RECOMMENDATIONS FOR SELECTION EFFORTS TO IMPROVE THE THERAPEUTIC QUALITY OF *ECHINACEA ANGUSTIFOLIA* CROPS IN BRITISH COLUMBIA

by

Alain Boucher

B.Sc. University of Ottawa, 1997
M.Sc. University of Ottawa, 2001

A THESIS SUBMITTED IN PARTIAL FULLFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies

(Plant Science)

The University of British Columbia
(Vancouver)

August 2008

ABSTRACT

For over a century, documented scientific research and debate has revolved around the therapeutic properties of the medicinal plant *Echinacea angustifolia*. With overwhelming evidence demonstrating the biological activity of its root phytochemical constituents, the genetic improvement of *E. angustifolia* by selecting phytochemically rich genotypes has garnered both scientific and commercial interest. This dissertation presents results of multi-disciplinary experiments intended to help establish scientifically based guidelines for breeding efforts aimed at developing therapeutically superior varieties of *E. angustifolia* in British Columbia.

Cultivated *E. angustifolia* populations from British Columbia and Washington were grown in a common greenhouse environment to identify possible genetically superior populations with respect to root concentrations of therapeutically relevant caffeic acid derivatives (CAD) and alkamides. However, none of the studied cultivated *E. angustifolia* populations showed significant genetic differences in terms of root phytochemical traits. In the second part, an investigation into correlations between root and shoot phytochemical concentrations in field- and greenhouse-grown plants revealed that concentrations of therapeutically relevant marker compounds in shoots were generally poor predictors of concentrations in roots. Some weak yet significant positive correlations were observed between root and shoot concentrations of CADs but were inconsistent between the two environments. Significant genotype by environment interactions were documented for the first time in phytochemical traits of *E. angustifolia* in a study of five genetically homogeneous populations grown in three different

environments, including 1 greenhouse and 2 field sites in British Columbia. For the final objective, *in vitro* bioassays showed that environmentally and genotypically related differences in concentrations of CADs and alkamides in *E. angustifolia* ethanolic root extracts did not translate into significant differences in their anti-inflammatory potential as measured by pro-inflammatory interleukin (IL-6 and IL-8) secretion in human bronchial epithelial (BEAS-2B) cells challenged with rhinovirus. When used in isolation however, pure tetraene alkamide showed a significant inhibitory effect on secretion, thereby further supporting the use of high alkamide production as a selection criterion for therapeutic *E. angustifolia* cultivar development. A series of recommendations derived from these findings are presented along with ideas for important future studies in the field of *Echinacea* research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

I would like to thank my research advisor, Dr. Shannon Cowan, for her contributions to my research and the time that she devoted to the thoughtful revision of my work. I am indebted to the members of my research committee, Dr. Andrew Riseman, Dr. Jörg Bohlmann, and Dr. Murray Isman, for their insightful advice and valuable direction.

I am grateful to Mr. David Kaplan, the staff of the UBC horticultural greenhouse, and Mr. Sean Trehearne at Totem Field for their technical assistance and recommendations in a matter of true importance: growing plants.

This project would have been entirely fruitless had it not been for the participation of growers and their generous contributions of resources. I am especially grateful to Mrs. Tracy and Mr. Warren Schimpf from the Flying-Two Ranch along with Mr. Gary Ward from Walachin Walnuts who so generously allowed us to use their land for multiple growing seasons.

Thanks to the participation of Dr. Sidney Katz and Dr. Robert Harris, this project was able to take on a broader and more meaningful scope. Their contributions of time, laboratory resources, and expert advice are acknowledged and immensely appreciated.

I thank Dr. Mahesh Upadhyaya and Dr. Bob Copeman for agreeing to have me as a teaching assistant in their courses. My work with these two esteemed professors has greatly enriched my experience and education at UBC.

I am grateful to Dr. Thor Arnason of the University of Ottawa for selflessly allowing me to use his equipment and resources without conditions of compensation or benefit. I am also thankful to him for his reviews and comments on parts of this dissertation.

Soon-to-be Dr. Diane Edwards is owed many thanks from me for numerous reasons, not the least of which are her willingness to converse, moral support and friendship. What I lacked in quantity of student colleagues, she made up in quality.

I am most grateful to my family. My parents gave me a brand new laptop at the beginning of my program and unwavering encouragement throughout. Both of these contributions have been instrumental in accomplishing my work.

Nothing that can be written on paper could ever adequately reflect the gratitude that I feel towards my wife Avital. Unlike my hairline, her love and support for me endured throughout my time as a doctoral student. She was a source of much-needed motivation and constant understanding. Without her I would not have been able to bring this project to a successful conclusion nor would I have my beautiful daughter Navah, and for this I will be eternally grateful.

To Avital and Navah,

whose therapeutic values are indisputable

CO-AUTHORSHIP STATEMENT

Chapter 2: Alain Boucher was involved with the identification and design of the research program, performing the research, data analyses and manuscript preparation. Dr. Shannon Cowan was involved in the identification of the research program and manuscript revision.

Chapter 3: Alain Boucher was involved in the identification and design of the research program, performing the research, data analyses and manuscript preparation. Dr. Shannon Cowan was involved in the identification of the research program and manuscript revision. Dr. John Arnason facilitated the research and assisted with manuscript revision. Paula Brown was involved in performing the research.

Chapter 4: Alain Boucher was involved with the identification and design of the research program, performing the research, data analyses and manuscript preparation. Dr. Shannon Cowan was involved in the identification of the research program and manuscript revision.

Chapter 5: Alain Boucher was involved with the identification and design of the research program, performing the research, data analyses and manuscript preparation. Shannon Cowan was involved in the identification of the research program and manuscript revision. Drs. Sid Katz and Robert Harris facilitated the performance of tissue culture experiments and contributed significant advice during manuscript preparation.

Chapter One

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 THE GENUS *ECHINACEA*

Plants from the North American native genus *Echinacea* have become international icons of a fluctuating herbal industry. Members of the large Asteraceae family, these herbaceous perennials are commonly recognized by ray florets which vary between shades of purple to light pink or white. *E. paradoxa* is a notable exception in having yellow ray florets. For many species, factors other than taxonomic identity are likely to be important determinants of ligule colour, including developmental stages of the capitula (McKeown 1999) and the influence of a latitudinal colour cline as described by McGregor (1968). The taxonomy of the genus *Echinacea* has recently been the subject of considerable research and discussion. Findings from an extensive morphometric and chemotaxonomic revision of the entire genus resulted in a proposed reclassification (Table 1.1) of the previously accepted nine species (McGregor 1968). The reclassified taxa fall into four species with eight varieties (Binns et al. 2002a).

The three commercially important *Echinacea* taxa are placed either into three distinct species, as described by McGregor (1968); i.e. *E. purpurea* (L.) Moench, *E. angustifolia* DC, and *E. pallida* (Nutt.) Nutt. or into two species groups, as classified by

Binns et al. (2002a); i.e. *E. purpurea*, *E. pallida* var. *angustifolia*, and *E. pallida* var. *pallida*. Because of the long-standing and continued use of the McGregor designations for the commercial taxa in question and the enduring debate over the adoption of the revised classification (Blumenthal & Urbatsch 2006), the original nomenclature from McGregor (1968) will be employed in this thesis.

The *Echinacea* market

Echinacea has not eluded the downward trend in the North American herbal market (Blumenthal 2005). However, despite a year-over-year decline in sales of 14.9% in 2005, *Echinacea* based products are still the second highest grossing herbal remedy in North America, with sales in the United States estimated at over $23 million (Blumenthal 2005). *Echinacea* cultivation is not tracked by any central agency or organization in North America, let alone globally. In addition, the market for cultivated *Echinacea* raw material is supplied in large part by small-scale growers whose venture in *Echinacea* production is often short lived. A reliable determination of total hectarage allocated to *E. angustifolia* cultivation, or for any of the commercial species for that matter, is made very difficult because of these facts. What is clear, however, is that *E. angustifolia* roots have consistently commanded the highest price among all parts of any *Echinacea* species and continue to do so despite significant fluctuations in the market. In 2002, the North American price for dried *E. angustifolia* root averaged about $10/kg whereas the price for *E. purpurea* and *E. pallida* roots averaged about $7/kg and $6/kg respectively (San Francisco Herb and Natural Food Co. 2002).

Traditional medicinal uses of *Echinacea*

North American First Nation tribes, whose territories lie between the Appalachian Mountains and the Rocky Mountains, have a long and rich tradition of using *Echinacea* plants for a wide range of applications. Traditional use of *Echinacea* has primarily been focused on the roots of *E. angustifolia* and, to a lesser degree, *E. purpurea* and *E. pallida* (Bauer & Wagner 1991). Interestingly, the natural geographic range of *E. angustifolia* closely coincides with the traditional limits of the tribal territories of the groups with documented use of the plant (Bauer 1998). Native American peoples have relied heavily on *Echinacea* to treat a very wide range of ailments, perhaps more than on any other plant (Gilmore 1913, Borchers et al. 2000). These ailments included venomous bites, various aches, wounds, respiratory infections (Kindscher 1989), as well as venereal diseases such as syphilis (Hobbs 1994). Topical applications of *Echinacea* preparations were used to treat burns, stings and animal bites (Foster & Tyler 1999).

It was the usage of *Echinacea* by European colonists that lead to the introduction of the traditional North American remedy into the European pharmacopoeia. Reports of North Americans of European origin using *Echinacea* for medicinal purposes have been documented as far back as the 18th century (Bauer & Wagner 1991) with widespread use observed in the mid-19th century at which time it garnered tremendous interest within the Eclectic medical movement. The popularity of the herb in North America persisted up until a drastic reduction in its use occurred in the early part of the 20th century. It was almost completely abandoned in the late 1920's. This period of disfavour followed a stern repudiation of the drug by the American Medical Association in response to claims that were seen as extravagant and exaggerated (McGregor 1968). Coincidentally, it was

around this time that European interest in the plant increased when German researchers undertook important groundwork towards describing and understanding the therapeutic effects of *Echinacea*.

Current medicinal uses of *Echinacea*

Some of the popularly accepted indications for the use of *Echinacea* have been strongly supported by a significant body of scientific evidence and clinical trials (reviewed in Blumenthal et al. 2003). Despite persistent debate over the efficacy of *Echinacea* products (Shah et al. 2007, Schoop et al. 2006, Turner et al. 2005, Barrett 2003), specific claims of its use as an effective treatment against upper respiratory tract infections (URTI) and other cold and flu symptoms is supported by clinical evidence (Shah et al. 2007, Goel et al. 2004). Significant reductions in symptom severity and duration in patients treated with various *Echinacea* preparations have been demonstrated in comparison with control groups receiving placebo (Shah et al. 2007, Goel et al. 2004, Brinkeborn et al. 1999). In total, eleven of the twelve studies dealing with the treatment of URTI symptoms that have been reviewed in the recently published American Botanical Council *Echinacea* monograph (Blumenthal et al. 2003) showed positive results of URTI treatment with *Echinacea* preparations alone or in combination with other herbal remedies.

The efficacy of prophylactic use of *Echinacea* preparations to prevent the occurrence of URTI's on the other hand is not strongly supported by clinical trial results (Grimm & Muller 1999). In fact, some reports have raised the possibility that long term use of *Echinacea* could lead to immune compromising conditions such as leukopenia

(Kemp & Franco 2002). The Commission E monograph recommends its use for a duration of no longer than 8 weeks (Blumenthal et al. 1998). *Echinacea* has also been reported as an effective antimycotic agent, (Roesler et al. 1991, Steinmuller et al. 1993, Binns et al. 2000) with potential clinical use as an adjunct treatment of reoccurring candidiasis in female patients (Coeugniet & Kuhnast 1986). In addition, the topical application of *Echinacea* preparations to wounds and abrasions promotes healing by reducing inflammation and cicatrization (Tubaro et al. 1987, Speroni et al. 2002).

A significant body of published research describes various *in vitro* effects of *Echinacea* extracts. Much of the information derived from these laboratory assays demonstrate the effects of *Echinacea* on two specific physiological processes, namely the inflammatory response and cellular oxidative damage (Barrett 2003). The demonstrated anti-inflammatory potential of *Echinacea* extracts has received particular attention given its putative role in *Echinacea*'s moderating effects on URTI symptoms.

1.2 PHYTOCHEMISTRY OF *ECHINACEA*

Considerable work has been done to elucidate the specific activity of chemical constituents in *Echinacea*. A wide array of compounds ranging from highly polar to lipophillic has been isolated and assessed for their bioactivity. Important variations have been demonstrated in the chemical constitution of *Echinacea* depending on species, developmental stage, plant part, genotype and environmental influence, with potential interactions between all of these factors (Binns et al. 2002b, Letchamo et al. 1999).

Phenolic compounds

On the polar end of the spectrum, the phenolic caffeic acid derivatives constitute one of the two major commercially important classes of *Echinacea* phytochemicals. A number of these compounds are commonly used as chemical markers to verify product identity and quality. Echinacoside (Figure 1.1), a phenylpropanoid glycoside, is found in relatively high amounts in the roots and aerial parts of two of the three medicinally used *Echinacea* species, *E. angustifolia* and *E. pallida*, with the highest concentrations being reported in roots of *E. angustifolia* (Binns et al. 2002b). The apparently negligible contribution of echinacoside to the immunomodulating properties of *Echinacea* (Bauer & Wagner 1991) has lead to debate over its value as a marker of quality. Nonetheless, there is ample evidence demonstrating it as a highly effective antioxidant (Pellati et al. 2004, Speroni et al. 2002) and a potential contributor to anti-inflammatory activity (Speroni et al. 2002) through nitric oxide scavenging (Xiong et al. 2000). It is still used as an important industry marker and is especially useful in differentiating *E. angustifolia* products from *E. purpurea* products given its negligible and usually undetectable accumulation in the latter species.

Cynarin (1,3-Dicaffeloyl-quinic acid) is another caffeoyl derivative (Figure 1.1) that is important when verifying specific product identity since it is characteristically found, among the commercial species, only in roots of *E. angustifolia*. It is a quinic acid derivative present in other species of the Asteraceae family including artichokes (*Cynara cardunculus*). Chlorogenic acid (3-O-Caffeoyl-quinic acid), another quinic acid-containing phenolic, is accumulated in significant concentrations in *E. angustifolia* roots and is often measured for purposes of phytochemical evaluations. It has well

documented biological activities, including, most notably, antioxidative potential (Kono et al. 1997). It is doubtful however that any *Echinacea*-specific properties can be ascribed to this compound individually given its cosmopolitan occurrence in species from other distantly related families including the Rubiaceae and the Solanaceae.

Cichoric acid (Figure 1.1) accumulation, although reported in all of the medicinal *Echinacea* species (Binns et al. 2002b), is usually very low and often below detectable limits in *E. angustifolia* roots. Its greatest concentrations in *Echinacea* are found in aerial parts and roots of *E. purpurea* with lower levels reported in the flowerheads of *E. angustifolia* and *E. pallida* (Binns et al. 2002b). Zheng et al. (2006) reported increased production of cichoric acid in roots of *E. angustifolia* grown under deep-flow hydroponic conditions. Evidence for cichoric acid bioactivity includes *in vitro* and *in vivo* observations of phagocytosis stimulation (Bauer & Wagner 1991), inhibition of hyaluronidase activity (Soicke et al. 1988) and protection of type III collagen from degradation by free radical attack (Facino et al. 1995).

Standardisation and identification of *Echinacea* products based on the above caffeic acid derivatives have been facilitated by the development of a number of validated HPLC methods (eg. Laasonen et al. 2002, Perry et al. 2001, Bergeron et al. 2000).

