3.3, 3.5 & Fig. 3.1). This decrease in leaf biomass due to water stress is the expected physiological response due to reduced carbon fixation and is a common growth trade-off for survival when plants experience a water deficit. Artificially excluding ambient levels of UV-B radiation in a temperate climate increased the foliar concentration of the dominant monoterpene, geranial (*trans*-citral) but did not have a significant effect on the foliar concentration or emissions of other types of mono- or sesquiterpenes; furthermore, though UV-B exclusion decreased stem length significantly, it did not affect leaf biomass yield or fresh weight to dry weight ratios (Table 3.2, 3.4 & Fig. 3.2). One would expect to find decreased stem length in plants exposed to UV-B radiation based on the results of previous studies relating UV-B and plant architecture (Day and Neale 2002). Our results indicate that some level of ambient UV-B radiation may be necessary for proper stem elongation since many studies regarding UV-B and plant architecture were investigating increased rather than ambient UV-B. The general impact of UV-B on plant biomass is less clear, however, as results of studies had varying outcomes (Jansen et al. 1998, Day and Neale 2002). Phytochemistry results (emissions and foliar concentrations of mono- and sesquiterpenes) in response to UV-B and water stress are discussed below.

Emissions. Terpene emissions did not demonstrate significant responses for either abiotic stressor. Emissions have not been previously reported for this species, and it is interesting to note that the quality (constituents) of the emitted terpenes differed slightly from the foliar terpene pool (Table 3.3 vs. 3.1, Table 3.5). Most mono- and sesquiterpenes in lemon verbena are

hydrophobic and relatively small molecules, thus they can move through the cuticle of the leaf and those surrounding special storage/secretory structures via simple diffusion. This is supported by the lack of significant differences between those terpenes emitted in water stressed versus non-stressed plants. If any of the measured terpenes from lemon verbena were escaping from the interior of the leaf through the stomata, their emission would have been affected by the stomatal closure associated with water stress, however, the majority of studies involving terpenoid emission in "storing" species have shown that there is no relationship between emissions and stomatal conductance (Kesselmeier and Staudt 1999). Monoterpene emissions that emanate from storage sites (e.g., glandular trichomes, resin pools) have been shown to be temperature dependent and related to vapor pressure and transport resistance along their diffusion path. The exceptions to this are those terpenes that are emitted, but are not found in the terpene pool. The emission rates for non-stored compounds are thought to be directly related to biosynthesis and conversion. Light may have a stronger effect on the emission of non-stored terpenes while water stress affects the emission of other compounds (e.g., organic acids) that exit the plant via the stomata (Kesselmeier et al. 1997, Llusia and Penuelas 1998, Cinege et al. 2009). If one examines the ratio of emitted compounds to the foliar concentrations of the same compound, regardless of treatment (Fig. 3.3), these do not seem to be following the ratios one would expect for consistent passive diffusion from a stored pool as suggested by (Dudareva and Pichersky 2000). These patterns indicate that some mechanism of differential release is operating. While these mechanisms are commonly discussed when investigating plants' response to wounding or production of pollination attractants, differential release has not been previously examined experimentally in relation to abiotic factors. The emitted compounds represent a very small fraction of the foliar terpene pool (1.0E-6 to 1.0E-9 %) yet even this small a fraction may factor

strongly into herbivore detection and/or preference (Carroll and Hoffman 1980, von Dahl et al. 2006, Zavala and Baldwin 2006, Laothawornkitkul et al. 2008, Loivamaki et al. 2008).

UV-B. Our findings of higher levels of select monoterpenes are similar to those results found, in terms of foliar phytochemistry, in artificially increased levels of UV-B for *Achillea millefolium* with the lower UV-B levels producing slightly higher concentrations of foliar terpenes (Thines et al. 2007). Zavala and Ravetta (2002) found the opposite results in terms of whole plant resin and biomass yield in a field experiment with *Grindelia chiloensis* (Asteraceae) both of which were significantly higher in plants exposed to ambient UV-B. Their report indicates the terpenes typically found in the resin of *G. chiloensis* but does not quantify individual terpene responses to UV-B. Karousou et al. (1998) found similar results in terms of non-significant differences in response to UV-B, but they did not have any terpenes that were higher in the low UV-B treatment. Lemon verbena is adapted to a naturally high-light environment, and it is possible that the "ambient" levels of UV-B radiation were not sufficient to cause abiotic stress that would lead to enhanced terpene production, according to the model put forth in Vickers, et al. (2009). This is additionally supported by equivalent quantum yields, chlorophyll A fluorescence, from PSII between the two treatments. The increased stem growth in plants exposed to UV-B radiation could indicate a trade-off in carbon allocation; the trade-off between carbon-based terpenes and growth would be explained according to the optimal defense hypothesis (as phenotypic plasticity), the carbon-nutrient balance hypothesis, and the growth: differentiation balance hypothesis. The biomass yield per plant, however, does not indicate that leaf biomass per plant was significantly affected, so "growth" would be carbon allocated to stem elongation. The plants not exposed to UV-B were allocating significantly more carbon resources to geranial production, producing 150% of the geranial found in the plants exposed to UV-B.

This is at a significant metabolic cost to the plant. According to the method employed by Gershenzon (Gershenzon 1994) for terpenes whose biosynthetic pathway was well known, the average monoterpene "cost" can be estimated based on glucose as a unit of cellular currency. Considering substrates and cofactors the cost of monoterpenes ranges from 3.12 grams of glucose per gram monoterpene for linalool ($C_{10}H_{18}O$) to 3.54 g glucose/g α-pinene ($C_{10}H_{16}$). A similar range exists for the sesquiterpenes with cost varying inversely with the degree of oxygenation (Gershenzon 1994). Geranial is the most prevalent monoterpene produced by the lemon-scented variety of *A. citriodora* and may play a dominant role in its phytochemistry. Loss of ambient UV-B seems to act as a stress, either increasing geranial production at the cost of stem elongation or in its absence plants are not receiving appropriate growth cues and build up higher levels of geranial due to the excess assimilated carbon.

Water Stress. The significant increase in foliar terpene concentration of a number of mono- and sesquiterpenes (on a μg/g dry weight basis) indicates that plants are producing mono- and sesquiterpenes when exposed to prolonged drought stress rather than their increase being purely due to reduced leaf expansion. The difference in biomass (reduced in water-stressed plants) could indicate a resource allocation tradeoff, such as those explained in the carbon-nutrient balance hypothesis, with excess carbon being allocated to carbon-based secondary metabolites (Bryant et al. 1983, Bryant et al. 1985, Tuomi 1992). Yet this hypothesis is largely based on nitrogen-deficiencies; if there is excess carbon it means that the plant is fixing sufficient carbon to increase growth if there is not a deficiency in other limiting nutrients. Plants were fertilized throughout the experiment, thus it is unlikely the plants were suffering from growth reducing fertility deficits. The quantum yields indicate a slight decrease in PSII efficiency in well-watered plants, but the values are not considered low enough to cause a

biologically relevant reduction in PSII efficiency. Low weight mono- and sesquiterpenes, like isoprene, have a number of reactive conjugated and terminal double bonds which could contribute to the regeneration of $NADP^+$ and phosphate levels in chloroplasts when CO_2 assimilation is compromised by the reduction in stomatal conductance associated with water stress. This regeneration of $NADP^+$ and phosphate levels would help protect the photosynthetic apparatus from damage due to accompanying ambient or even high light environment. This protection would be conferred by increased terpene concentrations at the leaf's surface or in the air spaces of the leaf mesophyll.

The results of lemon verbena's exposure to water stress are in line with those found for other terpene-storing species (e.g., *Origanum majorana, Mentha piperita*, and *Satureja douglasii*) where water-stress was shown to increase concentration in the essential oils (Gershenzon et al. 1978, Clark and Menary 1980, Rhizopoulou and Diamantoglou 1991). Some of the terpenes follow the response found by Gershenzon (1978, 1984) who suggested that the increased concentration could be due to decreased production of leaf area, resulting in an increase in concentration, but a decrease in overall yield per plant. This is not quite the case as, for the majority of the terpenes, when the concentrations are extrapolated to a per plant basis the yield does not become lower in the water stressed plants. Terpene concentration per plant is significantly greater in water stressed plants for the monoterpene, sabinene, which is significantly higher in water-stressed plants on a per gram dry weight or per plant basis.

Responses to water stress reported in the literature seem to be very species specific and related to certain environments. Peñuelas and Llusiá (1997) found no significant effects of water stress on the accumulation of essential oils in *Rosmarinus officinalis* and postulated that this was due to this species being adaptation to semi-arid environments. Llusiá and Peñuelas (1998) later

demonstrated decreased emission rates in a variety of species (*Pinus halepensis, Pistacia lentiscus, Cistus albidus, C.monspeliensis, Quercus ilex, Phyllyrea latifolia, P. angustifolia,* and *Arbutus unedo*) studied under severe drought. In contrast we found drought stress had a significant effect on foliar terpene yield per gram but not on emissions in lemon verbena, a species adapted to semi-arid, drought prone environments. Simon et al. (1987) found that while total plant biomass and leaf area decreased with increasing levels of stress, the essential oil content of *Ocimum basilicum* tended to increase; furthermore, percent composition of specific oils was affected with some oils (i.e. chavicol) increasing and others (i.e. linalool) decreasing in response to water stress (Simon et al. 1987). This trend is also true for *A. citriodora,* though a different species, thus its oil is composed of different constituents. *Cymbopogon* spp., which shares a similar aromatic profile, with seemingly minor differences in constituent monoterpenes, and grows in similar environments to *A.citriodora,* exhibited a very different response under mild and moderate water-stress regimes with no significant difference in terpene yield but significant differences in biomass yield serving to increase overall yield of essential oils per plant (Singhsangwan et al. 1994). *Cymbopogon* spp. are, for the most part, C4 plants with the associated mesophyll/chloroplast arrangements to overcome high-light low water stresses, thus it may have no need for a additional terpene response to abiotic stress (Bertea et al. 2003).