Lipophilic compounds

Alkamides are nitrogenous lipid-derived compounds accumulated, among the three commercial species, predominantly in *E. angustifolia* and *E. purpurea* with only trace amounts detectable in *E. pallida* (Binns et al. 2002b). They constitute a highly

diverse class of compounds with over 20 different alkamides isolated from the *Echinacea* genus (Harborne & Williams 2004). Alkamides are found in at least ten different plant families with more than 200 different related compounds identified so far. Families with species producing characteristically high levels of alkamides, aside from the Asteraceae, include the Piperaceae and the Rutaceae (Ramirez-Chavez et al. 2004). *Echinacea* alkamides are characterised by fatty acid-derived carbon chains of varying lengths (11-16) bound to either an isobutyl- or methylbutyl-amide group (Figure 1.2). The different alkamides also vary from one another by the number and position of double and/or triple bonds along their carbon chain. There is evidence to show that the biological activity and bioavailability of *Echinacea* alkamides are affected by the pattern of unsaturation in the carbon chain (Matthias et al. 2007, Chen et al. 2005, Woelkart et al. 2005, Matthias et al. 2005).

Some of the carbon chain variations are characteristic to particular species thereby making alkamides valuable standardisation markers (Binns et al. 2002a). Alkamides with only one double bond are major constituents of *E. angustifolia* lipophilic fractions whereas in *E. purpurea* the major constituents have two double bonds (Bauer 1998). However, the most abundant alkamides found in both *E. purpurea* and *E. angustifolia* are the isomeric marker compounds Dodeca-2E, 4E,8Z,10E/Z-tetraenoic acid isobutylamides (tetraenes) which are found to accumulate mainly in roots and inflorescence (Binns et al. 2002b). The alkamides are responsible for the characteristic local anaesthetic effect that produces a numbing sensation of the tongue when *Echinacea* roots are chewed on (Bauer & Wagner 1991). There is growing evidence to support claims that alkamides constitute a major component of *Echinacea* biological activity (Woelkart & Bauer 2007, Barrett

2003). Effects specific to *Echinacea* alkamides have been demonstrated on various processes including macrophage activation (Goel et al. 2002), inflammation (Chen et al. 2005), and T-cell response to rhinovirus infection (Sasagawa et al. 2006). However, it appears that the various alkamides may not all be contributing equally to the various actions of *Echinacea*. In a study examining the immunomodulatory effects of individually isolated *Echinaceae* alkamides, Raduner et al. (2006) demonstrated differences in binding affinity to cannabinoid receptors among the three tested alkamides and their ability to modulate the secretion of the pro-inflammatory cytokine interleukin(IL)-6 in human cell cultures. Differences among alkamides have also been reported in their effect on the production of IL-2 (Sasagawa et al. 2006), nitric oxide (Chen et al. 2005), and the nuclear transcription factor NFκB (Matthias et al. 2007), as well as in their bioavailability following oral administration (Woelkart et al. 2005).

As is commonly found in other species of the Asteraceae family, certain *Echinacea* species produce polyacetylenes (ketoalkenes/ynes) as characteristic constituents of their lipophilic phytochemical profiles. These compounds have demonstrated photo-activated anti-fungal properties (Binns et al. 2000), but appear to be largely irrelevant to *E. angustifolia* given the extremely low concentrations reported in the roots of this species (Binns et al. 2002b, Bauer & Wagner 1991). Characteristically high levels of ketoalkynes/enes (Figure 1.3) accumulate in the roots of *E. pallida*, as well as a few of the non-commercial species (Binns et al. 2002b). Because of their specificity, among the commercial species, to *E. pallida*, the ketoalkynes/enes have been regarded as useful chemical markers for the purpose of identification and standardisation of *E. pallida* preparations (Bauer et al. 1988).

Polysaccharides, glycoproteins and other compounds

The highly polar polysaccharides and glycoproteins found in *Echinacea* have garnered considerable interest given the mounting evidence that supports their roles in the *Echinacea*'s non-specific immunostimulation activity (Alban et al. 2002). A variety of polysaccharides have been identified in all parts of *E. angustifolia* and *E. purpurea* as well as in cell cultures and expressed flowerhead juices from *E. purpurea* (reviewed in Bauer 1998). Immunological assays have clearly demonstrated the stimulatory effects of some these polysaccharide fractions. An acidic arabinogalactan, derived from *E. purpurea* cell culture, was reported to be an effective and selective stimulator of macrophage cells with a dose dependant increase of oxygen radical release (Luettig et al. 1989).

The major formulation method currently used for *Echinacea* preparations in North America, namely ethanolic extraction, is largely ineffective in extracting polysaccharide and glycoprotein fractions from raw material. Pressing juices out of fresh *E. purpurea* flowerheads, a mode of preparation most popular in Europe (Galambosi 2004), is considered to be the most effective way of obtaining extracts with higher concentrations of polysaccharides and glycoproteins in preparations.

Other chemical constituents have been isolated and identified including flavonoids (mainly quercetin, kaempferol, and rutoside derivatives), essential oils and alkaloids (Bauer 1998). However, no commercial and/or therapeutic value has yet been clearly demonstrated for these compounds in isolation or in combination with other compounds, and their potential contribution to the bioactive effects specific to *Echinacea* preparations has not been investigated.

Biosynthetic regulation of phytochemical markers of *E. angustifolia*

Little is known about the downstream steps in the metabolic pathways involved in the synthesis of some of the more important active compounds. Although pathways leading to the formation of caffeic acid have been well described, the enzymes and coenzymes regulating the downstream steps involved in the production of its derivatives in *E. angustifolia* are currently unknown. It is however probable that enzymes involved in the early steps of the shikimate metabolic pathway play important roles in regulating accumulation of the caffeic acid derivatives. Such enzymes include phenylalanine ammonia lyase (PAL), which catalyzes the first committed step in phenylpropanoid metabolism by converting L-phenylalanine into *trans*-cinnamic acid. In light of the well known influence of various environmental factors on its activity (Dixon & Paiva 1995), PAL is likely a contributing factor in the accumulation of CADs in *E. angustifolia*.

The biosynthetic pathway leading to the production of alkamides is entirely unknown at this time. They have conventionally been thought of as desaturation products of long-chain fatty acids such as oleic acid. Recently, alkamides have been hypothesized to share common early biosynthetic steps with N-acylethanolamides (López-Bucio et al. 2007). N-acylethanolamides, which interestingly include the endogenous animal cannabinoid ligand anandamide, are derived through hydrolysis of the minor membrane phospholipid N-acylated phosphatidylethanolamine by phospholipase D (Chapman 2004). Both of these structurally related groups of compounds have been demonstrated as novel signalling molecules involved in regulating plant growth and morphogenesis (López-Bucio et al. 2006).

1.3 *ECHINACEA* CULTIVATION AND BREEDING

Internationally, medicinal and horticultural *Echinacea* cultivation has almost exclusively depended on *E. purpurea* (Galambosi 2004). The greater focus on *E. purpurea* as a cultivated species has historical and agronomic reasons (Foster & Tyler 1991). The agronomic reasons include its higher disease resistance, the presence of active compounds in its aerial parts that can be harvested annually, and its adaptability to a wider range of growing conditions. It is also much more of a horticulturally important species than *E. angustifolia*. The profitability of *E. purpurea* as a specialty cut-flower has been attributed to its higher yields, longer stem lengths and relative absence of major pests (Starman et al. 1995), contrary to *E. angustifolia*. Several *E. purpurea* cultivars of proven ornamental value have been released commercially. However, the selections have been based on traits of horticultural relevance with little or no information available on their secondary metabolites.

All of the herbal market demand for *E. purpurea* can now be met through cultivation (Letchamo et al. 2002). On the other hand, wild-harvesting of *E. angustifolia* is still actively practised (Price & Kindscher 2007) in part because of the perceived higher potency of naturally grown roots. This is of grave concern to conservation efforts given the unsustainable nature of the practise. *E. purpurea* is considered to be extirpated in Michigan and *E. pallida* is listed as a threatened species in Tennessee and Wisconsin (USDA 2002). Perhaps more importantly, conservation efforts aimed at other *Echinacea* species, such as *E. tennesseensis* (Beadle) Small and *E. laevigata* (Boynton & Beadle) Blake, both of which have been listed as endangered (USDA 2002), could be severely impacted by accidental harvesting because of misidentification given their resemblance to

E. angustifolia. Moreover, the published assessments of phytochemical concentrations in *E. angustifolia* roots, from the wild and under cultivation, do not support the idea that levels of bioactive compounds are reduced in cultivated material (Zheng et al. 2006). Despite these considerations, the development of a medicinal *E. angustifolia* crop lags far behind *E. purpurea*.

Published recommendations for *Echinacea* growing conditions in Canada vary little among cultivated species (Table 1.2). However, these recommendations are being refined through research aimed at determining optimal cultivation requirements for individual species (Galambosi 2004). With the advent of relatively simple and efficient procedures for the analyses of secondary compounds in *Echinacea* (eg. Laasonen et al. 2002, Perry et al. 2001, Bergeron et al. 2000), it is now feasible to make recommendations that will improve not only overall biomass yield but also the production of the therapeutic metabolites.

Considerable work remains to establish refined recommendations for the cultivation of each species of *Echinacea*. Although a number of factors have been investigated for their effects on growth, yield and the production of secondary metabolites, the majority of these reported experiments, again, are focused on *E. purpurea*.

The question of spacing and planting density has been investigated for *E. purpurea* and *E. angustifolia*. On one hand, Shalaby et al. (1997) demonstrated beneficial effects of increased inter-plant spacing (up to 60 cm apart) on *E. purpurea* growth. The increased spacing however resulted in a reduction of total biomass per unit production area. On the other hand, Parmenter and Littlejohn (1997) observed maximum

root yield per area for *E. purpurea* at planting densities of 20 plants/m of 1.5 m wide beds. In their design, this density corresponded to inter-row and inter-plant distances of less than 0.2 m and 0.22 m respectively. They also noted density dependent changes in the rhizome:root ratio leading them to speculate that spacing may influence phytochemical quality of cultivated products. From their investigations into *E. angustifolia* spacing, Little (1999) recommended inter-plant spacing of no more than 23 cm between *E. angustifolia* plants under cultivation. This recommendation was based on the maximum leaf rosette diameter observed in South Dakota garden trials. Although aboveground competition for light would be minimised by such spacing, Little (1999) does not address the question of belowground interactions between plants. Other than competition for available soil nutrients and water, allelopathy may also play an important role in belowground interactions between plants. Viles and Reese (1996) demonstrated allelopathic potential of volatile compounds and aqueous extracts from *E. angustifolia* roots on *Lactuca sativa* and *Panicum virgatum* seedlings. There is currently no direct evidence supporting or refuting allelopathic interactions between plants of *E. angustifolia*.

A surprisingly small number of published studies have addressed the question of *Echinacea* response to applications of fertilizer and differing nutrient solutions. Among those that have examined the question, their main focus has been on *E. purpurea* with very little specific attention on *E. angustifolia*. El-Gengaihi et al. (1998) found that applications of inorganic fertilisers with high levels of nitrogen and low levels of potassium to *E. purpurea* crops in Egypt slightly increased biomass as well as alkamide levels. Dufault et al. (2003), on the other hand, did not find significant differences in

levels of caffeic acid derivatives between fertilised and unfertilised plants of *E. purpurea* or *E. pallida*. In a Chilean field trial, none of the tested macro-nutrients (N, P, or K) showed significant effects on either root yield or alkamide concentration in *E. angustifolia* (Berti et al. 2002). Echinacoside was slightly higher with the addition 100 kg ha^{-1} of K in older plants only. Under controlled greenhouse conditions, the phytochemical concentrations of *E. angustifolia* plants were similar to each other despite varying NO^{3-}/NH^{4+} ratios (Zheng et al. 2006). The available evidence, albeit limited at this point, suggests that overall increased macro-nutrient availability has little influence on phytochemical production in cultivated *E. angustifolia*. Similar inferences cannot be extended to micro-nutrient availability given the absence of reliable evidence.

The different *Echinacea* species show varying degrees of susceptibility to a range of pests and pathogens in cultivation. *E. angustifolia* has the poorest response of the three commercial species to cultivation with erratic achene germination (Shalaby et al. 1997) and a reportedly high susceptibility to fungal pathogens (Letchamo et al. 2002). Some of the significant pathogenic agents reported in *Echinacea* crops include *Sclerotinia sclerotium* (Chang et al. 1997a), which causes stem rot and wilting, *Fusarium oxysporum* (Peichowski et al. 1997) and *Botrytis cinerea* (Chang et al. 1997b). Although no fungicides are currently registered in Canada for the control of *Echinacea* fungal diseases, Wang et al. (2000) have shown effective control of *Sclerotinia* stem rot with applications of benomyl, iprodione and vinclozolin. The effects of fungicides on the phytochemical profile of *Echinacea* plants are unknown. Viral infection of *Echinacea purpurea* by Cucumber Mosaic Virus has been shown to cause a drastic and significant

effect on relative concentrations of the various alkamides and an overall quantitative reduction of the lipophilic fraction (Hudaib et al. 2002).

Although molecular markers have been proposed as important tools in *E. angustifolia* breeding efforts, there are currently no reports of their actual application for breeding purposes. RAPDs and AFLPs have contributed valuable information towards related objectives including quantitative phytochemical predictions (Baum et al. 2001), population genetic diversity assessment (Kim et al. 2004), and identification of valuable and genetically distinct germplasm (Kapteyn et al. 2002).

1.4 PHYTOCHEMICAL TRAITS AS THE FOCUS OF BREEDING EFFORTS

Secondary metabolites, or natural products, have long been recognized as a means for plants to interact with their environments, whether it be as defenses, attractants or other types of chemical signaling agents (Rosenthal & Berenbaum 1992). Either by their abundance or by their absence, the potential of secondary metabolites to influence the commercial value of the plants that produce them is obvious. Because of this, these compounds have been targeted by breeding and selection efforts aimed at improving the quality and commercial value of numerous crop plants. A recent example of a successful effort to modify the concentrations of specific secondary metabolites through selection and breeding is the development of the 'Anna' and 'Bernardo' varieties of sweet basil (*Ocimum basilicum*) by the Nestle company. The varieties were selected on the basis of high levels of the monoterpene linalool and low levels of the phenylpropanoids eugenol and eugenol-methyl to impart a strong and desirable flavour to the cultivated plants. The use of these basil varieties as source material for the production of pesto sauce, as reported by the company (Nestlé CT Agriculture 2006), has led to a reduction in

production costs of almost 25%. Parallels between breeding desirable basil chemotypes and the development of phytochemically rich *E. angustifolia* varieties are clear in that, for both cases, the desirability of a given genotype is dependent on a suite of secondary metabolites that can vary quantitatively and qualitatively (see Labra et al. 2004 for example of phytochemical variation in *O. basilicum*).

Despite the complexity of biosynthetic pathways from which stem secondary metabolites, distinct genotypes can arise in natural populations that result in apparently discrete differences between groups or classes of chemotypes. For instance, in a phytochemical examination of French *Cannabis sativa* cultivars, Fournier and Paris (1980) observed distinct chemotypes, differentiable by marked differences of ratios in which two major cannabinoids, tetrahydrocannabinol (THC) and cannabidiol (CBD), accumulated in the plants. Similar observations were recorded earlier by Small & Beckstead (1973) where, in a systematic survey of 350 *Cannabis* genotypes, individual plants fell within one of three chemotypes depending on their cannabinoid profiles; namely the pure CBD chenotype, the pure THC chemotype, or an intermediate THC/CBD chemotype. The discrete differences between chemotypes were recently explained by a single locus model with codominant alleles (Meijer et al. 2003). This example illustrates how apparently complex phytochemical traits could realistically be fixed where phenotype-determining genes regulate downstream steps of complex biosynthetic pathways.

Other examples of naturally occurring chemotypes that present great potential and/or realized value to varietal improvement programs include naturally decaffeinated *Coffea arabica* plants (Silvarolla et al. 2004), *Rosmarinus* biotypes with high levels of

essential oils (Carruba et al. 2006) or *Origanum* chemotypes with distinct terpenoid profiles (Radusiene et al. 2005).

1.5 OBJECTIVES AND OVERVIEW OF THE THESIS

General objective and overall theme of the thesis

The research presented in this thesis is intended to answer a series of key questions which will help in establishing guidelines for efficient and effective selection strategies primarily aimed at genetically improving root concentrations of currently used quality markers and the therapeutic quality of the *E. angustifolia* crop. Many of the experiments presented in this thesis have a particular focus on *E. angustifolia* production in British Columbia.