It seems that our results cannot be simply explained by the prevailing theories of plant defense in terms of the carbon-nutrient balance hypothesis, as there is no shortage of nutrients driving the production of carbon-based metabolites. The growth-rate hypothesis also would not come into play here as it predicts a typical response that is constrained genetically with low levels of phenotypic plasticity. While they may have a role as feeding deterrents, there seems to be another force operating in the production of the mono- and sesquiterpenes of lemon verbena.

Our results (Table 3.1, Fig. 3.1) do fit into a model where the low molecular weight terpenes (mono- and sesquiterpenes) may act to ameliorate abiotic stress responses by scavenging and neutralizing the reactive oxygen species (ROS) associated with drought stress (Vickers 2009). These terpenes may also protect photosynthetic machinery (plastoquinones and other electron carriers and membranes) by providing a malleable pool of reducible compounds that can regenerate $NADP^+$ and ADP as electron acceptors (Chaves et al. 2009, Lawlor and Tezara 2009, Vickers et al. 2009a).

When the energy absorbed is higher than that which can be used in photosynthesis due to low carbon assimilation or other restrictions, various reactive oxygen species (ROS) can be formed in the thylakoid membranes at the photosystem which, in turn, activates a complex scavenging system (Apel and Hirt 2004, Krieger-Liszkay 2005, Vickers et al. 2009a). Isoprene has been intensively studied for its role in heat dissipation, membrane stabilization, and allowing for increased thermo- and photo-tolerance in some species of plants (Sharkey and Loreto 1993, Velikova and Loreto 2005, Velikova et al. 2005, Sasaki et al. 2007, Vickers et al. 2009a). As mono- and sesquiterpenes, also low molecular weight terpenes, have similar chemical structures (i.e. reactive conjugated and often terminal double bonds) they have also been proposed to confer phototolerance in the face of abiotic stress, quenching oxygen free radicals by allowing similar energy transfer and dissipation (Velikova et al. 2004, Peñuelas and Munné-Bosch 2005, Velikova et al. 2005, Vickers et al. 2009a). The observed increase in foliar concentrations of mono- and sesquiterpenes (Table 3.1) in response to water stress, which produces a variety of reactive oxygen species, lends support to the hypothesis that some terpene production can be induced by abiotic stressors (Chaves et al. 2009, Lawlor and Tezara 2009, Vickers et al. 2009a, Vickers et al. 2009b).

In conclusion, the increase in terpene concentrations we observed in response to water stress fits with a model of abiotic stress responses more than existing defense-based hypotheses. Further research into conferred fitness advantages, synergistic stress responses, as well as associated costs of production of these terpenes will be critical to expanding our understanding of the selection pressures that have been involved in past as well as current adaptability of terpene-producing plants. These data will also assist in developing more predictive models of plant chemistry and ecology that will have improved accuracy in the coming decades of global climate change. Our results indicate that ambient levels of UV-B radiation may be necessary for stem elongation and that there are tradeoffs in allocation to growth versus terpene accumulation in plants exposed to ambient UV-B. Rather than the detrimental effects presented by many studies in which existing amounts of UV-B have been augmented, this study indicates that ambient levels of UV-B may be important in proper plant development, especially in those species that have evolved in high UV-B radiation environments. This study does not negate the role of these compounds in defense against herbivory, but it does indicate that the cause and effect relationships assumed in several defense hypotheses may need to be reconsidered. As these compounds have been shown to be affected by abiotic stresses, these should be incorporated into existing models of plant defense and used to develop those models that emphasize the role of abiotic stresses as determinants of plant chemical response.

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Table 3.1: Foliar mono- and sesquiterpenes extracted from lemon verbena under different water conditions (stressed and not stressed) 2007[14].

Compound	Water-Stressed (µg/g dry weight)			No Water Stress (µg/g dry weight)		
α-Pinene**	73.73	±	5.38	51.89	±	4.32
β-Phellandrene***	337.70	±	21.08	239.19	±	13.29
δ-Limonene†	1561.41	±	95.59	1191.28	±	141.20
cis-β-Ocimene**	310.48	±	27.51	174.50	±	28.04
Sabinene***	98.15	±	6.64	22.27	±	9.76
Linalool	tr	±		tr	±	
Unknown 1	73.45	±	20.03	45.67	±	17.25
α-Terpineol†	280.45	±	22.82	225.39	±	15.73
Neral (*cis*-Citral)	3422.16	±	281.45	3073.81	±	178.90
Geranial (*trans*-Citral)	4058.20	±	435.71	3847.94	±	293.91
α-Cubebene**	2203.55	±	147.35	1607.13	±	120.79
β-Bourbonene*	91.16	±	17.58	36.18	±	15.64
Uknown 2	42.76	±	19.88	18.46	±	9.97
α-Cedrene†	44.65	±	19.47	5.80	±	5.79
Caryophyllene**	491.51	±	39.91	316.66	±	30.12
α-Caryophyllene*	589.44	±	171.07	154.56	±	104.14
Alloaromadendrene**	118.53	±	8.93	83.13	±	6.41
Germacrene-D†	766.57	±	48.04	584.99	±	73.85
α-Curcumene*	829.16	±	60.35	623.11	±	61.02
Bicyclogermacrene	521.86	±	54.79	401.95	±	68.20
α-Cedrene†	221.14	±	31.65	138.12	±	32.74
Unknown 3	107.32	±	7.39	88.73	±	12.00
(-) Spathulenol*	1680.30	±	133.91	1220.48	±	125.84
Total Mono & sesquiterpenes†	17816.34	±	1329.84	14062.51	±	995.12

Results are means ± Standard Error from 12 plants.
***** denotes significant differences at $\alpha < .05$
****** denotes significant differences at $\alpha < .01$
******* denotes significant differences at $\alpha = .001$ or less
† denotes a strong trend ($1.0 > \alpha > .05$)

[14] Biomass yield per plant was significantly lower in the plants submitted to water stress (8.86 g vs. 12.12 g, p=0.0279)

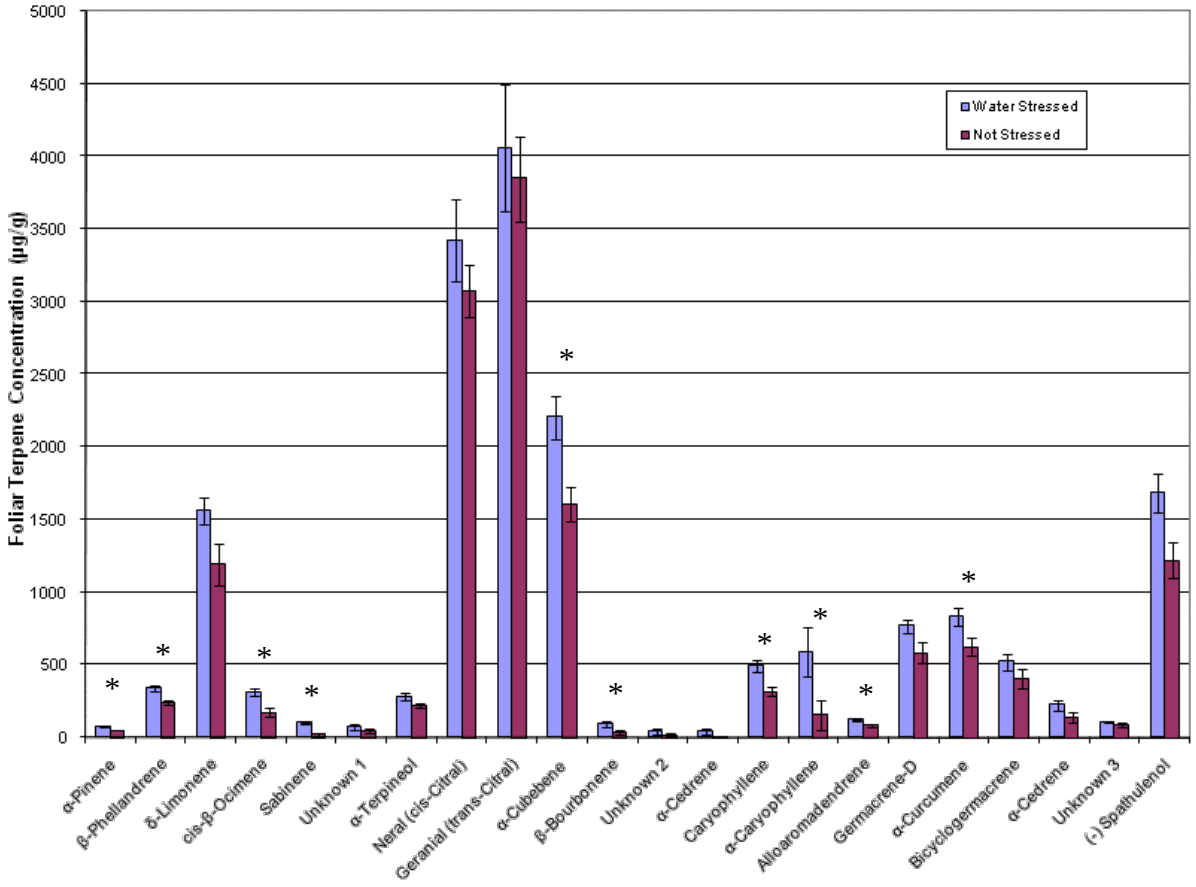

Figure 3.1: Average foliar terpene concentration was higher in plants that experience water stress than in non-stressed plants. An asterisk (*) indicates a significant difference in mean foliar terpene concentration at $\alpha < .05$.

Table 3.2: Foliar mono- and sesquiterpenes extracted from lemon verbena grown under different UV-B exposure conditions 2007[15].