Specific objectives

The underlying objective of the experiments presented in Chapter 2 was to assess cultivated *E. angustifolia* populations from British Columbia and Washington, to identify, at a population level, genetic differences expressed as significant phenotypic differences in root concentrations of phytochemical markers and selected agronomic traits. Significant phenotypic differences among populations grown in a controlled greenhouse experiment could justify focused selection efforts on specific populations with demonstrated agronomic performance outside the native range of *E. angustifolia*.

Chapter 3 presents a study that aims to identify phenotypic correlations of commercially and medicinally important phytochemical traits between different organs within individual *E. angustifolia* plants. The information obtained from this objective will be instrumental in assessing the potential value of a proposed indirect selection

strategy aiming to increase the root phytochemical production of an *E. angustifolia* crop. The experiments were conducted under greenhouse and field conditions to identify possible differences between the two environments.

The experiments described in Chapter 4 were intended to address questions pertaining to genotype x environment interaction effects (GE) on phytochemical and root yield traits in *E. angustifolia*. This study is unique in its use of *E. angustifolia* genotypes which had been clonally propagated to isolate the environmental effects on phenotypes of plants grown in three different environments representative of possible growing environments in British Columbia, including one greenhouse site and two distant field sites. Further, GGE biplot analyses (Yan et al. 2000) were conducted on phenotypic data to compare and evaluate the different sites as possible selection environments of *E. angustifolia* genotypes. This study is the first to directly address the question of GE on *E. angustifolia* phytochemistry and the potential for genotypic re-ranking across different growing environments.

The final objective in this thesis was to determine whether genotypically and environmentally related phytochemical differences in *E. angustifolia* extracts translate into significant differences in their anti-inflammatory potential. Chapter 5 presents the results of a series of bioassays based on rhinovirus-challenged cells treated, in a first experiment, with whole *E. angustifolia* extracts, and in a second experiment, with two isolated and pure *E. angustifolia* phytochemical markers; echinacoside and dodeca-2(E),4(E),8(Z),10(Z)-tetraenoic acid isobutylamide. This objective is intended to inform the development genotype-assessing bioassays to aid in the selection of desirable *E. angustifolia* genotypes.

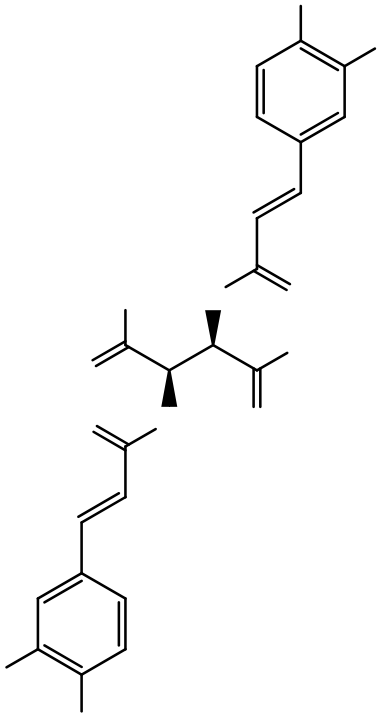
Table 1.1: Comparison of *Echinacea* classification according to McGregor (1968) with the proposed reclassification based on morphometric and phytochemical profile analysis of Binns et al. (2002a).

McGregor (1968)	Binns et al. (2002a)
E. purpurea	*E. purpurea*
E. angustifolia	*E. pallida* var. *angustifolia*
E. pallida	*E. pallida* var. *pallida*
E. sanguinea	*E. pallida* var. *sanguinea*
E. simulata	*E. pallida* var. *simulata*
E. tennesseensis	*E. pallida* var. *tennesseensis*
E. atrorubens	*E. atrorubens* var. *atrorubens*
E. paradoxa var. *neglecta*	*E. atrorubens* var. *neglecta*
E. paradoxa	*E. atrorubens* var. *paradoxa*
E. laevigata	*E. laevigata*

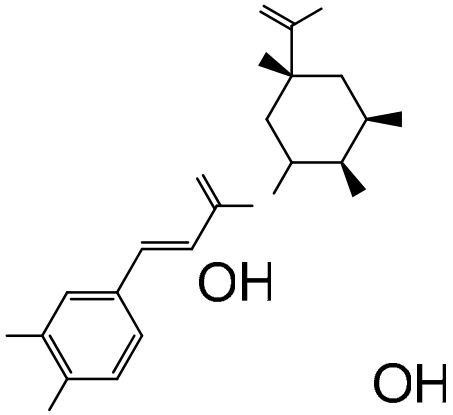
Table 1.2: General cultivation requirements and disease susceptibility of the three commercial *Echinacea* species (Li, 2000).

Spacing	Location	Soil conditions	Disease susceptibility
30 cm	Sunny	Rich, well-drained loam and sandy loam pH 6-7 Irrigation required	Leaf spot Root spot Aster yellows Stem rot Botrytis blight Wilt Virus diseases (CMV, broad bean wilt) Sclerotinia

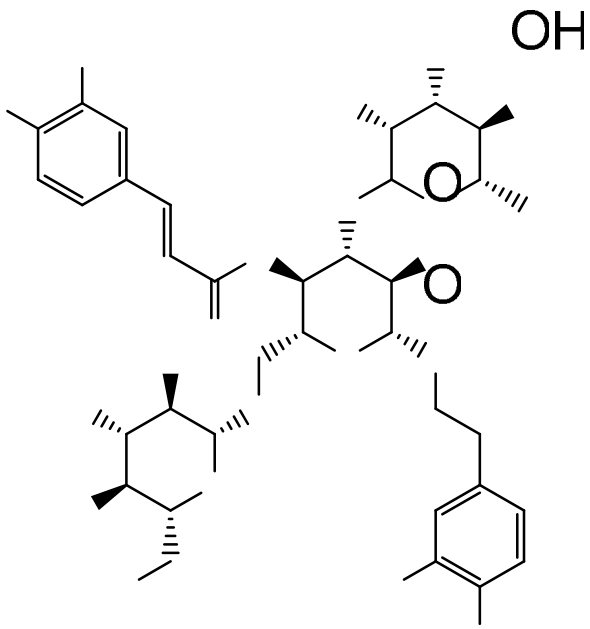
Figure 1.1: Chemical structures of caffeic acid derivatives found in *Echinacea*.



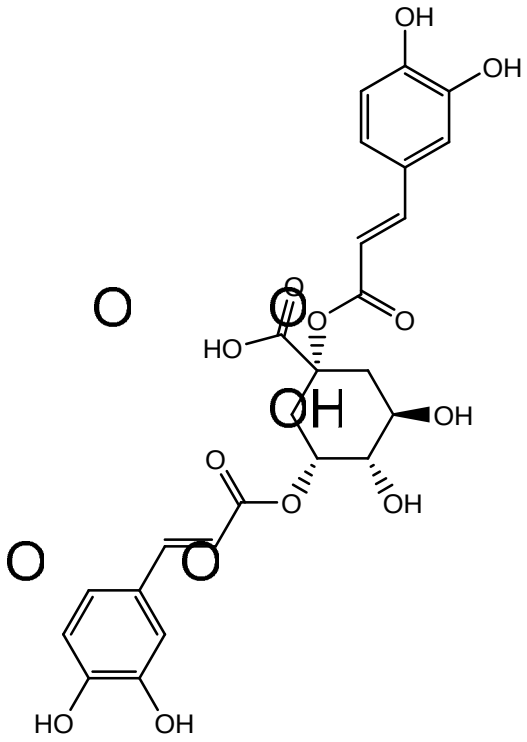
CICHORIC ACID



CHLOROGENIC ACID



ECHINACOSIDE

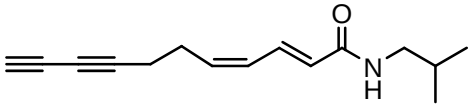


CYNARIN

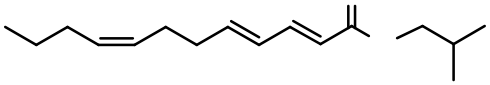
HO

OH

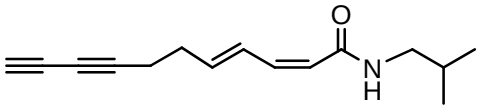
Figure 1.2: Chemical structures of *Echinacea* alkamides, numbered according to Bauer and Remiger (1989).



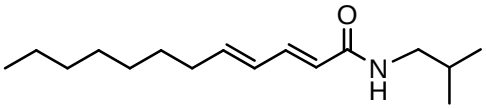
Alkamide 1



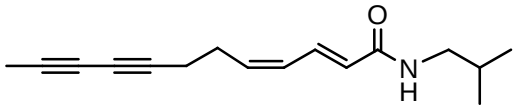
Alkamide 10



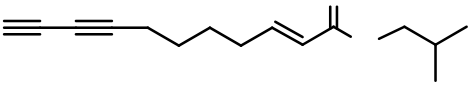
Alkamide 2



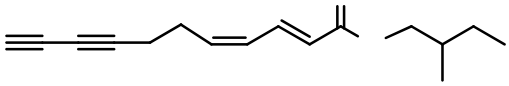
Alkamide 11



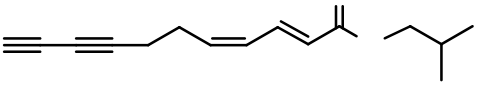
Alkamide 3



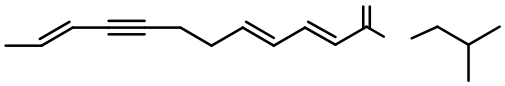
Alkamide 12



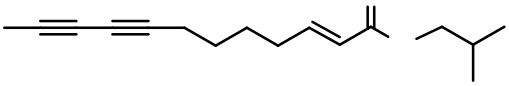
Alkamide 4



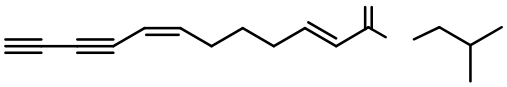
Alkamide 13



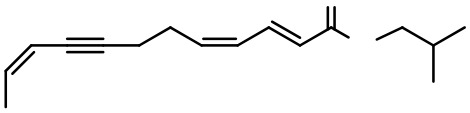
Alkamide 5



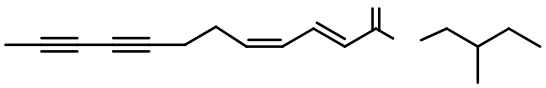
Alkamide 14



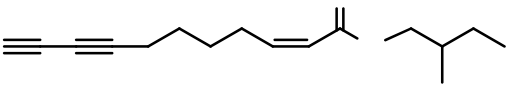
Alkamide 6



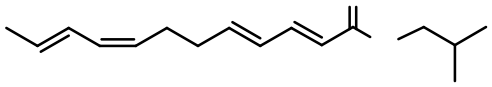
Alkamide 15



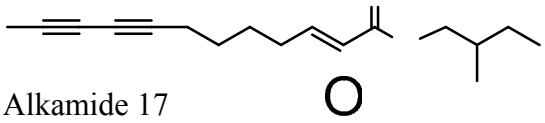
Alkamide 7



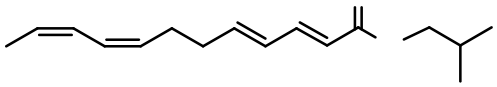
Alkamide 16



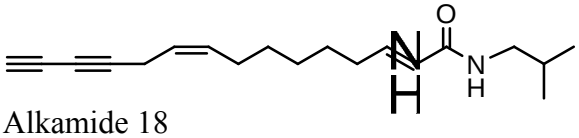
Alkamide 8*



Alkamide 17



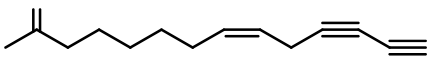
Alkamide 9*



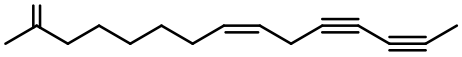
Alkamide 18

*The isomers alkamides 8 and 9 are collectively reported as tetraenes and constitute the highest concentration of alkamides in *E. angustifolia* roots.

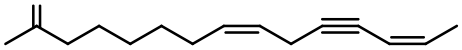
Figure 1.3: Major ketoalkenes/ynes accumulated in *E. pallida* roots. From Binns et al. 2002b and Bauer & Wagner 1991.



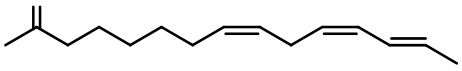
Tetradeca-8Z-ene-11,13-diyne-2one



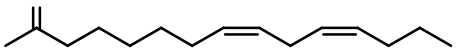
Pentadeca-8Z-ene-11,13-diyne-2-one



Pentadeca-8Z,13Z-diene-11-yne-2-one



Pentadeca-8Z,11Z,13E-triene-2-one



Pentadeca-8Z,11Z-diene-2-one

1.6 REFERENCES

Alban, S., B. Classen, G. Brunner, and W. Blaschek. 2002. Differentiation between the complement modulating effects of an arabinogalactan-protein from *Echinacea purpurea* and heparin. *Planta Medica*. 68: 1118-1124.

Barrett,B. 2003. Medicinal properties of *Echinacea*: A critical review. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology*. 10: 66-86.

Bauer, R. 1998. *Echinacea*: Biological effects and active principles. In: Lawson and Bauer (eds). Phytomedicines of Europe. American Chemical Society. Washington. pp. 140-157.

Bauer, R., P. Remiger. 1989. TLC and HPLC analysis of alkamides in *Echinacea* drugs. *Planta Medica*. 55:367- 371.

Bauer, R. and H. Wagner. 1991. *Echinacea* species as potential immunostimulatory drugs. *Planta Medica*. 55: 367-371.

Bauer, R., A. Kahn, and H. Wagner. 1988. Alkamides from the roots of *Echinacea pallida* and *E. angustifolia* roots. *Planta Medica*. 54: 426-430.

Baum, B.R., S. Mechanda, J.F. Livesey, S.E. Binns, and J.T. Arnason. 2001. Predicting quantitative phytochemical markers in single *Echinacea* plants or clones from their DNA fingerprints. *Phytochemistry*. 56: 543-549.

Bergeron, C., J.F. Livesey, D.V.C. Awang, J.T. Arnason, J. Rana, B.R. Baum, and W. Letchamo. 2000. A quantitative HPLC method for the quality assurance of *Echinacea* products on the North American market. *Phytochemical Analysis*. 11: 207-215.

Berti, M., R. Wilckens, S. Fischer, and F. Hevia. 2002. Effect of harvest season, nitrogen, phosphorus and potassium on root yield, echinacoside and alkylamides in *Echinacea angustifolia* L. in Chile. *Acta Horticutlurae*. 576: 303-310.

Binns, S.E., B. Purgina, C. Bergeron, M.L. Smith, L. Ball, B.R. Baum, and J.T. Arnason. 2000. Light-mediated antifungal activity of *Echinacea* extracts. *Planta Medica*. 66: 241-244.

Binns, S.E., B.R. Baum, and J.T. Arnason. 2002a. A taxonomic revision of *Echinacea* (Asteraceae: Heliantheae). *Systematic Botany*. 27: 610-632.

Binns, S.E., J.F. Livesey, J.T. Arnason, and B.R. Baum. 2002b. Phytochemical variation in *Echinacea* from roots and flowerheads of wild and cultivated populations. *Journal of Agricultural and Food Chemistry*. 50: 3673-3687.

Blumenthal, M. 2005. Herb sales down 7.4 percent in mainstream market. *HerbalGram*. 66: 63.

Blumenthal, M. and L.E. Urbatsch. 2006: *Echinacea* taxonomy – Is the re-classification of the genus warranted? *HerbalGram*. 72:30-31.

Blumenthal, M., J. Brinkman, and B. Wollschlaeger. 2003. *Echinacea* monograph. In: Blumenthal (ed.), The American Botanical Council Guide to Herbs. American Botanical Council. Austin. pp. 85-96.

Blumenthal, M., W.R. Busse, A. Goldberg, J. Gruenwald, T. Hall, C.W. Riggins, and R.S. Rister. 1998. The complete German commission E monographs- Therapeutic guide to herbal medicines. Klein and Rister (trans). American Botanical Council. Austin. pp. 122-123.