Compound	UV-B excluded (µg/g dry weight)			UV-B control (µg/g dry weight)		
α-Pinene	51.30	±	3.09	43.53	±	2.88
β-Phellandrene†	213.01	±	18.01	170.35	±	10.50
δ-Limonene	1146.83	±	61.04	1029.11	±	60.18
cis-β-Ocimene	276.02	±	116.08	221.76	±	63.11
Sabinene	63.47	±	4.60	61.42	±	6.04
Linalool*	44.14	±	4.38	tr		
Unknown 1	65.31	±	11.08	89.43	±	10.90
α-Terpineol	197.65	±	11.05	181.94	±	10.76
Neral (*cis*-Citral)†	2521.21	±	153.13	2123.06	±	114.97
Geranial (*trans*-Citral)*	5001.78	±	552.54	3323.43	±	389.70
α-Cubebene†	1560.43	±	66.23	1326.72	±	76.83
β-Bourbonene*	tr			66.77	±	8.09
Unknown 2†	98.72	±	7.82	77.48	±	4.93
α-Cedrene	74.93	±	4.08	59.12	±	5.95
Caryophyllene	254.27	±	26.48	200.15	±	10.89
α-Caryophyllene	42.28	±	4.91	41.34	±	4.24
Alloaromadendrene	79.14	±	4.91	71.48	±	5.57
Germacrene-D	398.55	±	75.46	248.95	±	48.77
α-Curcumene	722.01	±	76.05	619.18	±	59.90
Bicyclogermacrene	251.42	±	54.11	431.33	±	134.78
α-Cedrene†	287.67	±	64.79	81.29	±	70.40
Unknown 3	63.62	±	2.60	71.00	±	9.06
unknown 4	206.00	±	21.64	185.02	±	42.23
(-)- Spathulenol	1281.56	±	153.26	1225.55	±	151.20
Total mono- and sesquiterpenes	13073.83	±	741.97	11346.43	±	908.96

Results are means ± standard error, n=24.
***** denotes significant differences at $\alpha < .05$
† denotes a strong trend ($1.0 > \alpha > .05$)

[15] Biomass yield per plant was not significantly affected by UV-B (UV-B excluded = 16.3 g, UV present = 17.2 g)

Table 3.3: Mono- and sesquiterpenes emitted from leaves of lemon verbena under different water conditions (stressed and not stressed) 2007[16].

Compound	Water-Stressed (ng/g dry weight)			No Water Stress (ng/g dry weight)		
β-Phellandrene	0.000232	±	5.99E-05	0.000183	±	0.000124
β-Myrcene	0.000581	±	0.00013	0.000506	±	0.000159
Limonene	0.003839	±	0.000718	0.003827	±	0.000887
trans-β-Ocimene	0.000732	±	0.000128	0.000662	±	0.000154
cis-β-Ocimene	0.01176	±	0.00177	0.011254	±	0.002382
Linalool	0.000599	±	0.000173	0.000861	±	0.000198
Photocitral	0.000319	±	0.000122	0.000551	±	0.00011
α-Terpineol	0.000832	±	0.000346	0.000954	±	0.000328
Nerol	tr			tr		
Neral (*cis*-Citral)	0.001483	±	0.000463	0.001865	±	0.000453
Piperitone	0.000389	±	0.000148	0.000748	±	0.000344
Geranial	0.001091	±	0.000362	0.001652	±	0.00032
α-Cubebene	0.000653	±	0.000174	0.0009	±	0.000143
β-Bourbonene	0.00019	±	8.74E-05	0.000193	±	0.000101
α-Cedrene	0.000306	±	0.000105	0.00041	±	0.000101
Caryophyllene	0.001098	±	0.000205	0.001679	±	0.000268
α-*trans*-Bergamotene	0.000104	±	7.07E-05	0.00014	±	0.000102
Azulene	0.000172	±	9.57E-05	0.000103	±	8.71E-05
β-Caryophyllene	0.000359	±	0.000255	0.000268	±	0.000131
Aromadendrene	tr			tr		
Germacrene-D	0.000458	±	0.000123	0.000488	±	0.00012
α-Curcumene	0.000212	±	0.000128	0.000135	±	9.99E-05
α-Cedrene	8.11E-06	±	5.48E-05	tr		
α-Farnesene	0.001904	±	0.0007	0.002239	±	0.000452

Results are means ± Standard Error from 12 plants.
"tr" indicates that trace amounts were found, but in concentrations too low to be detected by GC/MS

[16] Biomass yield per plant was significantly lower in the plants submitted to water stress (8.86 g vs. 12.12 g, p=0.0279)

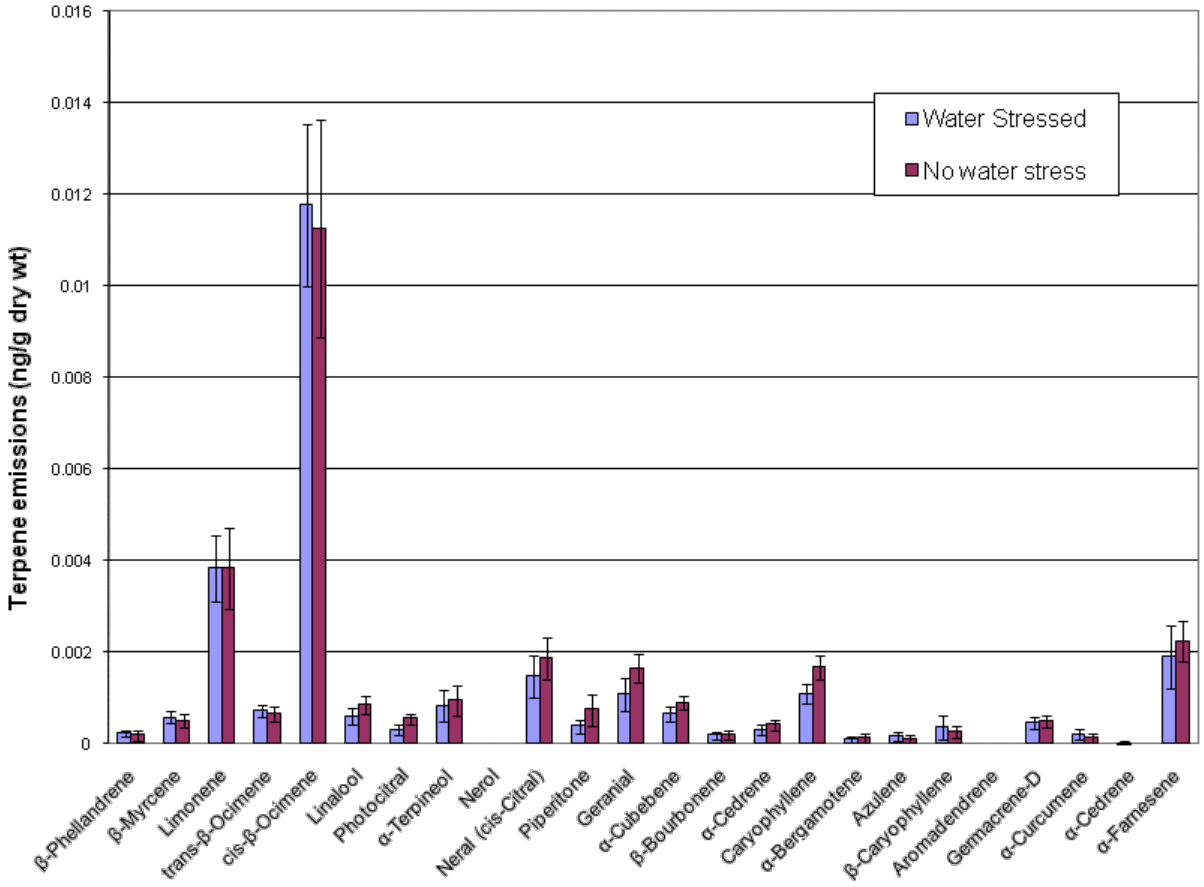

Figure 3.2: Average terpene emission was not significantly affected by water stress. Emissions are qualitatively different than foliar terpene concentrations in some cases and relative concentrations also seem to differ among the mono- and sesquiterpenes.

Table 3.4: Mono- and sesquiterpenes emitted from leaves of lemon verbena under different UV-B exposure conditions 2007[17].

Compound	UV-B excluded (µg/g dry weight)			UV-B control (µg/g dry weight)		
β-Phellandrene	1.13E-04	±	1.82E-05	1.15E-04	±	1.22E-05
β-Myrcene	2.48E-04	±	5.31E-05	2.27E-04	±	1.98E-05
D-Limonene	1.31E-03	±	2.18E-04	1.24E-03	±	8.30E-05
trans-β-Ocimene	2.59E-04	±	6.44E-05	2.47E-04	±	3.32E-05
cis-β-Ocimene	4.71E-03	±	1.40E-03	4.00E-03	±	5.28E-04
cis-β-Terpineol	0.00E+00	±	0.00E+00	7.06E-06	±	6.28E-06
trans-Sabinenehydrate	9.15E-06	±	6.27E-06	0.00E+00	±	0.00E+00
Linalool	1.95E-04	±	2.97E-05	1.67E-04	±	3.27E-05
Photocitral a	5.57E-05	±	2.36E-05	1.07E-05	±	6.07E-06
Nerol	4.41E-05	±	4.41E-05	0.00E+00	±	0.00E+00
Neral (*cis*-Citral)	2.91E-04	±	1.28E-04	1.40E-04	±	3.37E-05
Geraniol	1.13E-04	±	5.98E-05	0.00E+00	±	0.00E+00
Geranial (*trans*-Citral)	2.21E-04	±	1.48E-04	1.06E-04	±	2.48E-05
α-Cubebene	1.79E-04	±	5.86E-05	1.17E-04	±	2.16E-05
β-Bourbonene	2.21E-05	±	1.86E-05	0.00E+00	±	0.00E+00
α-Cedrene	4.91E-05	±	1.76E-05	2.37E-05	±	1.37E-05
Caryophyllene	3.04E-04	±	1.21E-04	1.49E-04	±	3.30E-05
α-trans-Bergamotene	4.26E-05	±	4.26E-05	0.00E+00	±	0.00E+00
Germacrene-D	1.59E-04	±	1.03E-04	5.98E-05	±	1.80E-05
α-Curcumene	2.86E-05	±	2.13E-05	0.00E+00	±	0.00E+00
α-Cedrene	3.45E-05	±	3.45E-05	0.00E+00	±	0.00E+00
α-Farnesene	3.35E-05	±	3.35E-05	0.00E+00	±	0.00E+00
Caryophyllene oxide	5.78E-05	±	5.78E-05	0.00E+00	±	0.00E+00
β-Pregnane	0.00E+00	±	0.00E+00	2.29E-06	±	1.95E-06
(-)-*trans*-Pinane	4.46E-07	±	4.46E-07	0.00E+00	±	0.00E+00
τ-Cadinol	3.12E-06	±	3.12E-06	0.00E+00	±	0.00E+00

Results are means ± Standard Error from 24 plants.
"tr" indicates that trace amounts were found, but in concentrations too low to be detected by GC/MS

[17] Biomass yield per plant was not significantly affected by UV-B (UV-B excluded = 16.3g, UV present = 17.2g)

Table 3.5: The percentage of emitted mono- and sesquiterpenes relative to their foliar mono- and sesquiterpenes extracted from the leaves of lemon verbena under different water conditions (stressed and not stressed) 2007[18].

Compound	Water-Stressed (μg/g dry weight)	No Water Stress (μg/g dry weight)
α-Pinene	NE	NE
β-Phellandrene	6.87E-08	7.65E-08
β-Myrcene	NS	NS
Limonene	2.46E-07	3.21E-07
trans-β-Ocimene	NS	NS
cis-β-Ocimene	3.79E-06	6.45E-06
Sabinene	NE	NE
Linalool	TR	TR
Photocitral	NS	NS
Unknown 1	NE	NE
α-Terpineol	2.97E-07	4.23E-07
Nerol	NS	NS
Neral (*cis*-Citral)	4.33E-08	6.07E-08
Piperitone	NS	NS
Geranial	2.69E-08	4.29E-08
α-Cubebene	2.96E-08	5.60E-08
β-Bourbonene	2.08E-07	5.33E-07
Uknown 2	NE	NE
α-Cedrene	6.85E-07	7.07E-06
Caryophyllene	2.23E-07	5.30E-07
α-*trans*-Bergamotene	NS	NS
Azulene	NS	NS
β-Caryophyllene	NS	NS
α-Caryophyllene	NE	NE
Aromadendrene	NE	NE
Germacrene-D	5.97E-08	8.34E-08
α-Curcumene	2.56E-08	2.17E-08
Bicyclogermacrene	NE	NE
α-Cedrene2	3.67E-09	tr
Unknown 3	NE	NE
α-Farnesene	1.13E-07	1.83E-07

Results are means of emitted terpenes divided by their corresponding foliar extracts from 12 plants.
NE denotes terpenes that were not emitted
NS denotes terpenes that were not stored (not found in foliar terpene extracts)
TR denotes those that were emitted and stored in trace amounts

[18] Biomass yield per plant was significantly lower in the plants submitted to water stress (8.86 vs. 12.12, p=0.0279)

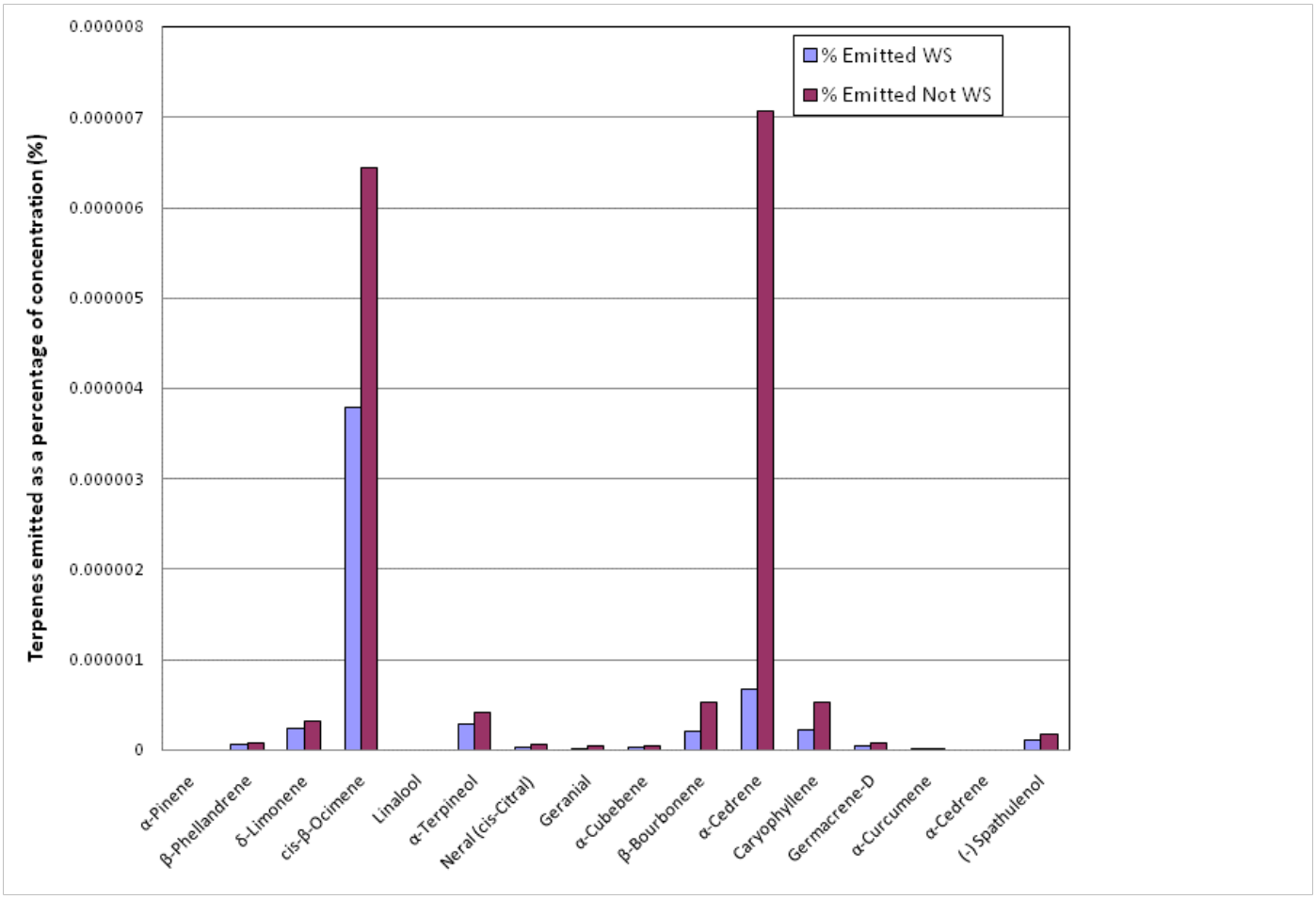

Figure 3.3: Percent emission of mono- and sesquiterpenes from plants subjected to water stress.

CHAPTER 4

LOW-COST, RAPID SCREENING OF WILD POPULATIONS OF AROMATIC PLANTS WITH THIN LAYER CHROMATOGRAPHY (TLC): A TEST CASE WITH ARGENTINE LEMON VEBERNA (*ALOYSIA CITRIODORA)*[19]

[19] Chapman, R.E., J. M. Affolter, D.E. Giannasi, M. Lagrotteria. To be submitted to *Society for Economic Botany.*

ABSTRACT

Thin layer chromatography (TLC) is evaluated as a simple, cost-effective means for surveying the chemotypic diversity in wild populations of essential oil plants. We tested this method with an aromatic plant species of increasing importance in its domestic and global market, lemon verbena (*Aloysia citriodora* Paláu), Verbenaceae, which is native to Argentina, Chile, and Peru. *Aloysia citriodora* is an aromatic, perennial shrub that is valued for its medicinal use (in tincture or tea form) as well as for its aromatic properties in culinary and cosmetic industries. We conducted a survey of populations of *A. citriodora* in three provinces in Argentina: Salta, Catamarca, and La Rioja. We extracted the essential oils using methanol and diethyl ether in the field, and analyzed the monoterpenes using thin layer chromatography (TLC) on silica gel layers developed in touluene:ethyl acetate (97:3). Monoterpenes were visualized using vanillin-sulfuric acid spray and heat. These were identified using TLC images, comparing plates to authentic standards, and by comparison with GC-MS. We identified 15 of the 17 compounds and analyzed the correlation of these compounds to source population. We found certain compounds to be ubiquitous while other compounds were highly indicative of the source population. Argentine *A. citriodora* exhibits some variation within populations and a great deal of chemotypic variation among populations. TLC is an effective, low-cost means to rapidly survey wild plant populations for presence of specific compounds as well as chemotypes.

KEYWORDS: *Aloysia citriodora*; *Aloysia triphylla*, lemon verbena; essential oils; monoterpene; TLC; chemotype

INTRODUCTION

International trade in aromatic plants, such as lemon verbena (*Aloysia citriodora* Paláu), is increasing in both volume and value. These species, and the essential oils they contain, have been identified as a renewable resource and source of supplemental economic product in many rural areas and developing nations. Monoterpenoids are the primary constituents of essential oils, and their volatility is responsible for the characteristic odors of many aromatic plants. The United Nations Industrial Development Organization (UNIDO), considers essential oils from medicinal and aromatic plants a renewable resource that could be developed industrially for economic benefit (Silva 2006). According to the United Nations Commodity Trade Statistics Database (COMTRADE 2009) the world import market of essential oils and aromatic plants in the perfumery and cosmetic industry has grown from 11.6 billion US dollars in 2003 to 17.5 billion US dollars in 2007. There is a need for access to simple technologies and transfer of expertise to many developing countries and rural communities so that they may reap the financial benefit of producing the value added products (e.g., essential oils, tinctures, and herbal mixtures) rather than simple sale of the raw plant materials. Included in all recommendations for sustainable use of medicinal and aromatic plants (MAPs) is the need to: 1) develop management practices for collection and/or cultivation, 2) determine the effects of agronomic and environmental variables on MAPs in their native habitats, and 3) conduct research related to the conservation and sustainable utilization of MAPs (WHO 2003, Bogers et al. 2006, Silva 2006, Affolter and Pengelly 2007).