Borchers A.T., C.L. Keen, J.S. Stern, and M.E. Gershwin. 2000. Inflammation and Native American medicine: the role of botanicals. *The American Journal of Clinical Nutrition*. 73:339-347.

Brinkeborn, R.M., D.V. Shah, and F.H. Degenring. 1999. Echinaforce and other *Echinacea* fresh plant preparations in the treatment of the common cold. A randomized, placebo controlled, double-blind clinical trial. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology*. 6: 1-6.

Carruba, A., R. La Torre, R. Piccaglia, and S. Grandi. 2006. Chemical and botanical characterization of a *Rosmarinus officinalis* biotype from Sicily. *Acta Horticulturae*. 723: 197-202.

Chang, K.F., R.J. Howard, and S.F. Hwang. 1997a. First report of *Sclerotinia sclerotiorum* on coneflower. *Plant Disease*. 81:1093.

Chang, K.F., R.J. Howard, R.G. Gaudiel, and S.F. Hwang. 1997b. First report of Botrytis blight, caused by *Botrytis cinerea*, on coneflowers. *Plant Disease*. 81: 1461.

Chapman, K.D. 2004. Occurrence, metabolism, and prospective functions of N-acylethanolamines in plants. *Progress in Lipid Research*. 43: 302-327.

Chen,Y., T. Fu, T. Tao, J. Yang, Y. Chang, M. Wang, L. Kim, L. Qu, J. Cassady, R. Scalzo, and X. Wang. 2005. Macrophage activating effects of new alkamides from the roots of *Echinacea* species. *Journal of Natural Products*. 68: 773-776.

Coeugniet E.G. and R. Kuhnast. 1986. Recurrent candidiasis: adjuvant immunotherapy with different formulations of Echinacin. *Therapiewoche*. 36: 3352-3358.

Dixon R.A. and N.L. Paiva. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell*. 7: 1085-1097.

Dufault, R.J., J. Rushing, R. Hassell, B.M. Shepard, G. McCutcheon, and B. Ward. 2003. Influence of fertilizer on growth and marker compound of field-grown *Echinacea* species and feverfew. *Scientia Horticulturae* . 98: 61-69.

El-Gengaihi, S.E., A.S. Shalaby, E.A. Agina, and S.F. Hendawy. 1998. Alkylamides of *Echinacea purpurea* L. as influenced by plant ontogony [*sic*] and fertilization. *Journal of Herbs, Spices and Medicinal Plants*. 5:35-41.

Facino, R.M., M. Carini, G. Aldini, L. Saibene, P. Pietta, and P. Mauri. 1995. Echinacoside and caffeoyl conjugates protect collagen from free radical-induced degradation: A potential use of *Echinacea* extracts in the prevention of skin photodamage. *Planta Medica*. 61: 510-514.

Foster, S. and V.E. Tyler. 1999. Tyler's Honest Herbal: A sensible guide to the use of herbs and related remedies; 4th edition. The Hawthorn Press. New York. 441pp.

Galambosi, B. 2004: Cultivation in Europe. In: S.C. Miller & H. Yu (eds) *Echinacea*: The genus *Echinacea*. CRC Press. Boca Raton, FL. pp.29-52.

Gilmore, M.R. 1913. A study in the ethnobotany of the Omaha Indians. *Collections of the Nebraska State Historical Soc*iety. 17:358-370.

Goel, V., C. Chang, J.V. Slama, R. Barton, R. Bauer, R. Gahler, and T.K. Basu. 2002. Alkylamides of *Echinacea purpurea* stimulate alveolar macrophage function in normal rats. *International Immunopharmacology*. 2: 381-387.

Goel,V., R. Lovlin, R. Barton, M.R. Lyon, R. Bauer, T.D.G. Lee, and T.K. Basu. 2004. Efficacy of a standardized *Echinacea* preparation (EchinilinTM) for the treatment of the common cold: A randomized, double-blind, placebo-controlled trial. *Journal of Clinical Pharmacy & Therapeutics*. 29: 75-83.

Grimm, W. and H.H. Muller. 1999. A randomized controlled trial of the effect of fluid extract of *Echinacea purpurea* on the incidence and severity of colds and respiratory infections. *The American Journal of Medicine*. 106: 138-143.

Harborne, J.B. and C.A. Williams. 2004. Phytochemistry of the genus *Echinacea*. In: S.C. Miller & H. Yu (eds) *Echinacea*: The genus *Echinacea*. CRC Press. Boca Raton, FL. pp.59-71.

Hobbs, C. 1994. Echinacea: A Literature Review. *Herbal Gram*. 30: 33-48.

Hudaib, M., J. Fiori, M.G. Bellardi, C. Rubies-Autonell, and V. Cavrini. 2002. GC-MS analysis of the lipophilic principles of *Echinacea purpurea* and evaluation of cucumber mosaic cucumovirus infection. *Journal of Pharmaceutical and Biomedical Analysis*. 29: 1053-1060.

Kapteyn, J., P.B. Goldsbrough, and J.E. Simon. 2002. Genetic relationships and diversity of commercially relevant *Echinacea* species. *Theoretical & Applied Genetics*. 105: 369-376.

Kemp, D.E. and K.N. Franco. 2002. Possible leukopenia associated with long-term use of *Echinacea*. *The Journal of the American Board of Family*. 15: 417-419.

Kim, D., D. Heber, and D.W. Still. 2004. Genetic diversity of *Echinacea* species based upon amplified fragment length polymorphism markers. *Genome*. 47: 102-111.

Kindscher, K. 1989. Ethnobotany of purple coneflower *Echinacea angustifolia* Asteraceae and other *Echinacea* species. *Economic Botany*. 43: 498-507.

Kono,Y., K. Kobayashi, S. Tagawa, K. Adachi, A. Ueda, Y. Sawa, and H. Shibata. 1997. Antioxidant activity of polyphenolics in diets. Rate constants of reactions of chlorogenic acid and caffeic acid with reactive species of oxygen and nitrogen. *Biochimica Et Biophysica Acta*. 1335: 335-342.

Laasonen, M., T. Wennberg, T. Harmia-Pulkkinen, and H. Vuorela. 2002. Simultaneous analysis of alkamides and caffeic acid derivatives for the identification of *Echinacea purpurea*, *Echinacea angustifolia*, *Echinacea pallida* and *Parthenium integrifolium* roots. *Planta Medica*. 68: 572-574.

Labra, M., M. Miele, B. Ledda, F. Grassi, M. Mazzei, and F. Sala. 2004. Morphological characterization, essential oil composition and DNA genotyping of *Ocimum basilicum* L. cultivars. *Plant Science* 167: 725–731.

Letchamo, W., J. Livesey, T.J. Arnason, C. Bergeron, and V.S. Krutilina. 1999. Cichoric acid and isobutylamide content in *Echinacea purpurea* as influenced by flower developmental stages. In: Jannick (ed.). *Perspectives on new crops and new uses*. ASHS Press, Alexandria VA. pp.494-498.

Letchamo, W., Polydeonny, L.V., Gladisheva, N.O., Arnason, T.J., Livesey, J., and Awang, D.V.C. 2002. Factors affecting *Echinacea* quality. In: Jannick and Wipckey (ed.) *Trends in New Crops and New Uses*. ASHS Press, Alexandria VA. pp.514-521.

Li, T.S.C. 2000. Medicinal Plants: Culture, Utilization and Phytopharmacology. Techomic. Lancaster PA. 517pp.

Little, R. 1999. Taming *Echinacea angustifolia*: Research at SDSU and insights from a grower. South Dakota State University. Retrieved on March 18, 2008 <http://biomicro.sdstate. edu/reesen/Echinaca/newsletter.htm>

Lopez-Bucio, J., G. Acevedo-Hernandez, E. Ramirez-Chavez, J. Molina-Torres, and L. Herrera-Estrella. 2006. Novel signals for plant development. *Current Opinion in Plant Biology*. 9: 523-529.

Lopez-Bucio, J., M. Millan-Godinez, A. Mendez-Bravo, A. Morquecho-Contreras, E. Ramirez-Chavez, J. Molina-Torres, A. Perez-Torres, M. Higuchi, T. Kakimoto, and L. Herrera-Estrella. 2007. Cytokinin receptors are involved in alkamide regulation of root and shoot development in *Arabidopsis*. *Plant Physiology*. 145: 1703-1713.

Luettig, B., C. Steinmueller, G.E. Gifford, H. Wagner, and M. Lohmann-Matthes. 1989. Macrophage activation by the polysaccharide arabinogalactan isolated from plant cell cultures of *Echinacea purpurea. Journal of the National Cancer Institute*. 81: 669-675.

Nestlé CT Agriculture. 2006. Proprietary basil varieties for green sauces (Pesto). *Nestlé's Sustainable Agriculture Initiatives: Project News Report.*

Matthias, A., L. Banbury, L.M. Stevenson, K.M. Bone, D.N. Leach, and R.P. Lehmann. 2007. Alkylamides from *Echinacea* modulate induced immune responses in macrophages. *Immunological Investigations*. 36: 117-130.

Matthias, A., R.S. Addison, K.G. Penman, R.G. Dickinson, K.M. Bone, and R.P. Lehmann. 2005. *Echinacea* alkamide disposition and pharmacokinetics in humans after tablet ingestion. *Life Sciences*. 77: 2018-2029.

McGregor R.L., 1968. The Taxonomy of the Genus *Echinacea* (Compositae). *The University of Kansas Science Bulletin*. 48: 113-142.

McKeown, K.A. 1999. A review of the taxonomy of the genus *Echinacea*. In: Janick (ed.), Perspectives on new crops and new uses. ASHS Press, Alexandria, VA. pp. 482-489.

Meijer, E.P.M, M. Bagatta, A. Carboni, P Cruciti, V.M.C. Moliterni, P. Ranalli, and G. Mandolino. 2003. The inheritance of chemical phenotype in *Cannabis sativa* L. *Genetics*. 163:335-346.

Parmenter, G.A. and R.P. Littlejohn. 1997. Planting density effects on root yield of purple coneflower (*Echinacea purpurea* (L.) Moench*). New Zealand Journal of Crop & Horticultural Science*. 25: 169-175.

Pellati, F., S. Benvenuti, L. Magro, M. Melegari, and F. Soragni. 2004. Analysis of phenolic compounds and radical scavenging activity of *Echinacea* spp. *Journal of Pharmaceutical and Biomedical Analysis*. 35: 289-301.

Perry, N.B., E.J. Burgess, and V.l. Glennie. 2001. *Echinacea* standardization: Analytical methods for phenolic compounds and typical levels in medicinal species. *Journal of Agricultural & Food Chemistry*. 49: 1702-1706.

Piechowski, K., S. Rizvi, and R.N. Reese. 1997. First report of *Fusarium oxisporum* on purple coneflower. *Plant Disease*. 81:227.

Price, D.M. and K. Kindscher. 2007. One hundred years of *Echinacea angustifolia* harvest in the smoky hills of Kansas, USA. *Economic Botany*. 61: 86-95.

Raduner, S., A. Majewska, J.Z. Chen, X.Q. Xie, J. Hamon, B. Faller, K.H. Altmann, and J. Gertsch. 2006. Alkylamides from *Echinacea* are a new class of cannabinomimetics. Cannabinoid type 2 receptor-dependent and -independent immunomodulatory effects. *The Journal of Biological Chemistry*. 281: 14192-14206.

Radusiene, J., D. Stankeviciene, R. Venskutonis. 2005. Morphological and chemical variation of *Origanum vulgare* L. from Lithuania. *Acta Horticulturae*. 675: 197-203.

Ramirez-Chavez, E., J. Lopez-Bucio, L. Herrera-Estrella, and J. Molina-Torres. 2004. Alkamides isolated from plants promote growth and alter root development in arabidopsis. *Plant Physiology*. 134: 1058-1068.

Roesler, J., A. Emmendoerffer, C. Steinmueller, B. Luettig, H. Wagner, and M. Lohmann-Matthes. 1991. Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to test subjects mediates activation of the phagocyte system. *International Journal of Immunopharmacology*. 13: 931-942.

Rosenthal, G.A. and M.R. Berenbaum. 1992. Herbivores: Their interactions with secondary plant metabolites, ecological and evolutionary processes. Academic Press, San Diego CA. 493pp.

San Francisco Herb and Natural Food Co. 2002. Wholesale herbs, spices and teas. Fremont CA. pp. 15–42.

Sasagawa, M., N.B. Cech, D.E. Gray, G.W. Elmer, and C.A. Wenner. 2006. *Echinacea* alkylamides inhibit interleukin-2 production by jurkat T cells. *International Immunopharmacology*. 6: 1214-1221.

Schoop, R., P. Klein, A. Suter, S.L. Johnston. 2006. *Echinacea* in the prevention of induced rhinovirus colds: a meta-analysis. *Clinical Therapeutics*. 28:174-183.

Shah, S., S. Sander, C.M. White, M. Rinaldi, and C. Coleman. 2007. Evaluation of *Echinacea* for the prevention and treatment of the common cold: a meta-analysis. *The Lancet Infectious Diseases*. 7: 473-480.

Shalaby, A.S., E.A. Agina, S.E. El-Gengaihi, A.S. El-Khayat, and S.F. Hindawy. 1997. Response of *Echinacea* to some agricultural practices. *Journal of Herbs, Spices, and Medicinal Plants*. 4:59–67.

Silvarolla, M.B., P. Mazzafera, and L.C. Fazuoli. 2004. A naturally decaffeinated arabica coffee. *Nature*. 429: 826.

Small, E., and H.D. Beckstead. 1973. Common cannabionoid phenotypes in 350 stocks of *Cannabis*. *Llodyia*. 36:144-165.

Soicke, H., G. Al-Hassan, and K. Gorler. 1988. Further derivatives of caffeic acid from *Echinacea purpurea*. *Planta Medica*. 54: 175-176.

Speroni, E., P. Govoni, S. Guizzardi, C. Renzulli, and M.C. Guerra. 2002. Anti-inflammatory and cicatrizing activity of *Echinacea pallida* nutt. root extract. *Journal of Ethnopharmacology*. 79: 265-272.

Starman, T.W., T.A. Cerny, and A.J. MacKenzie. 1995. Productivity and profitability of some field-grown specialty cut flowers. *HortScience*. 30: 1217-1220.

Steinmueler, C., J. Roesler, E. Groettrup, G. Franke, H. Wagner, and M. Lohmann-Matthes. 1993. Polysaccharides isolated from plant cell cultures of *Echinacea purpurea* enhance the resistance of immunosuppressed mice against systemic infections with *Candida albicans* and *Listeria monocytogenes*. *International Journal of Immunopharmacology*. 15: 605-614.

Tubaro, A., E. Tragni, P. Del Negro, C.L. Galli, and R. Della Loggia. 1987. Anti-inflammatory activity of a polysaccharidic fraction of *Echinacea angustifolia*. *The Journal of Pharmacy and Pharmacology*. 39: 567-569.

Turner R.B., R. Bauer, K. Woelkart, T.C. Hulsey, and J.D. Gangemi. 2005. An evaluation of *Echinacea angustifolia* in experimental rhinovirus infections. *The New England Journal of Medicine*. 353: 341-348.

USDA, NRCS. 2002. The PLANTS database, Version 3.5. National Plant Data Center. Baton Rouge LA. Retrieved on March 18, 2008 <http://plants.usda.gov>.

Viles, A.L. and R.N. Reese. 1996. Allelopathic potential of *Echinacea angustifolia* D.C. *Environmental & Experimental Botany*. 36: 39-43.

Wang, H., K.F. Chang, S.F. Hwang, G.D. Turnbull, and R.J. Howard. 2000. Effects of root inoculation and fungicide soil drenches on *Sclerotinia* blight of coneflower. *Canadian Journal of Plant Science*. 80: 909-915.

Woelkart, K. and R. Bauer. 2007. The role of alkamides as an active principle of *Echinacea*. *Planta Medica*. 73: 615-623.