Genetic, biochemical, and ecological factors determine the occurrence of monoterpenes in the essential oils of aromatic plants. Chemical profiles have long been used in chemotaxonomy to distinguish between species and varieties of plants, and there is evidence for

infraspecific geographic variation in monoterpene composition for many species. The existence of chemical "races" or chemotypes within species of plants is supported by current research regarding the biosynthetic pathways of terpenes which indicate that while many biotic and abiotic factors affect the quantitative expression of certain genes regulating biosynthesis, the qualitative ability to produce certain compounds are under tight genetic control (Gershenzon et al. 2000, Dewick 2002, Mahmoud and Croteau 2002). It is this great deal of qualitative variation in chemical composition that is most significant within and among geographically distinct populations (Lincoln and Langenheim 1981, Comer et al. 1982, Segal et al. 1987, Mathela et al. 1988, Von Rudloff et al. 1988, Gil et al. 2007). Many medicinal and aromatic plants are sold as bulk plant material (leaves, roots, stems), thus the majority of quality control has focused on reducing contamination with other species of plants. Quality control to the level of chemical constituents is often overlooked. Improving the efficacy of a plant medicine and the market-value of an end-product (e.g., extracted essential oils) based on chemical composition is of primary importance. It follows, therefore, that some level of quality control that allows for the rapid qualitative assessment of compounds present in intact plants, before collection or cultivar development, should be implemented.

We propose a simple, cost-effective means for surveying the chemotypic diversity at the collection/selection phase in wild populations of essential oil plants, the use of thin layer chromatography (TLC). TLC has been used to detect active principles in plant-based and synthetic drug compounds and is often recommended due to its simplicity, low cost, and sensitivity (Yuen and Laucam 1985, Roy et al. 1997, Siek et al. 1997, Oprean et al. 1998, Pascual et al. 2002). Coupled with selective GC-MS to identify pre-screened compounds, TLC could be used to rapidly survey wild populations, allowing researchers to identify: 1) the

spectrum of chemotypes present, 2) the range of these chemotypes, and 3) their prevalence in natural and cultivated populations.

As a test species we studied wild populations of *Aloysia citriodora* Paláu (=*Aloysia triphylla* (L'Hér.) Britton; synonyms include: *Lippia citriodora* Kunth, *Lippia triphylla* (L'Hér.) Kuntze), Verbenaceae, which is native to Argentina, Chile and Peru and is grown throughout Latin America as well as North Africa (Morocco), southern Europe, and parts of Asia (Botta 1979, Armada and Barra 1992, Carnat et al. 1999, Rotman and Mulgura de Romero 1999). *Aloysia citriodora* is an aromatic, perennial shrub which is valued for its medicinal use (in tincture or tea form) as a digestive aid, antimicrobial, antispasmodic, analgesic, and diuretic as well as being used as a treatment for colds, insomnia and anxiety (Botta 1979, Carnat et al. 1999, Rotman and Mulgura de Romero 1999). Essential oil extracts of the leaves are also used extensively in the flavoring and cosmetic industries.

The major compounds responsible for *A. citriodora's* aroma and flavor are the mono- and sesquiterpenes found in its leaves and flowers. Reports on the essential oil composition of *A. citriodora* growing in its native range in South America using traditional techniques of steam distillation followed by GC-FID and GC-MS indicate the presence of multiple chemotypes[20] of this species (Gil et al. 2007). The commonly cultivated chemotype of *A. citriodora* worldwide is known as lemon verbena due to the dominance of lemon-scented monoterpenes, especially citral; however, even within this chemotype there is a great deal of variation in the composition of minor constituents and the proportion of essential oil components (Bandoni et al. 2008). The oil of lemon verbena is composed primarily of isomers of citral (i.e. neral and geranial, 10-40% depending on chemotype), accompanied by sabinene, limonene, cineole, geraniol, caryophyllene,

[20] A chemotype is a chemical phenotype, based on the presence of certain complements of compounds (e.g., terpenes)

linalool and spathulenol in varying proportions (Carnat et al. 1999, Crabas et al. 2003, Argyropoulou et al. 2007, Gil et al. 2007, Pereira and Meireles 2007, Bandoni et al. 2008).

The quality of the essential oil of lemon verbena is largely determined by the concentration of the two isomers of citral (neral and geranial) and limonene. Studies have shown that these compounds are present in most chemotypes of *A. citriodora*, but their concentrations vary considerably based on source of origin, plant part sampled, and plant developmental stage (Argyropoulou et al. 2007, Gil et al. 2007, Bandoni et al. 2008). Lemon verbena is easily propagated and grows well in cultivation under a broad range of climatic conditions and has an extensive natural range. Gil et al (2007) showed clear and consistent infraspecific variability of lemon verbena collected from multiple provinces in Argentina and Chile grown in a common garden. They identified cultivars for optimizing the current target oil components of high yields of neral and geranial with the typical lemony and fresh herbaceous flavor. Bandoni et al. (2008) analyzed multiple samples of lemon verbena from Argentina and found that the majority fit the dominant profile and clear markers could by determined for typical Argentine source lemon verbena essential oils. Both Bandoni et al. (2008) and Gil et al (2007) also indicate the presence of other chemotypes in native populations of *A. citriodora* that deviate from the citral-dominated variety.

We propose the use of thin layer chromatography as a low-cost means to rapidly survey aromatic medicinal plant populations for desirable cultivars, collection sources, chemotypic abnormalities, and rare or novel chemotypes worthy of conservation efforts based on monoterpene profiles. To assess the practicality and effectiveness of this technique we surveyed wild populations of *A. citriodora* in three provinces in its native range in Argentina. We propose that 1) TLC will be a sufficiently accurate means to distinguish distinct chemical profiles; 2)

chemotypes[21] will vary in frequency throughout the natural range of *A. citriodora* (cedrón) in Argentina; and 3) that specific terpene chemotypes will cluster, with higher frequencies of certain chemicals in distinct regions (represented by provinces).

MATERIALS AND METHODS

This research was conducted in February, 2001. Leaves from *A. citriodora* (lemon verbena, cedrón) were collected from three provinces in Argentina: Salta, Catamarca, and La Rioja. Fresh leaves were removed from stems and packed to fill a 20 mL glass scintillation vial (Fisher Scientific, Pittsburgh, PA). The vials were filled with methanol and allowed to oscillate with the leaf material for 24 hours. Upon return to the field-lab terpenes were separated from the methanol extracts using diethyl ether. The ether was evaporated and these samples were transported back to the U.S., where they were stored at -20 °C until analyzed. Fifteen individuals were sampled at 10 m intervals along a linear transect in the middle of a population in each province (growing at approximately the same altitude of 1500 m). Samples were reconstituted in 2.5 mL redistilled hexane (HPLC grade, Fischer Scientific); 150 µL were spotted onto TLC plates (Whatman TLC plates 60 Å). Nine samples were run per plate. Samples were applied 1.5 cm from the bottom with 2.0 cm between applied spots and 2.0 cm between samples and the edge of the plate to minimize distortion. All runs were stopped when the solvent was 1.5 cm from the top of each plate to standardize R_f values. Plates were developed in touluene:ethyl acetate (TEA) at a ratio of 97:3. Monoterpenes were visualized with vanillin/sulfuric acid spray and heat (10 min at 100 °C) to determine terpene identity and create a profile of monoterpenes for each plant. Color and R_f values for each spot on the TLC plate were compared against

[21] This use of the term chemotype is not in strict keeping with its definition as "true-breeding," as we have done no common-garden experiments with these plants to date.

known standards for positive identification following methods outlined in Wagner et al. (1984). TLC identification of terpenes was confirmed using gas chromatography coupled with a mass spectrometer (GC-MS).

Gas chromatography analysis was performed on 1 μL of a subset of samples from each population to confirm the comprehensive set of terpenes using a Hewlett Packard model 5985B GC-MS system. Mass spectrometer conditions were: ion source 200 °C; electron energy 70 eV; multiplier voltage 220 V; GC/MS interface zone 300 °C; scan range 40-400 atomic mass units. Mass-spectral identification was done using the Wiley MS database. When available, analytical standards were used as reference compounds to compare retention times. Samples were hand-injected into a split-splitless injection port temperature of 225 °C. Separations were made on a 15 m x .53 mm i.d. fused silica column coated with DB-5 (Alltech Associates, Deerfield, IL). The samples were injected in the splitless mode (50 °C) with a purge time of 0.5 min and held at that temperature for 2 min, after which the oven temperature was increased to 130 °C at a rate of 2 °C /min followed by another increase in temperature of 10 °C /min to a final temperature of 280 °C, holding the column at 280 °C for 15 min.

Chemical Standards. Analytical standards used were: citral mixture of isomers (61 % cis, 36 % trans), nerol, geraniol, R-(+)-limonene (Sigma, St. Louis, MO); β-citronellol, citronellal, citronellol, linalool oxide, eugenol (TCI America, Portland, OR); α-pinene, myrcene, , (-)-carvone, (-)-menthone, menthol, *cis*-myrtanol, germacrene-D (Fluka Chem. Co., Milwaukee, WI); α-cubebene, S-(-)-limonene, α-terpineol, δ-(+)-3-Carene, δ-Cadinene, *trans*-nerolidol (Aldrich Chem. Co., Milwaukee, WI); and β-pinene and piperitone (ICN, Plainview, NY).

Statistical Analysis. Principle component analysis (PCA) was used to summarize chemotypic variation among provinces and to determine which chemicals contribute most to

these differences, or may serve as markers for certain chemotypes/populations. Canonical correlation analysis (CCA) was used to analyze of the correlation of the compounds within and among samples to be plotted as variables and compared to source populations. This enabled us to test the statistical relationship of the populations as independent variables with correlation of monoterpenes as dependent variables. These are represented spatially along the two canonical axes. Statistics were calculated using JMP v.7.0 and SAS v. 9.1 statistical packages.

RESULTS & DISCUSSION

Seventeen distinct spots, representing monoterpenes were separated by thin layer chromatography (Fig. 4.1). The best separation was achieved by using TEA as a developing solvent and the best visualization method was a vanillin – sulfuric acid spray followed by 10 min at 100 ºC (R.E. Chapman unpublished results). Spots were identified using R_f and color profile according to standard references under identical plating processes and were confirmed with GC-MS analysis of select samples (Wagner et al. 1984). Using this method we were able to identify 15 of the 17 monoterpenes (Table 4.1).

Canonical Correlation Analysis. Figure 4.2 shows the canonical plot according to the first two dimensions that best separate individual samples into groups based on their chemical composition. The three populations (Salta, Catamarca, and La Rioja) have significantly different combinations of compounds (Fig. 4.2a; Wilks-Lambda: df=34, $F<0.0001$). The first canonical correlation separates the chemotypes of the La Rioja population from Catamarca and Salta, accounting for 90.8 % of the variance. The second canonical correlation separates the Catamarca and Salta populations from each other, accounting for the remaining 9.2 % of the variance. The scoring coefficients for the different compounds are given in Table 4.2 and are represented

visually by the bi-plot rays (Fig. 4.2c). These show the relationship between the compounds and the first and second canonical discriminant factors. Both components reflect variability within populations. Salta and Catamarca showed some degree of overlap of chemotypes (Fig. 4.2), but only for a few samples. One plant from the Catamarca population differed from predicted canonical correlations and, based on the model, had a composition of compounds that would predict Salta as the population of origin.

Principle Component Analysis. Principle component analysis allowed us to portray the relationships among populations and the importance of specific chemicals for discriminating population source and associated chemotype(s). The three geographically distinct populations separated via PCA according to chemotypic variation are shown in Figure 4.3.The first principle component accounts for 24.5 % of the variation, separating the La Rioja and Salta populations. The second principle component accounts for 17.9 % of the total variance and separates the Catamarca and Salta populations. If one compares the principle component loading plot (Fig. 4.4) and the PCA score plot (Fig. 4.3) of the populations it is possible to determine which compounds are most prevalent in the geographically distinct populations. The PCA loading plot demonstrates that unknown 1 and 2, citral and *trans*-sabinene hydrate have a high positive correlation with PC1 indicating they are distinctive of the La Rioja population. Carvacrol has a positive correlation with PC2 and neither a negative nor positive correlation with PC1 making it highly indicative of the Catamarca population, and phytol is indicative of the Salta population.

CONCLUSIONS

Aloysia citriodora demonstrates the presence of a high degree of infraspecific variation in chemical constituents both within and among populations (Table 4.1; Figs. 4.2a, 4.3, 4.4).

Samples clustered into geographically distinct groups based on chemotype, and indicator compounds for each group were identified (Figs. 4.2a-c, 4.3, 4.4). While it did not capture all of the monoterpenes in *A. citriodora*, TLC was a low-cost method that allowed the rapid visualization of sufficient monoterpenes to distinguish chemotypes of interest and possible chemotypes to avoid (Fig. 4.1, Table 4.1).

The compounds found in our study are not all consistent with the typical lemon-scented variety of *A. citriodora*. Two of the three populations studied (i.e. Catamarca and Salta) had few individuals with citral as a component and limonene was not able to be confirmed by our method (Carnat et al. 1999, Crabas et al. 2003, Argyropoulou et al. 2007, Gil et al. 2007, Pereira and Meireles 2007, Bandoni et al. 2008). We found a strong occurrence of β-thujone and citronellal which are not typical but have been reported for Argentine *A. citriodora* by Gil et al. (2007) and Bandoni et al. (2008). They reported the thujone, citronellal, sabinene chemotype as originating from Salta, but not being common. We found a prevalence of citronellal (and β-citronellol) in many individuals from Salta while β-thujone was a marker for plants from Catamarca (Fig. 4.3, 4.4). These monoterpenes are considered "negative" for essential oil qualities of this species, ranging from toxic (i.e. β-thujone) to less desirable (i.e. citronellal and β-citronellol), and are not the compounds associated with market value or cultivar development for *A. citriodora*. Our samples from La Rioja are more consistent with the typical oils of lemon verbena and are clustered very distinctly from the Catamarca and Salta populations (Fig. 4.2 a,b; 4.3) (Carnat et al. 1999, Crabas et al. 2003, Argyropoulou et al. 2007, Gil et al. 2007, Pereira and Meireles 2007, Bandoni et al. 2008). The geographic trend in our data, when compared with populations sampled and grown in a common garden by Gil et al. (2007) indicate that there may be latitudinal clines that show a prevalence of citronellal and thujone chemotypes in the northwest,

followed by increased prevalence of citral in La Rioja (Fig. 4.2), and citral-dominated chemotypes in San Luis and Mendoza (Gil et al. 2007, Bandoni et al. 2008).

The variation we captured using TLC is an important consideration for brokers who buy the raw material from many provinces and sell the resulting mixture for manufacturing teas, tinctures or essential oils. The absence of citral in many of the Salta and Catamarca plants could make those less valuable for essential oil production. The presence of toxic compounds such as thujone could present serious problems. These could be avoided at the level of collection by simple pre-screening that could determine which areas contain populations or individual plants with undesirable compounds. In Catamarca local herbalists and residents report differential use of the chemotypes; using *A. citriodora* plants with specific aromas to treat certain ailments (C. Juri unpublished report). These chemotypes could represent future development of additional cultivars or varieties of *A. citriodora* with medicinal utility. Their range and prevalence throughout populations in these northwestern provinces should be documented.

Thin layer chromatography was a useful tool for distinguishing chemotypes from different source populations of *A. citriodora* in Argentina. It captured a significant portion of the monoterpene variability in the essential oils of *A. citriodora*. This method allowed for the identification of negative components (i.e. citronellal and β-thujone) described in other studies (Gil et al. 2007, Bandoni et al. 2008). TLC also determined those plants containing desired compounds (e.g., citral) for inclusion in possible common garden experiments for further cultivar development or for wild harvest. Our method did not capture some of the low weight or highly volatile monoterpenes (e.g. pinene and myrcene), and we would need to develop the plates along a second axis to separate those monoterpenes at the solvent front (e.g. limonene).

Conducting analysis near the site of collection could greatly improve the detection of the more volatile monoterpenes, as these were lost in drying the samples for transport and reconstituting them in the lab. We achieved slightly better results when we using diethyl ether or methylene chloride as the extraction solvent, but that is not practical for field work in many cases as the former is explosive and the latter is very toxic and should not be released into the environment. The solvent extraction system is important, with nonpolar solvents yielding the best terpene extracts. Pascual et al. (2002) conducted a study to determine the efficacy of TLC in identifying class of compounds in medicinal plants (e.g., alkaloids, flavonoids). They indicated that TLC was not as useful in determining essential oils from herbal drug mixtures. Their extraction protocol, however, involved a nonpolar wash to remove leaf waxes, thus they removed many of the mono- and sesquiterpenes from the herbal mixture before they began their analysis.

The TLC method outlined in this paper captured infraspecific variation within and among populations and is a low-cost means to survey wild populations for known and novel chemotypes that requires very little time or analytical equipment. We used GC-MS to analyze only those samples necessary to confirm the identity of each of the spots and assess monoterpenes that we were not capturing on TLC (e.g. limonene). In areas with limited access to analytical equipment and/or limited funding, as is the case for many countries interested in developing their essential oil market (Silva 2006), TLC can serve as a starting point in assessing the chemotypic diversity of essential oil plants for sustainable development and conservation initiatives.