Woelkart, K., C. Koidl, A. Grisold, J.D. Gangemi, R.B. Turner, E. Marth, and R. Bauer. 2005. Bioavailability and pharmacokinetics of alkamides from the roots of *Echinacea angustifolia* in humans. *Journal of Clinical Pharmacology*. 45:683-689

Xiong, Q., Y. Tezuka, T. Kaneko, H. Li, L.Q. Tran, K. Hase, T. Namba, S. Kadota. 2000. Inhibition of nitric oxide by phenylethanoids in activated macrophages. *European Journal of Pharmacology*. 400: 137–144.

Yan, W., L.A. Hunt, Q. Sheng, and Z. Szlavnics. 2000. Cultivar evaluation and mega-environment investigation based on the GGE biplot. *Crop Science*. 40: 597-605.

Zheng, Y., M. Dixon, and P.K. Saxena. 2006. Growing environment and nutrient availability affect the content of some phenolic compounds in *Echinacea purpurea* and *Echinacea angustifolia*. *Planta Medica*. 72: 1407-1414.

Chapter Two

COMPARISONS OF PHYTOCHEMICAL AND AGRONOMICAL TRAITS OF CULTIVATED *ECHINACEA ANGUSTIFOLIA* POPULATIONS FROM THE PACIFIC NORTHWEST[1]

2.1 INTRODUCTION

The cultivation of the North American group of medicinal plants in the genus *Echinacea* is relatively recent and has focused primarily on the faster growing, widely adaptable species *E. purpurea*. This species has been garnering considerable agricultural interest internationally for nearly a century (Bauer & Wagner 1991). On the other hand, until recently, commercial *E. angustifolia* preparations were produced almost exclusively from wild-sourced material. Increased attention in the higher-valued *E. angustifolia* crop has lead to the establishment of cultivated populations in Canada within and outside of its native range (Sari et al. 1999). Depending on the species, different parts of the *Echinacea* plant can be used for the production of herbal products. Contrary to *E. purpurea* where roots, leaves and flowerheads are regularly harvested, and as a whole, is of great ornamental and horticultural value (McGregor 1968, Sari et al. 1999), the

[1] A version of this chapter will be submitted for publication. Boucher, A.Y. and S.E. Cowan. Comparisons of Phytochemical and Agronomical Traits of Cultivated *Echinacea angustifolia* Populations From the Pacific Northwest.

commercial interest in *E. angustifolia* lies primarily in its roots as a highly prized source of natural product.

Even though links between reported *Echinacea* bioactivity and its commercially relevant marker compounds remain to be clearly convincingly demonstrated, there is mounting evidence supporting their role in the therapeutic qualities of the plant (Barnes et al. 2005). Two classes of compounds are primarily used as commercial markers; the lipophilic alkamides, usually represented in *E. angustifolia* by the cumulative value of the two principal isomeric compounds collectively referred to as tetraenes, and the hydrophilic caffeic acid derivatives, which include cynarin, echinacoside, cichoric acid and chlorogenic acid. In addition to their therapeutic relevance, these compounds are of great importance in assessing taxonomic identity of plant material given their qualitative and quantitative differences among *Echinacea* species (Binns et al. 2002a).

The introduction of *E. angustifolia* into cultivation is relatively recent (Letchamo et al. 2002), especially outside of its native range on the western side of the Rocky Mountains. This may be a contributing factor to the high levels of genetic variation in cultivated populations, which is observable in the form of phenotypical heterogeneity. Established *E. angustifolia* cultivars with clearly described superior agronomic and/or phytochemical characters have not been widely reported. Letchamo et al. (2002) present findings that demonstrate increases in levels of marker compounds following selection from the *E. angustifolia* cultivar 'Ergogo'. Although no information was given on the nature of the selection procedures (eg. selection criteria, number of cycles) or specific growing conditions in which the trials were conducted, this report supports the potential

for increased phytochemical yield in selected lines of vegetatively propagated *E. angustifolia*.

Due to the difficulties and heavy resource requirements associated with vegetative propagation of *E. angustifolia*, and in the absence of commercial cultivars, growers must rely on seed or seedlings obtained from unselected or wild populations to establish any medium or large-scale production, often with little to no information on the source population. These cultivated populations are likely to have similarly high levels of genotypic and phenotypic variation as those observed in natural populations (Binns et al. 2002b, Kim et al. 2004).

Nonetheless, a number of factors, both genetic and environmental, could potentially lead to early differentiation of mean traits among cultivated population. Some of the genetic factors potentially involved would be analogous to those involved in the early stages of speciation. Such factors include genetic differentiation due to a founder effect (Mayr 1963), whereby a new population is established by a limited number of individuals that are not representative of the full genetic spectrum of the source population. Other potential reasons for genetic differentiation are unintentional or indirect selection, and conceivably even local natural selection pressure depending on the age of the cultivated population (in terms of generations) and a grower's reseeding practices. Environmental effects demonstrated to impact *Echinacea* agronomic and phytochemical traits include soil fertility (Shalaby et al. 1997, El-Gengaihi et al. 1998), biotic (Chang et al. 1999) and abiotic stresses (Gray et al. 2003) and cultivation practices, such as planting density (Shalaby et al. 1997). Differences between cultivated populations due to environmental factors however are of little value in the early stages of

selective breeding. Preliminary assessments of cultivated populations under more homogeneous conditions such as those found in a greenhouse will help reduce environmental variation and give a better idea of the genetic potential of a population. Of course, this assumes that greenhouse observations correlate reasonably well with those from the field.

The objective of this study was to conduct an initial assessment of cultivated *E. angustifolia* populations from British Columbia and Washington with demonstrated agronomic performance outside of its native range in order to determine, at a population level, genetically related and significant phenotypic differences. Such differences could potentially justify increased focus on specific populations as a source of valuable germplasm with higher genetic potential for future selective breeding work.

I hypothesize that genetic differentiation of Northwestern cultivated populations will result in significant differences in mean phytochemical and agronomic traits among plants randomly selected from these populations when grown under a common greenhouse environment. Furthermore, if any type of artificial selection has occurred within these populations, mean levels of measured traits and their variability should differ from those found in wild populations.

2.2 MATERIALS AND METHODS

Plant material

Source populations

The five cultivated populations compared in this work (populations 1-5) were obtained through contributions of randomly selected lots of achenes from 4 cultivated

populations located in British Columbia and 1 in southern Washington state. Achenes from three wild *E. angustifolia* populations were obtained through the USDA's National Plant Germplasm System (NPGS) and were relabelled for the purpose of this experiment as populations 6-8. Corresponding USDA NPGS accession numbers were as follows: population 6 = PI 631288; population 7 = PI 631318; population 8 = PI 631319. All of the achenes were harvested within 1-3 years of the start of the experiment and had been stored at 4C in the dark.

Germination and seedling growth

Thirty randomly selected achenes from each population were surface sterilised in a 20% solution of commercial bleach with 0.1% Tween 20 for 30 minutes with occasional swirling. These were rinsed 3 times in dH_2O and planted in individual cells of 72-plug plastic tray inserts (Kord, Brampton, ON) filled with a mixture of Sunshine Mix #1 (Sun Gro Horticulture, Vancouver, BC) and sand in a 4:1 (v/v) ratio.

The trays were placed under continuous fluorescent lighting that consisted of 4 x 1.22m long GE 34 Watt, Cool White, Rapid Start, Watt-Miser® fluorescent bulbs with a per bulb lumen rating of 2280 mean lumens. The light source was placed 60 cm above the trays and the growing medium was kept moist until all of the germinated seedlings had at least three true leaves (21 days from start). Among these seedlings, 9 were randomly selected for each population (n = 72) to be transplanted and transferred to greenhouse conditions.

Growing conditions

Seedlings were transplanted into 150 x 180 mm black plastic pots containing a mixture of 80% Sunshine Mix #1 (Sun Gro Horticulture, Vancouver, BC), 10% steam pasteurized mineral soil and 10% Perlite amended with a slow release fertilizer (Osmocote 13-13-13 [Scotts Marysville, OH]) and dolomite lime at a rate of 32g per litre of growth medium to increase and maintain its pH to ca. 7.0. Pots were watered as required and no additional fertilizer was added. To better simulate natural diurnal fluctuation, no artificial lighting was provided in the greenhouse. To reduce pest pressure from aphids and mealy bugs, a horticultural grade insecticidal soap solution was applied by spraying every 2 weeks evenly to all plants.

The plants were placed on greenhouse flood benches in a randomized complete block design to account for any light gradient along the bench. Each population was replicated 3 times with 3 plants per replication.

Harvesting

After 190 days after initial germination, all plants were placed in the dark and harvested within 12 hours in a random order to minimize possible light-related diurnal fluctuations in phytochemical contents. Different morphological parameters of real or potential agronomic value were measured at harvest. Given the various stages in flowerhead development (from no flowerhead to anthesis) and the undue influence on the height parameter, plant height was determined by first removing any developing or fully developed flowerhead at the base of the stem and then gathering the leaves from the rosette and measuring the highest vertical point from the surface of the growing medium.

The number of shoots per plant was counted. Leaf trichome density (i.e. trichome #/cm^2) was assessed by counting the number of trichomes in a 0.25cm^2 square area adjacent to the mid-vein on the adaxial surface of fully expanded mature leaves and multiplying the count by 4. For each plant, an average trichome density was assessed from three different leaves. After rinsing off the substrate with tap water, root volume was determined by immersing the root system in a water-filled 1000 mL beaker and measuring the volume of water lost from overflow. Whole plants were blotted with paper towels to eliminate excessive moisture and placed in paper bags and left to dry at 26º C and 30% relative humidity in the dark until loss of mass from drying was no longer recorded (ca. 14 days). For each plant, dried material was divided among roots, shoots, and inflorescence (when present) and weighed separately to obtain dry masses on a Sartorius L420D (Gottingen, Germany) balance.

Extraction for phytochemical analysis

The whole dried root system of each plant was chopped up using an Osterizer blender fitted with a 250 mL glass jar. The root material was then ground to a powder in a mortar and pestle and sieved through a 40 mesh US Standard Sieve. Powdered root material was extracted individually using the following protocol based on Bergeron et al. (2000).

In 15 mL Falcon ™ tubes (Becton Dickinson Labware, Franklin Lakes, NJ), ca. 0.5 g of powder was weighed out precisely on a Sartorius 1602 MP8 analytical scale (Goettinburg, Germany). Ten mL of 70% EtOH was added to the powder and sonicated for 5 minutes in a Branson Model B-220 Ultrasonic Cleaner (Danbury, CT). The tubes

were then placed in a Sorvall GLC-1 benchtop centrifuge and spun for 5 minutes at 2500 rpm. The supernatant was poured off into 50 mL Falcon™ tubes. The extraction process was repeated two more times and the pooled supernatants were brought up to exactly 30 mL. All extracts were stored at –20 º C until analysis.

HPLC analysis of marker compounds

HPLC analyses for quantification of marker compounds were performed on an Agilent 1100 series chromatography system equipped with a photodiode array detector. Phenolic compounds (Chlorogenic acid, cichoric acid, cynarin, echinacoside, and caftaric acid) and alkylamides were analysed separately using different methods. Phenolic compound analysis was based on the INA method 106.000 (INA 2005). The stationary phase consisted of a Cosmosil 5C18-AR-II (4.6 mm x 150 mm, 5 µm) and the mobile phase consisted of a gradient of 0.1% Phosphoric Acid in Water (A), and Acetonitrile (B). The gradient program used was as follows: initial conditions 90% A; decrease to 78% A over 12 minutes; decrease to 60% over 14 min; hold at 60% A for 14.5 minutes; 4 minutes post time. Sample and standard injection volumes were 5.0 µl with a flow rate set at 1.5mL/minute and a column temperature maintained at a constant 35º C. Eluting compounds were detected at a wavelength of 330nm.

Alkylamide analysis was based on a USP method for dodecatetraenoic acid isobutylamide determination (USP 2004). The stationary phase consisted of a Phenomenex Prodigy ODS3 100A (4.6 mm x 250 mm, 5 µm) column. An isocratic mobile phase program consisting of Nanopure Water and Acetonitrile in a 55:45 ratio was used. Sample and standard injection volumes were 25.0 µl with a flow rate of

1.5mL/minute. Column temperature was maintained at a constant 30.0 C. Eluting compounds were detected at a wavelength of 254nm.

Comparisons of retention times and UV spectra of standards were used to determine peak identity in samples. Standards were obtained from ChromaDex Inc. (Santa Ana, CA, USA) for the purpose of quantifying the following compounds: chlorogenic acid, cichoric acid, cynarin, echinacoside, caftaric acid, dodeca-2-(E),4(E),8(Z),10(Z)-tetraenoic acid isobutylamide, undec-2-ene,8,10-diynoic acid isobutylamide, dodec-2-ene,8,10-diyonic acid isobutylamide, Dodeca-2(E),4(E)-dienoic acid isobutylamide. Dodeca-2-(E),4(E),8(Z),10(E)-tetraenoic acid isobutylamide, the second isomere included in the tetraenes mixture, was quantified on the basis of relative retention time and response factor obtained for its isomeric standard.

Statistical analysis

All of the statistical analyses described herein were performed with the SPSS statistical software package (Release 13.0, SPSS Inc. Chicago, IL). Statistical significance of the overall effect of source population was determined by one-way analysis of variance. Analyses were made on untransformed data given that the assumptions of variance of homogeneity and normal distribution of residuals were met. When overall differences were determined to be significant, pairwise differences between populations were determined by Tukey's HSD test. The above statistical analyses were obtained using the 'One-Way ANOVA' procedure. Significance of bivariate correlations were determined by Pearson correlation coefficients and two-tailed tests of significance. In the case of metabolite concentrations in the roots of flowering and non-flowering

plants, t-tests were applied for statistical comparisons between the two groups. P-values of less than 0.05 were considered statistically significant.

2.3 Results and Discussion

The results from this greenhouse study showed that significant differences in phytochemical and morphological traits do exist between wild and cultivated populations. Although statistically significant, the magnitude of the differences does not seem to warrant that particular focus be placed on any of the cultivated populations assessed in this study in any future breeding work. Source population had an overall effect on mean concentrations of some of the analysed marker compounds (Table 2.1), however, adequately conservative pairwise comparisons failed to show significance between individual populations.

A marked difference was observed in the relative proportions of the two tetraene isomers ($p<0.001$) among the populations. The strong population effect is explained by the fact that total tetraenes measured in population 8 had a much higher proportion of the ZZ tetraene isomer compared to all of the other populations, from wild or cultivated sources (Figure 2.1). Given that all of the plants were individually harvested, extracted and analysed in a random order, it is quite unlikely that this shift in tetraenes ratio is a processing artefact, due to differential sample degradation for example. Although the downstream steps in the tetraene biosynthetic pathway remain to be clearly elucidated, the conversion of one tetraene into its isomeric state may well be regulated by a single locus, perhaps coding for an isomerase or one of its regulatory elements. The assumed single- or few-gene nature of this trait would increase its probability of it being fixed, or

at least becoming predominant, in a given population. Matovic et al. (2007) synthesised and isolated by GC-MS a third tetraene isomer, dodeca-2-(E),4(E),8(E),10(Z)-tetraenoic acid, which had not been previously reported in *E. angustifolia* roots because of the inability to resolve its peak from the other tetraene peaks by the commonly used RP-HPLC methods. The reported findings show mean tetraene compositions of 10%, 80%, and 10% for the ZZ, ZE, and EZ isomers respectively. In light of this information, it is likely that one of the two tetraenes reported here are being overestimated due to the co-eluting EZ. Excluding population 8 in which there are significantly higher relative levels of the ZZ isomer, the mean percentages of ZZ in total measured tetraenes is 10.1%, which is almost identical to the percentage reported in Matovic et al. (2007). This observation, along with the fact that equivalent numbers of cis and trans bonds would have similar influences on their retention times, supports the idea that the ZE isomer is the tetraene that is potentially being overestimated with the current method that I used due to its co-elution with the EZ isomer. Interestingly, the percentage of the ZZ isomer observed in population 8 is actually closer to the proportions reported for *E. purpurea* (Lehmann et al. 2006).