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FIGURES AND TABLES

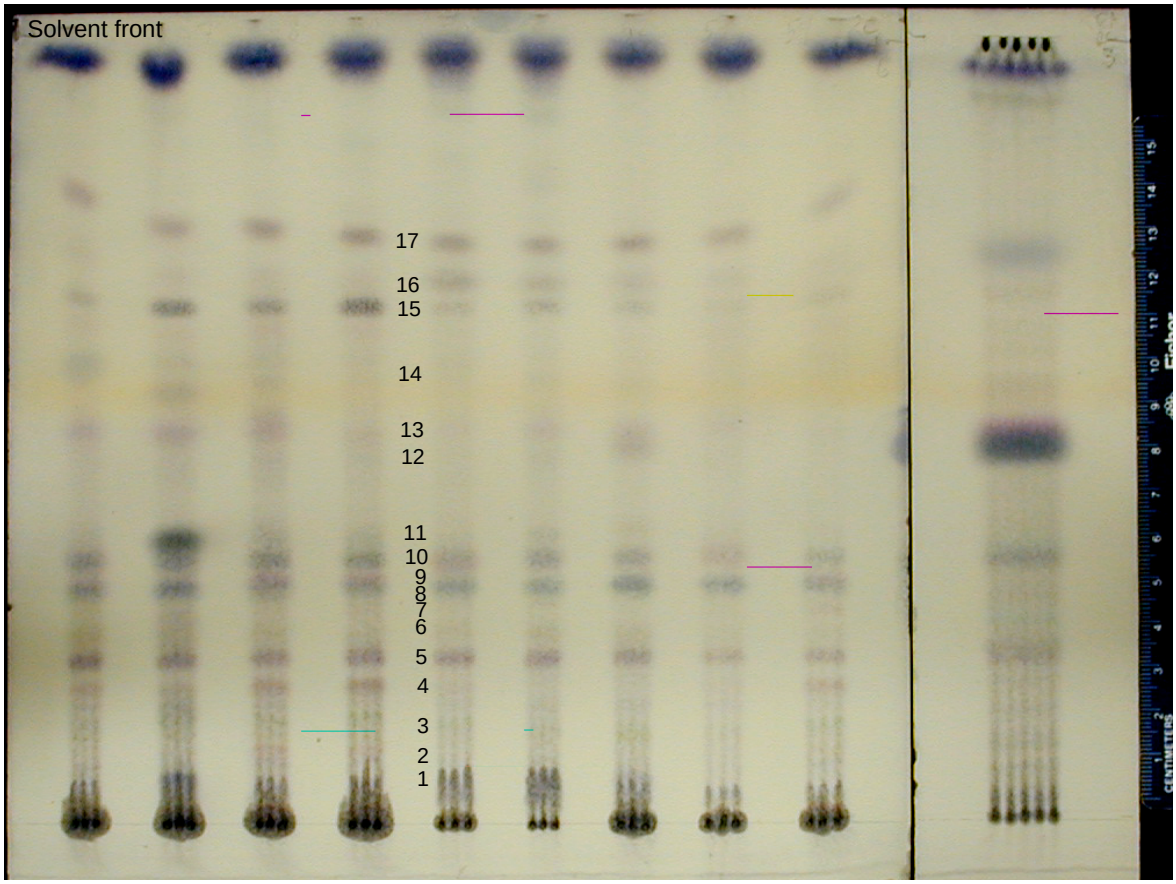
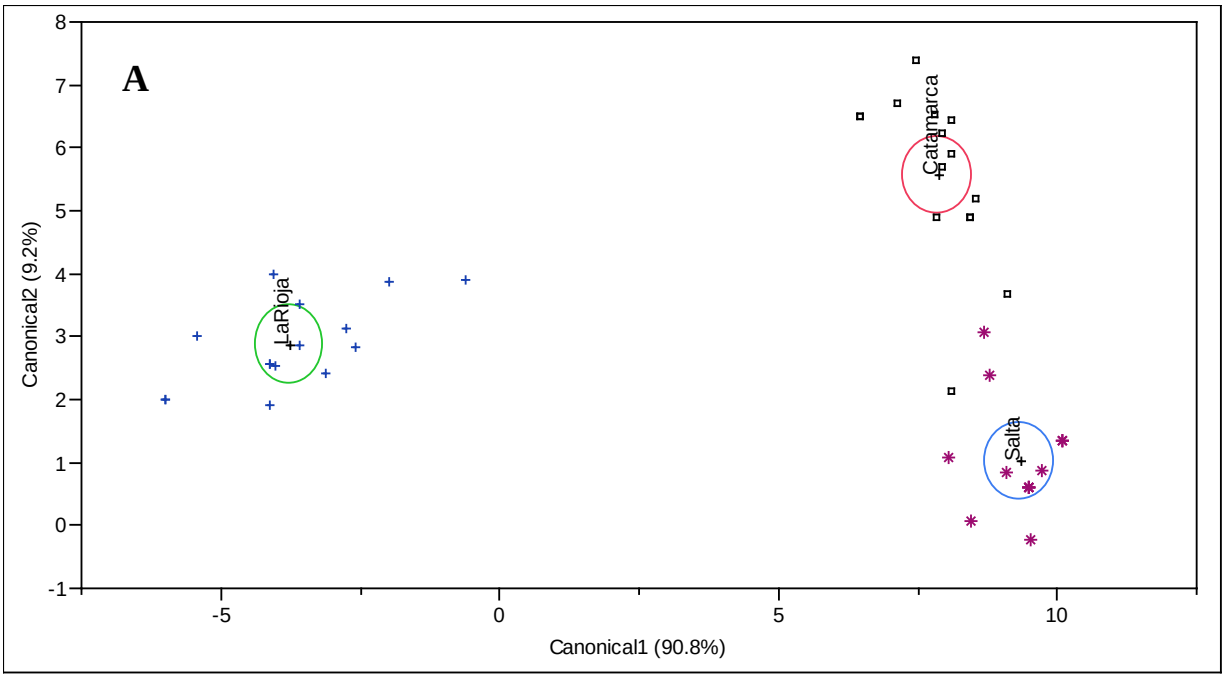
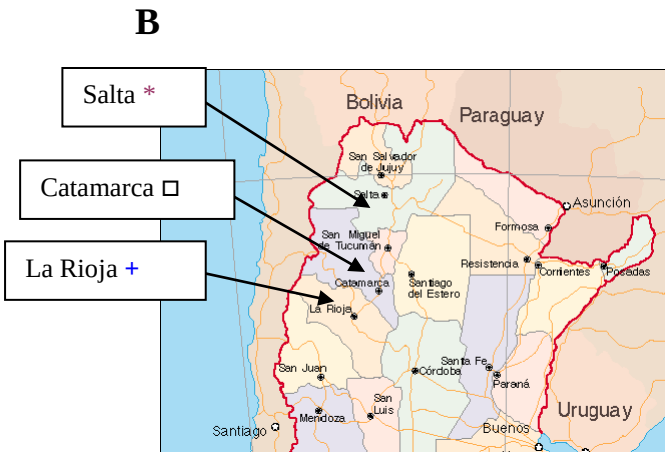


Figure 4.1: Example TLC plates with spots (1-17) numbered. Samples were reconstituted in hexane, 150 µL were applied to plates. The plates were developed in TEA and visualized with VS spray reagent and heat. The compound names are given in table 4.1.

Table 4.1: Monoterpenes identified by TLC from *Aloysia citriodora*.

No.	Compound	Rf value
1	Unknown 1	0.07
2	Unknown 2	0.12
3	Limonene oxide	0.15
4	Isoeugenol	0.17
5	Borneol	0.22
6	*trans*-Sabinene hydrate	0.26
7	Linalool	0.31
8	Phytol	0.33
9	Piperitone	0.33
10	β-Citronellol	0.40
11	Carene	0.35
12	Citral	0.47
13	Carvone	0.50
14	Carvacrol	0.56
15	β-Thujone	0.68
16	Citronellal	0.70
17	Sabinene	0.75





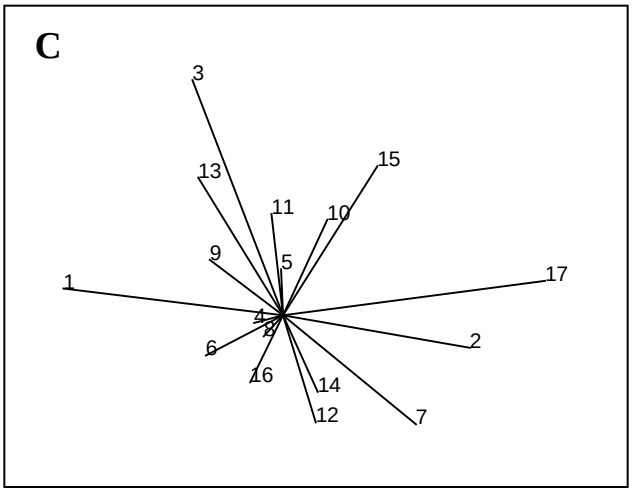


Figure 4.2: a) Canonical correlation plot of samples from three geographically distinct populations in Argentina. Canonical discriminant factor 1 accounts for 90.8 % of the variance and separates the Salta and Catamarca populations from La Rioja. Canonical 2 accounts for the remaining 9.2 % of the variance and separates the populations from Salta and Catamarca. Correlations of compounds are significantly different among populations (Wilks-Lambda: df=34, F<0.0001) b) Regions of collection of populations from Salta, Catamarca and La Rioja. c) Bi-plot rays for CCA in 4.2a. These rays indicate the direction of the variables in the canonical space.

Table 4.2: Contribution of compounds to the first two canonical variables.

		Scoring Coefficient	
No.	**Compound**	**Canon1**	**Canon2**
1	Unknown 1	-4.97	0.61
2	Unknown 2	2.87	-0.49
3	Limonene oxide	-0.95	2.49
4	Isoeugenol	-0.50	-0.13
5	Borneol	-0.04	0.84
6	*trans*-Sabinene hydrate	-1.03	-0.53
7	Linalool	1.64	-1.35
8	Phytol	-0.26	-0.26
9	Piperitone	-1.33	1.03
10	β-Citronellol	0.44	0.98
11	Carene	-0.36	3.17
12	Citral	0.54	-1.88
13	Carvone	-0.96	1.56
14	Carvacrol	0.63	-1.48
15	β-Thujone	1.07	1.73
16	Citronellal	-0.34	-0.69
17	Sabinene	8.05	1.08

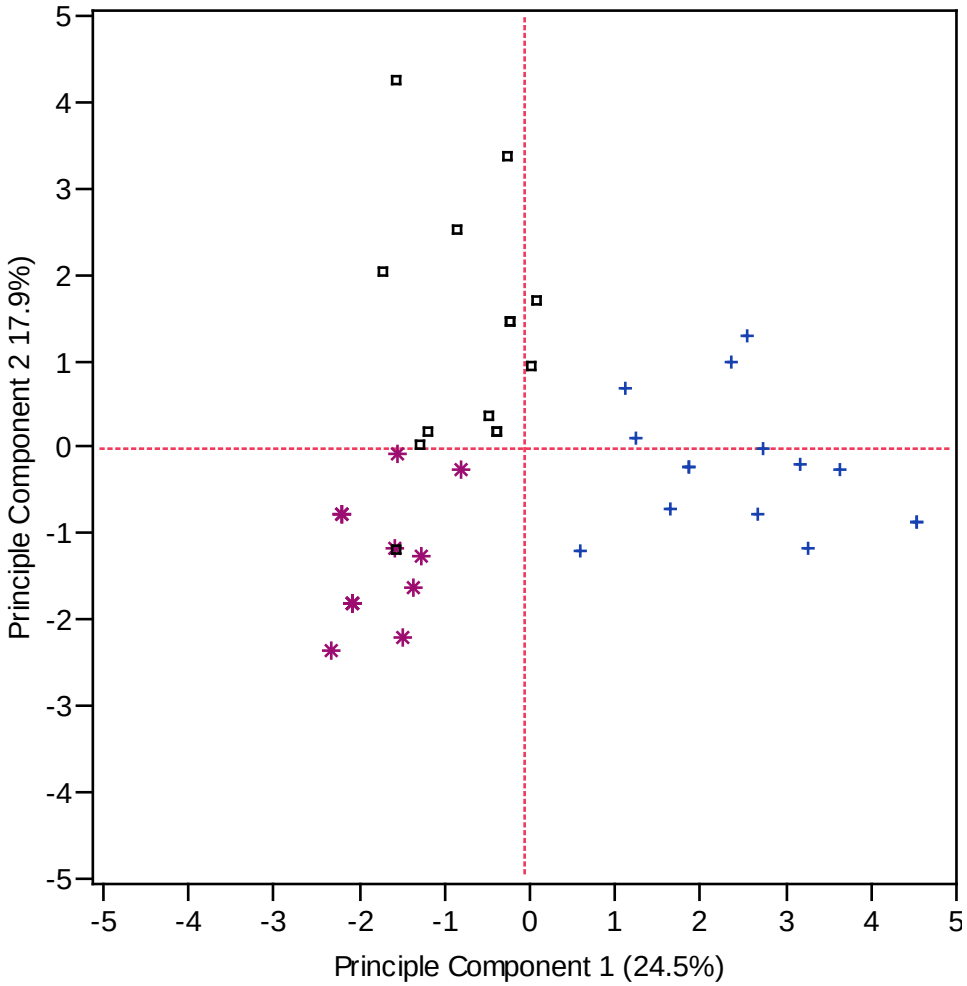


Figure 4.3: Principle component analysis for three geographically distinct populations in Argentina. Principle component 1 accounts for 24.5 % of the variance and separates La Rioja (+) and Salta (*) populations. Principle component 2 accounts for 17.9 % of the total variance and separates the Catamarca (□) and Salta (*) populations.