Even though alkamides from various sources have been demonstrated to have different biological activity, including antifungal and insecticidal properties, thereby supporting the ecological importance of this class of compound in plant-environment interactions, little is reported as to the possible differences in bioactivity specifically between the different tetraene isomers found in *Echinacea*. Matovic et al. (2007) demonstrated a much higher binding affinity to canabinoid receptors (CB2) for the ZZ isomer compared to the other two isomers present in *E. angustifolia*. Conversely, in a

study on LPS-mediated macrophage activation, Chen et al. (2005) describe equivalent inhibitory properties for the two tetraene isomers examined. Although recent evidence has highlighted the potential role of alkamides from other species in their resistance to insect pests (Tsao et al. 2005), our current understanding of the specific ecological importance of tetraenes to *Echinacea* remains rather limited. Any suggestions as to how a higher percentage of ZZ isomer might increase local fitness of an *Echinacea* population, if in fact it does, would be purely speculative. From a pharmacological standpoint, the demonstrated differences in the binding properties to CB2 receptors of the different tetraenes could also be indicative of differences in their respective immunomodulating properties. Future work should be done to verify this. In the case where the ZZ isomer would be shown to be of higher therapeutic value, plants obtained from population 8, or others with a similar tetraene composition, could be useful as a source of unique germplasm for increasing the percentage of the ZZ isomer in *E. angustifolia* breeding lines.

When the phytochemical data is pooled across populations, I observe a highly significant correlation between root volume and echinacoside concentration (Figure 2.2). This correlation holds true between echinacoside and root dry mass, which is unsurprising given that root volume and dry mass are very highly correlated. Root volume or dry mass however is uncorrelated with tetraenes, total alkamides or any of the other measured compounds. These relationships did not appear to differ among the populations. The presence or absence of such correlative relationships may be related to the developmental stage of the plant. Figure 2.3 shows the difference in root echinacoside concentration between plants that had reached the flowering stage and those

that had not. There is a significantly higher level of echinacoside in the flowering plants ($p=0.02$). None of the other measured compounds, including the tetraenes, showed any significant differences between the two flowering states (all p-values > 0.36). This finding strongly supports the hypothesis that echinacoside regulation is influenced by plant developmental stage and, specifically in this case, flowering. This could in part explain the significant correlation between root mass or root volume and echinacoside concentration as flowering plants across all populations had significantly higher dry root mass ($p=0.01$). Nonetheless, even when only non-flowering plants are considered in the analysis, the correlation remains significant ($R=0.313$; $p=0.02$) which suggests that echinacoside increase begins before floral initiation. Binns et al (2002a) observed a reduction of echinacoside within older roots whereas tetraenes increased. The opposite trend was observed in *E. atrorubens* roots, which more closely parallels what was found in this study on *E. angustifolia* assuming increased root mass with plant age.

Two agronomically important traits, shoot dry mass and leaf trichome density, showed significant differences among the populations studied (Table 2.2). In the case of shoot dry mass, the only two populations that differed significantly from each other were among the wild populations. None of the cultivated populations differed significantly from one another or from any of the wild populations. Surprisingly, an overall effect of source population was not observed in root dry mass despite a highly significant correlation with shoot dry mass ($R=0.8$; $p<0.001$). This may be due to the high levels of variation and the relatively small size of the sample. As for leaf trichome density, the highest level was observed in population 5, which was significantly higher than the lowest density measured in population 8. Higher trichome density is potentially an

important selection criterion given the anecdotal reports suggesting that leafhopper insects, which are the principal vectors of Asters Yellow disease, avoid plants with higher trichome densities. The link between pest resistance and leaf pubescence has clearly been demonstrated in a number of other plant-phytophagous insect systems (Levin 1973).

There is considerable variation in the measured traits described above, which suggests that statistical differences may have been determined with larger sample sizes. Analyses of microsatellite diversity or of other molecular markers offer a powerful tool to measure genetic relatedness and diversification in different populations. However, with the current state of knowledge on the genetic mechanisms regulating natural product synthesis in *E. angustifolia* and the relatively limited availability of sequence data, genetic differences observed in *Echinacea* populations, with few notable exceptions (Baum et al. 2001), have scarcely been relatable to phytochemical differentiation.

Conclusion

From these findings, I conclude that significant differences in marker compound concentrations in *E. angustifolia* products derived from cultivated populations grown in the American northwest would likely be due to environmental rather than genetic factors. These results support the idea that cultivated *E. angustifolia* populations, at least in the northwestern region of North America, and primarily in British Columbia, are not clearly differentiated in terms of commercially important traits. In fact, most of the statistically significant differences were observed among the wild populations likely due to local adaptation. It is therefore unlikely, at this stage of *E. angustifolia* cultivation in British Columbia, that focusing early selection efforts for breeding phytochemically rich *E.*

angustifolia cultivars on any of the assessed cultivated populations would provide any particular advantage over other cultivated or wild populations.

Table 2.1: Mean phytochemical concentrations (milligrams per gram of dry weight ± standard deviations) in *E. angustifolia* roots from 5 cultivated and 3 wild populations grown under common greenhouse conditions for 190 days (n=9 for each population). Means with different letter annotations were determined to be significantly different by Tukey's HSD test.

	Compound Concentration in Root Samples (mg/g)					
Source Population	Tetraenes	Total alkamides**	Cynarin	Echinacoside	Chlorogenic acid	Cichoric acid
1	5.20±1.59	7.01±2.07	1.55±0.50	3.73±2.31[ab]	0.28±0.25	0.06±0.03
2	3.95±1.53	5.56±1.97	1.44±0.57	2.61±1.56[b]	0.17±0.22	0.06±0.07
3	4.76 ±2.64	6.29±3.32	1.21±0.54	2.96±1.66[ab]	0.10±0.16	0.04±0.01
4	3.56 ±1.14	4.62 ±1.63	1.39±0.54	3.10±1.20[ab]	0.16±0.11	0.05±0.03
5	3.89 ±2.37	5.33±2.67	1.40±0.43	2.56±1.83[ab]	0.38±0.53	0.06 ±0.05
6[†]	5.12 ±1.30	6.49±1.74	1.55±0.67	5.18±2.30[a]	0.18±0.20	0.05 ±0.03
7[†]	3.39±1.16	4.71±1.57	1.48±0.77	2.52±1.31[b]	0.12±0.24	0.04 ±0.02
8[†]	3.10±0.88	4.28±1.15	0.93±0.31	1.24±0.72[b]	0.41±0.35	0.02 ±0.01
*p-value**	*0.06*	*0.07*	*0.29*	*0.00*	*0.17*	*0.27*

* p-value determined by one-way ANOVA

** Total alkamides represent the sum of measured concentrations for all available alkamide standards including both tetraene isomers

[†] wild populations

Table 2.2: Mean values of measured agronomic traits (± standard deviations) of individual *E. angustifolia* plants from 5 cultivated and 3 wild populations grown under common greenhouse conditions for 190 days (n=9 for each population). Means with different letter annotations were determined to be significantly different by Tukey's HSD test.

	Agronomic traits					
Source population	Shoot number	Shoot d.m. (g)	Leaf trichome # (/cm^2)	Height (cm)	Root d.m. (g)	Total root volume (mL)
1	3.56±2.13	7.43±3.56^{ab}	77.92±15.74^{ab}	27.23±3.86	8.50±4.51	37.89±20.46
2	3.44±1.13	6.57±3.19^{ab}	56.89±22.38^{ab}	28.01±7.88	5.14±2.51	28.61±15.84
3	3.11±1.45	7.41 ±4.90^{ab}	56.89±22.12^{ab}	27.74±6.44	7.34±3.96	29.56±14.28
4	4.78±1.72	8.02±1.98^{ab}	79.40±19.42^{a}	29.99±4.72	7.29±2.30	33.00±7.69
5	4.14±2.61	6.37±4.46^{ab}	64.57±28.96^{ab}	25.36±4.69	5.87±3.94	26.08 ±17.34
6†	4.89±2.15	11.71±5.31^{a}	69.62±15.02^{ab}	31.11±4.65	7.26±3.54	30.83±12.12
7†	4.38±2.00	5.08±3.08^{b}	65.67±17.95^{ab}	28.92±3.32	4.68±2.08	21.43±12.11
8†	3.33±1.12	5.20±4.08^{ab}	46.37±28.42^{b}	26.26±4.88	4.68±3.20	21.72±15.20
*p-value**	*0.29*	*0.03*	*0.03*	*0.39*	*0.15*	*0.32*

* p-value determined by one-way ANOVA
† wild populations

Figure 2.1: Differences among *E. angustifolia* populations in relative contribution by two tetraene isomers to total tetraene determinations (mg/g dry weight) in individual roots. Percentages indicated above each bar represent the proportion of the 8(Z)10(Z) isomer. Asterisks indicate highly significant differences ($p<0.01$) from other populations.

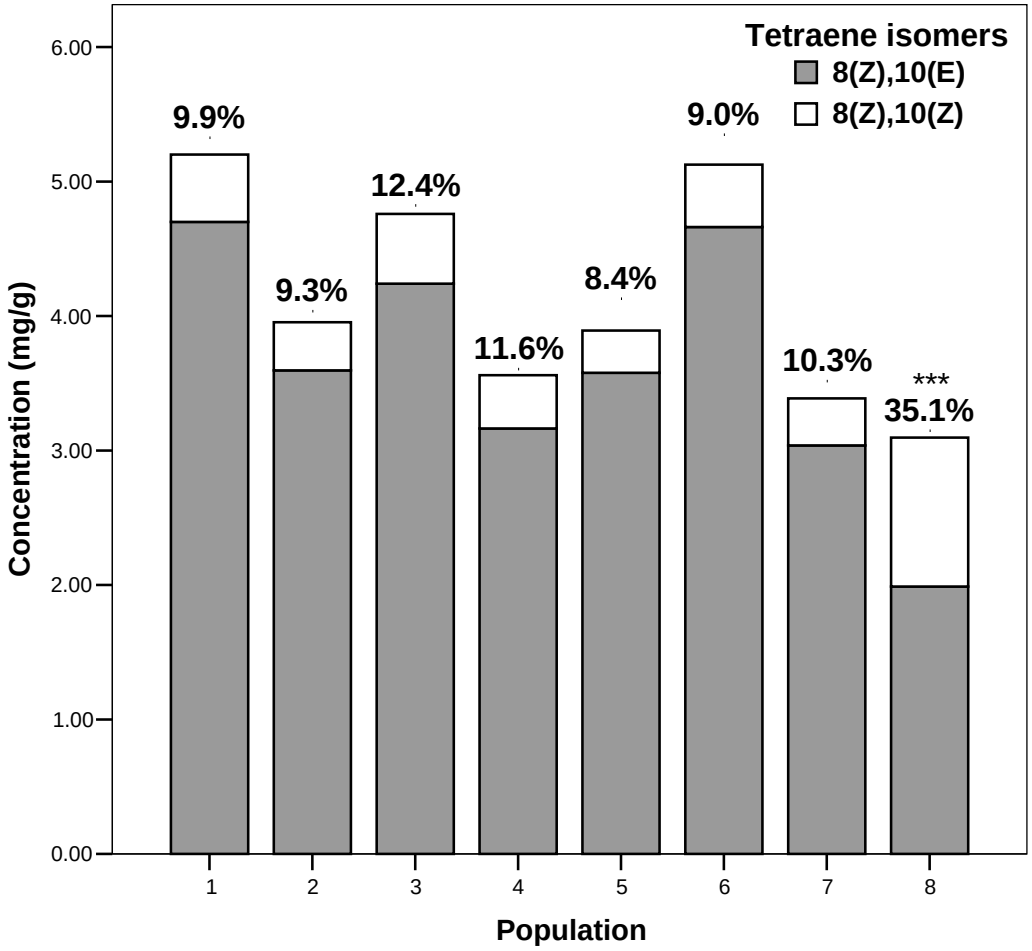


Figure 2.2: Scatter plot demonstrating a significant positive correlation (p=0.001) between concentration of echinacoside in roots (mg/g dry weight) and total root volume of individual *E. angustifolia* plants.

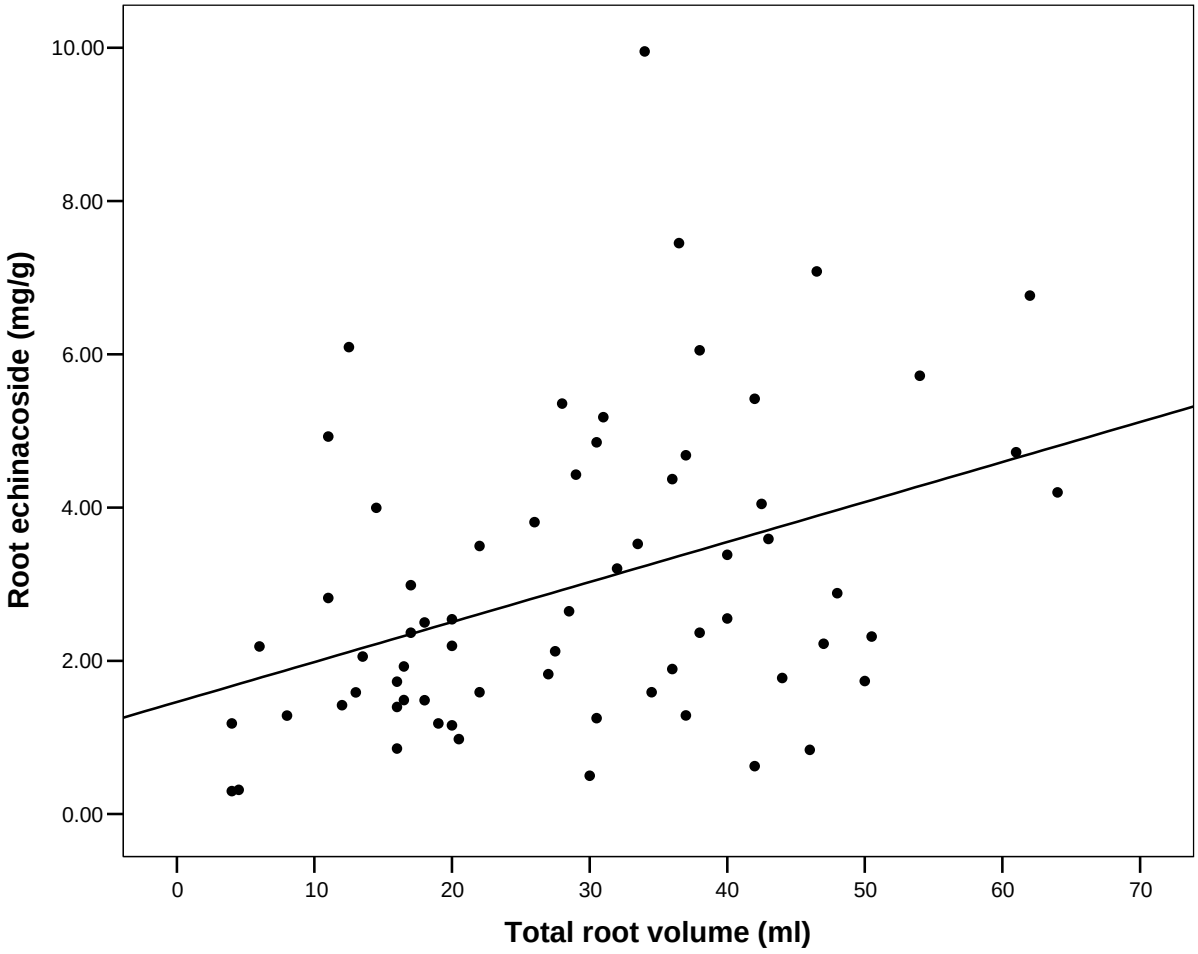
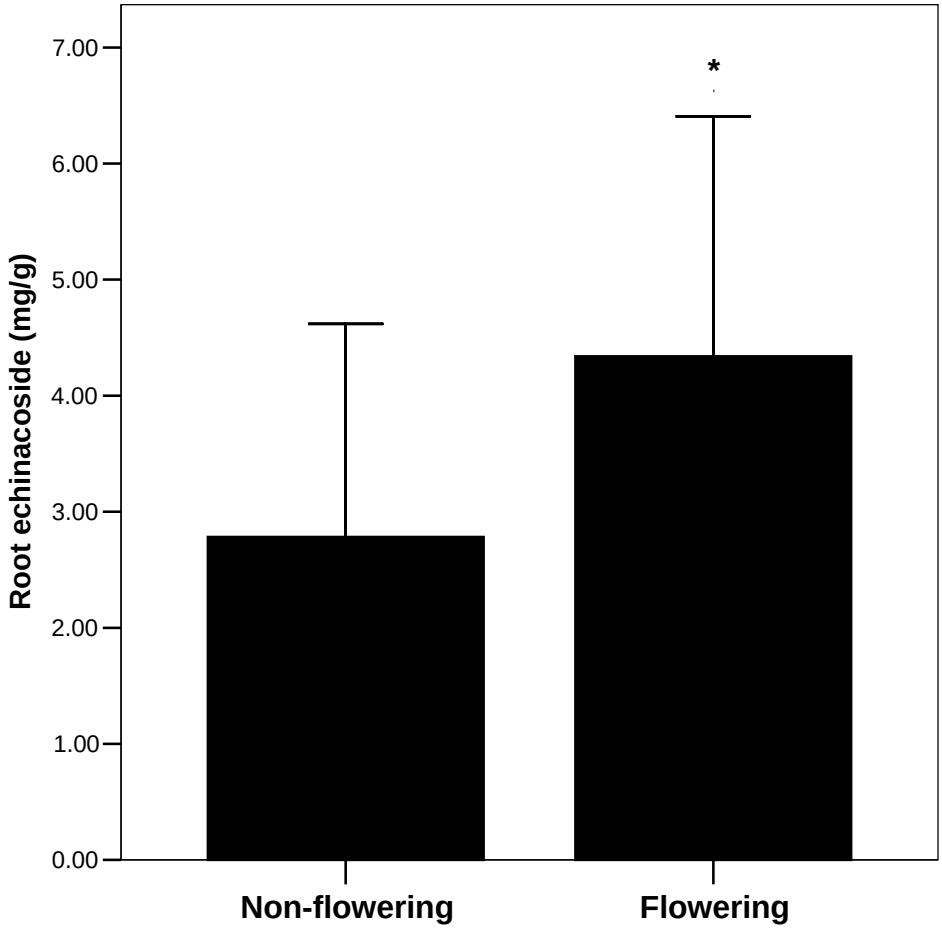