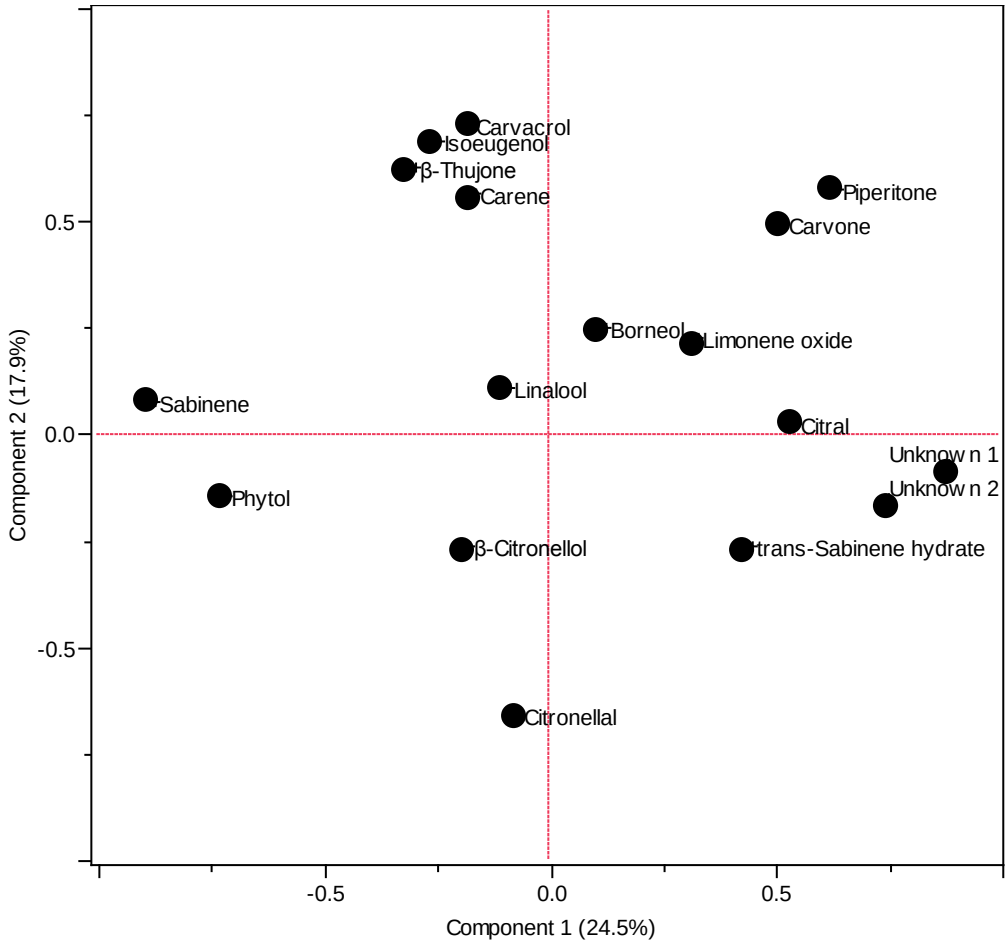


Figure 4.4: Principle component analysis loading plot demonstrates that unknown 1 & 2, citral and *trans*-sabinene hydrate have a high positive correlation with PC1 indicating they are distinctive of the La Rioja population. Carvacrol has a positive correlation with PC2 and neither a negative nor positive correlation with PC1 making it highly indicative of the Catamarca population and phytol is indicative of the Salta population.

CHAPTER 5

CONCLUSIONS

Conservation of medicinal and aromatic plants and sustainable economic development of these renewable global resources requires an approach that incorporates agronomic practices in an ecological context. It is critical to understand the chemical diversity of wild species and their role in native ecosystems and how that role and the production of desired constituents may be affected by collection or cultivation practices. The terpenes of aromatic plants play diverse ecological roles, and their volatility adds further complexity to the effects of changes in these constituents. Understanding their response to stress is critical to their conservation and sustainable use.

Foliar terpene composition in *Aloysia citriodora* is consistent throughout the growing season. The concentration of mono- and sesquiterpenes, however, changes significantly over the course of the harvest period. The terpenes increase up to the flowering period and decline thereafter. This has implications for collectors, as the harvest time for collecting maximum essential oils per leaf is highest early in the season. This also has implications ecologically. It has been suggested that the leaf could amplify the floral aroma, increasing pollinator attraction, or that defenses on leaves are highest through flowering and then these carbon sources are mobilized to be used in seed production and storage (Bianco et al. 1994, Dudareva et al. 2006). Terpenes are mobile carbon resources within the plant, and the initial decline in terpene concentration indicates a shift in the allocation of carbon resources mediated through the

catabolism of the mono- and sesquiterpenes as a carbon source (Croteau 1988, Mihaliak et al. 1991, Gershenzon 1994). The continued slower decline over time is likely due to continued leaf expansion without concurrent terpene biosynthesis. Based on the low percentage of the overall terpene pool that was demonstrated to be volatilized by lemon verbena, it is unlikely that the decline is due to continued release of the volatile mono- and sesquiterpenes from leaf trichomes (Gershenzon et al. 2000, Sangwan et al. 2001, Dudareva et al. 2006, Figueiredo et al. 2008).

The inverse relationship between plant density and foliar biomass in lemon verbena leads to an optimum plant spacing of 0.75 - 1.0 m between plants for highest biomass yield per hectare. Since foliar terpene content was not significantly affected by plant density, optimizing biomass yield should be the priority for production. Essential oil composition was not affected by either of the agronomic variables, and concentration per gram dry weight was highest in August, indicating that lemon verbena should be planted in stands of high to intermediate density and harvested at the start of flowering time (August in temperate climates of northern latitudes) to optimize the yield of essential oil from the leaves. If being used fresh or in teas, adjustments for seasonal variation should be made when determining the amount of leaf material used.

The increase in foliar terpene concentrations we observed in response to water stress fits with a model of abiotic stress responses better than existing defense-based hypotheses (Vickers et al. 2009). Further research into conferred fitness advantages, synergistic stress responses, and associated costs of production of these terpenes will be critical to expanding our understanding of the selection pressures that have been involved in past as well as current adaptability of terpene-producing plants. These data will also assist in developing more predictive models of plant chemistry and ecology that will have improved accuracy in the coming decades of global climate change. Our results indicate that ambient levels of ultraviolet-B solar radiation may be

necessary for stem elongation and that there are tradeoffs in allocation to growth versus terpene accumulation in plants exposed to ambient UV-B. This study suggests that ambient levels of UV-B may be important in proper plant development, especially in those species that have evolved in high UV-B radiation environments. Our research does not negate the role of these compounds in defense against herbivory and other pathogens, but it does imply that the cause and effect relationships assumed in several defense hypotheses may need to be reconsidered for mono- and sesquiterpenes. Volatile terpene emission rates did not have a significant response to changes in water availability or UV-B. Release of these compounds does not seem to be affected by stomatal closure or incident UV-B radiation.

Wild populations of *A. citriodora* show a great deal of infraspecific variation, both within and among populations in their native range. Chemotypes are significantly correlated with population of origin from different geographic regions in northwestern Argentina. TLC captures this variation and would allow collectors, growers, and conservationists to document chemotypic diversity and presence of certain compounds. These compounds include positive character impact compounds for lemon verbena (e.g., citral), negative compounds brokers avoid (i.e. citronellal and β-thujone), and novel compounds that may improve the quality of cultivars or be important for conservation efforts. TLC is an efficient, rapid, low-cost screening tool for wild populations of essential oil producing plants.

Many aromatic plant species have evolved in environments with multiple ecological stressors (e.g., drought, heat, high incident radiation, and low fertility). One of the responses to environmental stress is the production of the very compounds for which many of the species are valued economically. As global climate change alters water availability and temperature ranges in traditional agricultural areas, species adapted to these environmental stresses (i.e. aromatic

plants) may be viable alternative crops. Understanding the mechanism of adaptation may also allow us to improve crop development in other species to contend with changing water availability and increase in global temperatures. Future studies with terpene storing and emitting species should investigate how the chemotypes of these species respond to environmental stresses to test and further develop the "single biochemical mechanism for multiple physiological stressors" model proposed by Vickers et al. (Vickers et al. 2009). The geographic variability of chemotypes in *A. citriodora* could represent an adaptive advantage of certain chemical constituents in relation to the environmental parameters (e.g., water availability, incident radiation, and altitude) of the native populations. Future studies will investigate the geographic variation of other species of aromatic plants and how the chemotypes respond to these environmental stresses and the effects on associated herbivores and pollinators.

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