Figure 2.3: Echinacoside concentrations (mg/g dry weight ± standard deviations) in roots of flowering and non-flowering plants. The asterisk signifies statistically higher levels as determined by t-test ($p<0.05$).



2.4 REFERENCES

Barnes J, L.A. Anderson, S. Gibbons, J.D. Phillipson. 2005. *Echinacea* species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt.,*Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. *Journal of Pharmacy and Pharmacology*. 57: 929-954.

Bauer, R. & H. Wagner. 1991. *Echinacea* species as potential immunostimulatory drugs. In: Wagner & Farnsworth (eds) *Economic and Medicinal Plant Research*., Academic Press, New York. Vol. 5: pp.253-321.

Baum, B.R., S. Mechanda, J.F. Livesey, S.E. Binns, and J.T. Arnason, J.T. 2001. Predicting quantitative phytochemical markers in single *Echinacea* plants or clones from their DNA fingerprints. *Phytochemistry*. 56: 543-549.

Bergeron, C., J.F. Livesey, D.V.C. Awang, J.T. Arnason, J. Rana, B.R. Baum, and W. Letchamo. 2000. A quantitative HPLC method for the quality assurance of *Echinacea* products on the North American market. *Phytochemical Analysis*. 11: 207-215.

Binns, S.E., J.F. Livesey, J.T. Arnason, and B.R. Baum. 2002a. Phytochemical Variation in *Echinacea* from Roots and Flowerheads of Wild and Cultivated Populations. *Journal of Agricultural and Food Chemistry*. 50: 3673-3687.

Binns, S.E., J.T. Arnason, and B.R. Baum. 2002b. Phytochemical variation within populations of *Echinacea angustifolia* (Asteraceae). *Biochemical Systematics and Ecology* 30: 837–854.

Chang, K.F., R. Howard, S.F. Hwang, and S. Blade. 1999. Diseases of *Echinacea* on the Canadian Prairies. Agdex 630-2. Agri-fax, Alberta Agriculture, Food and Rural Development. 8pp.

Chen, Y., T. Fu, T. Tao, J. Yang, Y. Chang, M. Wang, L. Kim, L. Qu, J. Cassady, R. Scalzo, and X. Wang. 2005. Macrophage Activating Effects of New Alkamides from the Roots of *Echinacea* Species. *Journal of Natural Products*. 68: 773-776.

El-Gengaihi, S.E., A.S. Shalaby, E.A. Agina, and S.F. Hendawy. 1998. Alkylamides of *Echinacea purpurea* L. as Influenced by plant ontogony (*sic*) and fertilization. *Journal of Herbs, Spices & Medicinal Plants*. 5:35-41.

Gray, E.D., S.G. Pallardy, H.E. Garrett, and G.E. Rottinghaus. 2003. Acute drought stress and plant age effect on alkamide and phenolic acid content in purple coneflower roots. *Planta Medica*. 69: 50-55.

Institute for Neutraceutical Advancement. 2005. Phenolics in *Echinacea* by HPLC, INA method 106.000. Institute. for Neutraceutical Advancement. Ann Arbor, Mich. Retreived on July 2005 <http://www.nsf.org/business/ina/echinacea.asp?program =INA>.

Kim, D.H., D Heber, and D.W Still. 2004. Genetic diversity of *Echinacea* species based upon amplified fragment length polymorphism markers. *Genome*. 47: 102-111.

Lehmann, R.P., A. Matthias, N. Matovic, K.G. Penman, K.M Bone, and J.J De Voss. 2006. Prevalence of three tetraene alkamide isomers in *Echinacea angustifolia* and *Echinacea purpurea* roots. *Planta Medica*. 72: P_146.

Letchamo, W., L.V. Polydeonny, N.O. Gladisheva, J.T. Arnason, J.Livesey, and D.V.C. Awang,. 2002. Factors affecting *Echinacea* quality. In: Jannick and Wipckey (ed.) Trends in New Crops and New Uses. ASHS Press, Alexandria VA. pp.514-521.

Levin, D.A. 1973. The Role of Trichomes in Plant Defense. *The Quarterly Review of Biology*. 48(1): 3-15.

Matovic, N., A. Matthias, J. Gertsch, S. Raduner, K.M. Bone, R.P. Lehmann, and J.J. DeVoss. 2007. Stereoselective synthesis, natural occurrence and CB2 receptor binding. *Organic Biomolecular Chemistry*. 5: 169-174.

Mayr E. 1963. *Animal species and evolution*. Cambridge MA. Harvard Press. 797pp.

McGregor R.L., 1968. The Taxonomy of the Genus *Echinacea* (Compositae). *The University of Kansas Science Bulletin*. 48: 113-142.

Sari, A.O., M.R. Morales, and J.E. Simon. 1999 *Echinacea angustifolia*: An Emerging Medicinal. In: J. Janick (ed.) *Perspectives on new crops and new uses*. ASHS Press, Alexandria VA. pp.490-493.

Shalaby, A.S., S.E. El-Gengaihi, E.A.Agina, A.S. El-Khayat, and S.F. Hindawy. 1997. Growth and Yield of *Echinacea purpurea* L. as Influenced by planting density and fertilization. *Journal of Herbs, Spices & Medicinal Plants*. 5:69-76.

Tsao, R., C.H. Marvin, A.B. Broadbent, M.Friesen, W.Allen, and B.D. McGarvey. 2005. Evidence for an isobutylamide associated with host-plant resistance to western flower thrips, *Frankliniella occidentalis*, in Chrysanthemum. *Journal of Chemical Ecology*. 31:103-110.

United States Pharmacopeia. 2004. *Echinacea angustifolia* monograph, p.2075-2083. In: USP 28- National Formulary 23. USP Convention: Rockville, Md.

Chapter Three

CORRELATIONS OF MARKER COMPOUND CONCENTRATIONS IN DIFFERENT PARTS OF *ECHINACEA ANGUSTIFOLIA* AND THEIR IMPLICATIONS FOR INDIRECT SELECTION[1]

3.1 INTRODUCTION

Plants from the North American genus *Echinacea* are internationally recognized icons of a rapidly evolving herbal industry. There has been some debate over its clinical efficacy (Barnes et al. 2005, Schoop et al. 2006), but a recent meta-analysis shows overall significance in treatment of upper respiratory tract infections (Shah et al. 2007). Products derived from different *Echinacea* spp. remain among the top selling herbal supplements on the market (Blumenthal et al. 2006). There is increasing evidence that the bioactivity of *Echinacea* extracts stems from synergistic effects of multiple compounds rather than from the action of a single compound (Dalby-Brown et al. 2005). The bioactivities of isolated major secondary metabolites found in the various parts of *Echinacea* species have also been reported (Barnes et al. 2005).

[1] A version of this chapter has been submitted for publication. Boucher, A.Y., S.E. Cowan, J.T. Arnason, and P. Brown. Correlations Between Marker Concentrations in Different *Echinacea angustifolia* Parts and Their Implications for Indirect Selection.

Compounds from two main classes are commonly recognized as markers of identity and quality for *Echinacea* material intended for the manufacturing of natural health products. These two classes are the hydrophilic caffeic acid derivatives (CADs), and the lipophilic alkamides. The CADs include echinacoside, cichoric acid, chlorogenic acid, and cynarin while the main alkamides found in *E. angustifolia* are an isomeric mixture of two compounds, dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide and dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (hereafter abbreviated as the "tetraenes") (Bauer and Wagner 1991).

Echinacoside is frequently used as a measure of commercial product quality. Although current evidence refutes the role of echinacoside as an immunomodulator in *Echinacea* preparations, this compound does have demonstrated antioxidative (Pellati et al. 2004) and anti-inflammatory (Speroni et al. 2002) activities. Its overall medicinal value cannot be discounted. By contrast, the importance of alkamides as immunomodulatory agents is strongly supported by a mounting body of evidence that includes a recently proposed molecular mode of action (Bauer & Wagner 1991, Barrett 2003, Woelkart & Bauer 2007). Cynarin is important for taxonomic differentiation among the commercial *Echinacea* species; it is only found in *E. angustifolia*, while *E. purpurea* lacks cynarin but typically has high levels of cichoric acid and chlorogenic acid (Binns et al. 2002a).

In any breeding program to develop *E. angustifolia* cultivars with characteristically high levels of the commonly used marker compounds, fundamental questions regarding metabolite production and distribution within *Echinacea* plants must be addressed to develop efficient and effective selection strategies. Improved efficiency

can be achieved through indirect selection, which is feasible when more easily measured secondary traits are highly correlated with primary traits of interest. To be of breeding value, these correlations have to be genetic and the secondary trait must have higher heritability than the primary trait (Gallais 1983). Despite clearly demonstrated differences in marker compound concentrations between different parts of *E. angustifolia* (Binns et al. 2002a, Wu et al. 2004), there are no published reports, to my knowledge, of phytochemical correlations within individual plants of this species or on the heritability of their phytochemical traits.

In the absence of indirect selection approaches, the phytochemical assessment of *Echinacea* populations requires resource intensive harvesting and sample processing. Schulz et al. (2002) have proposed an analysis of roots using near infrared reflection (NIR) and attenuated total reflectance infrared (ATR-IR) spectroscopy that were effective in predicting root levels of echinacoside. However, a method to assess root phytochemistry has yet to be demonstrated that is inexpensive and time-efficient, and which does not require root harvest (and subsequent destruction or loss of crop quality through processing).

Indirect selection using concentrations of aerial parts as a secondary trait could be an effective alternative to direct phytochemical assessment of root material if the phytochemical levels found in aerial parts of individual plants correlate with those in their roots. Indirect assessment of root phytochemical concentrations would dramatically reduce the time and resources required for genotype evaluation, and could be used in combination with NIR and ATR-IR as described above. In addition, this approach would

allow for the preservation of desirable genotypes and the possibility for same-plant assessment at different ages.

This study was designed to determine how phytochemical traits of interest correlate between different plant parts of *E. angustifolia* to assess the value of indirect selection methods in crop selection to increase production of root marker phytochemicals. The correlations were measured in both greenhouse- and field-grown plants to determine if indirect selection would be effective in different environments.

3.2 MATERIALS AND METHODS

Plant production and harvest

All plants were grown from achenes randomly selected from a single lot harvested in 2002 from a cultivated *E. angustifolia* population in Savonah,British Columbia. Species identity of the population had been verified in November 2002 by the Saskatchewan Herb Research Program at the University of Saskatchewan. The achenes were stored at 4º C until used in 2003 and 2006 experiments. Prior to germination, I surface-sterilized the achenes in a 10% (v/v) solution of commercial sodium chloride with occasional agitation and rinsed them in sterilized distilled water (4X). I placed the achenes in a 9 cm Petri dish lined with three layers of Whatman no.1 filter paper that had been soaked with a 6.64 mM solution of ethephon (Ethrel, Bayer Crop Science, Calgary, Alta., Canada). Upon hypocotyl emergence, I transplanted the achenes to 72-plug plastic tray inserts (Kord, Brampton, Ont., Canada) that were filled with a mixture of Sunshine Mix #1 (Sun Gro Horticulture, Vancouver, B.C., Canada), steam pasteurized mineral soil and Perlite (8:1:1 by volume). I amended the growing medium with 5.9 kg m^{-3} Osmocote

13-13-13 fertilizer (Scotts, Marysville, Ohio). Every 3 to 4 days, I manually irrigated the trays until water started to flow out of the bottom holes. No additional fertilizer was added. Minimum day and night temperatures in the greenhouse were set at 20 and 24 ºC respectively with a mean relative humidity of ≈30%. No supplemental lighting was used. Midday measures of photosynthetically active radiation from natural light inside the greenhouse ranged from ≈156 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to 682 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under overcast and clear conditions respectively. Greenhouse: At the 3-leaf stage, I transplanted the seedlings to 150 x 180 mm black plastic pots filled with the same potting mix described above, and they were grown until 7 months old (Apr. 2003-Nov. 2003). From 250 *E. angustifolia* plants, 32 were randomly selected and were harvested in a single evening. Of the harvested plants, 14 had reached anthesis.

Field: The seedlings used in the field experiment were grown from achenes in the greenhouse (as described above) from the second week of Apr. 2006 to the second week of May 2006. At this stage, 68 seedlings were transplanted directly from plug trays to the Totem Field research station on the University of British Columbia Vancouver Campus (lat.49º15'26'N", long.123º14'59'W") in two rows of 34 plants with an inter-row spacing of 150 cm and an inter-plant spacing of 30 cm. Totem Field soil consisted of a sandy loam, and no additional fertilizer was added. After periods of 10 days without precipitation (26 June-8 July, 24-29 July, and 21 Aug.-8 Sep.), plants were manually irrigated every third day. Climate data from the Environment Canada weather station at Vancouver International Airport (8 km away) showed the mean relative humidity at the field site to be ≈74% with a total precipitation of 346.2 mm. All field trial plants were harvested (at 7 months of age) in a single evening in the first week of Nov. 2006,

following the first frost. There were 65 of 68 plants that survived to harvest, and 30 were randomly selected for analysis; those with mature capitula (15 plants) were analysed to determine phytochemical correlations between capitula and roots.

At harvest time, field soil samples were collected from the top 45 cm at 6 evenly distributed locations spanning the length of both rows, pooled into one sample, and sent for nutrient analysis (Norwest Labs, Edmonton, Alta., Canada). Greenhouse substrate samples were collected at harvest from 5 randomly selected pots, pooled into one sample, and sent for nutrient analysis. Results of soil analyses are summarized in Table 3.1.

At harvest, roots were rinsed with tap water to remove substrate and dried with paper towels. The plants were dried in paper bags in a dark open room (≈26 ºC, RH ≈30%) until loss of weight from drying was no longer recorded (≈14 days).

Phytochemical Extraction

For each plant, dried whole root systems, shoots, and, where applicable, bulked mature capitula (post-anthesis) were coarsley chopped in an Osterizer blender fitted with a 250 mL glass jar and ground to powder with a mortar and pestle. The powdered material was screened through 40 mesh (US standard). In all analyses, 'shoot' refers only to the leaf material from an individual plant; stems and capitula were removed. Capitular extractions were done separately. The protocol used for extraction was a modification of the one validated by Bergeron et al. (2000). Samples of 0.5 g were weighed in 15 mL polypropylene conical centrifuge tubes (Falcon 352097; Becton Dickinson Labware, Franklin Lakes, N.J.) and extracted three times with 10 mL of fresh 70% (v/v) ethanol by ultrasound in an ultrasonic cleaner (Branson B-220; Danbury, Conn.) for 5 minutes.

Between each extraction, samples were centrifuged, the supernatant was collected and the pellet was re-extracted (as above). The extract volume was brought up to 30 mL with ethanol. One mL of each sample was syringe filtered through a 0.22 μm polytetrafluoroethylene membrane filter (Nalgene, Rochester, N.Y.) directly into a vial before HPLC analysis.

RP-HPLC DAD protocol

All of the RP-HPLC analyses were performed on an Agilent system 1100 model (Agilent Technologies, Santa Clara, Calif.). The system was run using the Agilent Chemstation chromatography software (version 9.1, Santa Clara, Calif.). For the greenhouse material, the alkamides and the CADs were analysed simultaneously with the following method. A volume of 1 μL was injected by an autosampler (G1313A, Agilent Technologies; Santa Clara, Calif) fitted with a 100 μL loop. A quaternary pump (G1311A) was set at a constant flow rate of 0.5 $mL \cdot min^{-1}$ for the mobile phase, which consisted of varying combinations of the following solvents: A. Water with 0.05% (v/v) trifluoroacetic acid and B. Acetonitrile with 0.05% (v/v) trifluoroacetic acid. The mobile phase gradient was as follows: 5% to 20% B in 7 minutes, 20% to 50% B in 3 minutes, 50% to 90% B in 5 minutes, hold 90% B for 2 minutes. Between each run, there was an equilibration time of 5 minutes at initial conditions. The solvents were degassed with a solvent degasser (G1322A, Agilent Technologies; Santa Clara, Calif). Separation was done on a 3 μm, 100 X 2.0 mm YMC ODS-AM Waters column (Mississauga, Ont., Canada) kept at a constant temperature of 50 ºC in a column oven. Eluting compounds were detected using a photodiode array detector (G1315A, Agilent Technologies; Santa

Clara, Calif) at 210 nm, 260 nm and 326 nm detection wavelengths with a slit width of 4 nm. For the field-grown material, alkamides and CADs were analysed separately using published validated methods from the United States Pharmacopeia (USP 2004) and the Institute for Nutraceutical Advancement (INA 2005) respectively.

The rationale to use a different RP-HPLC method for the field trials was based on the methodological improvements that occurred for *Echinacea* RP-HPLC through publication of the USP and INA validated methods during the period between greenhouse and field trials reported here. Also, the HPLC system that was used for these experiments was located in one of the laboratories involved in the methods validation program for those methods. Due to the instability of extracts stored over a three year period, greenhouse-grown extracts were not re-analyzed with the newest validated methods, however, I proceeded to correlate the shoot and root phytochemistry for each trial separately.

I determined peak identity and quantification by relative retention times and calibration curves obtained from external standards for each of the reported compounds. The lower limit of quantification for all methods was 1 $\mu g \cdot mL^{-1}$. Standards were obtained from Chromadex (Santa Ana, Calif.). Compound identification was further confirmed by comparisons to published chromatograms and UV spectra (Bauer et al. 1988).

Statistical Analyses

All statistical analyses were performed with the SPSS 13.0 statistics software (SPSS Inc., Chicago, Ill.). Spearman's rho non-parametric correlations were computed to determine relationships between shoot concentrations and root concentrations of the

analysed metabolites. The non-parametric correlations were calculated to determine rank correlations between variables that have relatively large differences in their distributions and also to take into account disproportionate effects of outliers in the interpretation of results. Two-way analyses of variance were performed using the univariate general linear model procedure in SPSS with 'organ' and 'growing environment' as the main factors. Tukey *post hoc* comparisons were used to determine significant differences between phytochemical concentrations in different organs and/or growing environments. Independent-samples T-tests were used to compare mean concentrations of the analysed metabolites in flowering and non-flowering plants. The alpha level was set at 0.05 for significance and 0.10 for marginal significance.

3.3 RESULTS

Organ-specific marker compound levels: greenhouse and field

Roots of field-grown plants produced significantly higher levels of echinacoside, cynarin, chlorogenic acid and tetraenes compared to greenhouse plants (Table 3.2). The increase in root levels of field-grown plants was highest for cynarin (141%) and echinacoside (198%) (Table 3.2). Conversely, shoots of field-grown plants produced significantly higher concentrations of cichoric acid and caftaric acid, with increases of 210% and 164% respectively. Among the different organs of field-grown plants, roots were the highest source of tetraenes, cynarin and echinacoside and had a mean concentration of chlorogenic acid comparable to the one measured in mature capitula. The latter organ had the highest levels of cichoric acid whereas the highest levels of caftaric acid were measured in the shoots.

Correlations between roots, shoots, and capitula

My results indicate that significant correlations existed between root and shoot levels of some of the marker compounds in greenhouse-grown *E. angustifolia* (Table 3.3). There was a positive correlation between the roots and shoots of greenhouse-grown *E. angustifolia* plants for echinacoside and cynarin (Table 3.3). The correlation of root and shoot echinacoside concentrations was enhanced when only flowering greenhouse-grown plants were considered (Spearman's rho = 0.73 compared to 0.53 for total population) (Table 3.3 and Figure 3.1). In addition, echinacoside concentrations were significantly higher in roots of non-flowering plants compared to those in plants with capitula ($t=2.34$; $p=0.03$). Flowering stage-dependent differences were not observed for echinacoside concentrations in shoots nor for any of the other analysed metabolites. None of the root to shoot correlations for the other metabolites were improved by the presence of flowering capitula. Cichoric acid levels were very low to absent in *E. angustifolia* roots (Table 3.2) with the notable exception of a single plant in the greenhouse that had relatively high shoot and root concentrations (Figure 3.2). Tetreane levels in roots were not correlated with those in shoots (Figure 3.3). In the case of field-grown plants, there were no significant correlations between marker compound concentrations measured in roots and shoots, or between roots and capitula, except for the significant negative correlation between root and shoot levels of caftaric acid (note only 9 out of the total 30 analysed plants had measurable amounts of this metabolite in the roots) (Table 3.4).

Correlations between different marker compounds within roots

Within the same roots, concentrations of tetraenes were not correlated with any of the root concentrations of CADs (Figure 3.4). However, root concentrations of echinacoside in both growing environments were significantly correlated with root cichoric acid (r=0.37 in greenhouse, r=0.58 field) and with root cynarin (r=0.34 greenhouse, r=0.48 field) (Figure 3.4). Root cynarin concentrations were significantly correlated with levels of cichoric acid and with chlorogenic acid in the greenhouse (r=0.38 and r=0.57, respectively), whereas in the field, root cynarin levels were not significantly correlated to any of the other metabolites, yet chlorogenic acid was significantly correlated with cichoric acid and with caftaric acid (r= 0.67 and r= 0.38, respectively) (Figure 3.4).

3.4 DISCUSSION

Overall, the correlations suggest that indirect selection based on phytochemical levels of aerial organs would be most useful for gains in root echinacoside content. Despite reports of its unimportance in certain therapeutic properties of *E. angustifolia* (Bauer 1998, Rininger et al. 2000), echinacoside is an effective anti-inflammatory, wound-healing promoter (Speroni et al. 2002), and anti-oxidant (Pellati et al. 2004). Recent studies have shown that CADs used as quality markers of *Echinacea* have no bioavailability in humans following oral administration (Matthias et al. 2005, Woelkart et al. 2005). These findings, however, do not preclude their value in *Echinacea* products intended for non-oral use (e.g. topically applied preparations). An important target to improve the immunomodulatory qualities of *Echinacea* crops appears to be the alkamides

because of their immunological activity (Bauer & Wagner 1991, Barrett 2003, Woelkart & Bauer 2007) and bioavailability (Matthias et al. 2005, Woelkart et al. 2005). My results show no strong predictive value of aerial parts for the tetraene alkamides.

My results suggest that even moderate selection intensities applied to the greenhouse population using phytochemical levels in shoots as a secondary trait could eliminate plants producing some of the highest levels of tetraenes in roots. For instance, a selection intensity of over 30% would have been required to select the two highest root producers (Figure 3.3). Since many of the greenhouse plants did not produce any tetraenes, including the third highest root producer (Figure 3.3), selection based on shoot tetraene levels would likely not identify genotypes with high root production. Alkamide levels in *E. angustifolia* leaves are characteristically much lower than root levels (Bauer & Remiger 1989). In fact, shoot tetraenes in any of field plants were not detectable.

The mean levels of CADs and tetraenes in my root samples (Table 3.2) were within the ranges reported in 6 month old greenhouse-grown *E. angustifolia* plants by Binns et al. (2002b), with the exception of echinacoside levels from field grown roots which were more than 2.5x higher than those reported in their highest producing population. It was also well above the concentration of 2.03 $mg \cdot g^{-1}$ in wild harvested roots reported by Binns et al. (2002a) and those reported by Perry et al. (2001) for root material from field-harvested plants at 18-21 months of age (10.4 $mg \cdot g^{-1}$).

Despite the high variability in phytochemical levels measured in mature capitula, their mean levels were similar to previously reported data (Binns et al. 2002a), which showed significantly higher cichoric acid levels in capitula than in roots. The relatively low value of *E. angustifolia* leaf material in natural health products may be why this

report is one of only few describing marker compound concentrations in aerial parts. Bauer and Wagner (1991) also reported very low levels of tetraenes in aerial parts of *E. angustifolia* (<0.02 $mg \cdot g^{-1}$). The levels of cichoric acid that were very low to absent, as reported here in *E. angustifolia* roots were similar to those reported by Binns et al. (2002a). The lone outlier (Figure 3.2) may have arisen from a hybridization event as it had abnormal morphological traits for *E. angustifolia* (fibrous roots and wider leaves).

Field and Greenhouse Environmental Differences

The environments in this study were selected to represent two different, yet typical, growing conditions used in the production of *E. angustifolia* crops. The higher levels of root compounds observed here in field-grown plants compared to those from the greenhouse may be caused by several abiotic factors. Nutrient availability (Dufault et al. 2003, El-Gengaihi et al. 1998), and drought (Gray et al. 2002) appear to have little to no effect on the production of marker compounds in *Echinacea*. The markedly higher soil nutrient levels in the greenhouse-growth substrate (Table 3.1) are unlikely then to be the reasons for the differences in root concentrations. On the other hand, the effect of UV radiation on CAD accumulation in *E. angustifolia* remains untested. Increases in phenolic biosynthesis to mediate UV radiation damage are well documented in plants (Bergvinson et al. 1994, Jansen 1998). Higher CAD concentrations caused by higher light levels have been recently demonstrated in hairy root cultures of *E. purpurea* (Bilal et al. 2007). The low UV-B levels typically found in greenhouses, which are caused by the light filtering properties of standard greenhouse glass (Krizek et al. 2005), may explain the dramatic difference between echinacoside levels in my field and

greenhouse experiments. This hypothesis remains to be verified experimentally. Other factors that may also come into play include substrate texture and structure, root restriction in greenhouse pots, beneficial symbiotic associations (e.g. mycorrhizae), and differences in intensity and/or kinds of pest pressures.

My study, along with others, highlight the importance of considering plant age (Binns et al. 2002a, Wu et al. 2004) and developmental stages (Berti et al. 2002, Letchamo et al. 2002) during genotypic assessment of phytochemical performance. In the current study, all of the plants were harvested at the same age to reduce the effects of temporal and environmental factors on metabolite production. Variation in development stages among plants in this study may have contributed to the differences findings in the field and the greenhouse. Nevertheless, given widespread developmental and genetic variability within cultivated and wild *E. angustifolia* populations (Binns et al. 2002a), any simultaneous and wide-scale population assessment would likely be faced with such differences in developmental stages.

Impact of biosynthesis and location of marker compounds

Correlations between marker metabolite concentrations in different organs may be influenced by a variety of factors depending on the site of synthesis and what role, if any, translocation plays in the accumulation of the different metabolites. Very little is known about biosynthetic regulation and possible translocation of *Echinacea* metabolites. Temporal and age-related fluctuations have been reported in echinacoside contents of roots (Letchamo et al. 2002, Binns et al. 2002a). Alkamide increases in roots of *Echinacea pallida* have been induced by the plant defense signaling compound methyl

jasmonate (Binns et al. 2001). As the roots and shoots of a given plant contend with distinct sets of biotic and abiotic factors, secondary metabolite accumulation in the various organs may be affected differently and non-proportionately.

My data allowed the examination of correlations between compounds in root systems of individual plants. The information provided by this analysis gives insight into the likelihood of indirect genetic gain in the production of other marker compounds if selection were to be focused on a single compound. In unselected populations, I found that significant correlations between the examined marker compounds within the roots were not consistent in the two different growing environments (Figure 3.4). It is perhaps not surprising that root concentrations of tetraene alkamides were not correlated with any of the CADs given their two distinct biosynthetic origins. The correlation between CADs would logically be higher if the regulatory elements determining their accumulation acted on shared points of their biosynthetic pathway. The absence of consistent correlations present a potential complicating factor in *E. angustifolia* selection work intended to increase concentrations of an array of marker/bioactive compounds.

Marker concentrations in capitula were hypothesized to be better indicators of root concentrations since tetraenes and cichoric acid in shoots were shown previously at undetectable or trace concentrations, whereas capitula had comparable or higher marker levels than those of roots (Binns et al. 2002a, Wu et al. 2004). In this study however, capitula concentrations were not significantly correlated with root concentrations of any markers in field-grown plants (Table 3.4), indicating that capitula concentrations are poor indicators of root concentrations. Cichoric acid actually had a marginally significant ($p=0.07$) negative correlation between root and capitula concentrations (Table 3.4)

suggesting that selection for higher cichoric acid levels in capitula may in fact reduce already low root levels. Qu et al. (2005) found a negative correlation in *E. purpurea* between cichoric acid in roots and young tops, which consisted of undeveloped capitula, leaves and stems. They also found no significant correlation between concentrations in roots and nearly matured seed heads (defined as capitula with disk flowers past anthesis and green to yellowish styles) for both cichoric acid and alkamides in *E. purpurea*.

This was a first effort to develop an efficient indirect selection strategy useful in *E. angustifolia* breeding. Even though significant relationships were observed, especially in the case of echinacoside, the inconsistency of correlations across different environments makes the implementation of this selection strategy problematic. Direct evidence is needed to determine whether the negative relationship observed between root and shoot concentrations of caftaric acid (Table 3.4) and the lower levels of echinacoside in roots of flowering plants are due to translocation or other control mechanisms. Further work is also required to determine which environmental factors impact the strength of the relationships between marker compound concentrations in different parts of *E. angustifolia* plants. Additionally, a comprehensive evaluation of any selection strategy intended to increase the phytochemical traits of *E. angustifolia*, through means other than clonal propagation of selected genotypes, requires information on their heritability.

Conclusions

The results from these greenhouse and field trials suggest that direct phytochemical analyses of roots are required for effective genotypic selection in a greenhouse or field environment; aerial phytochemical concentration is not a reliable

indicator of genotype performance in root phytochemistry. Positive correlations of certain CAD concentrations in roots of greenhouse and field grown plants suggest that indirect genetic gain may be expected from selection for a single phenolic compound. Significant correlations between marker concentrations in roots and other organs in *E. angustifolia* were not consistent between the two environments. This experiment also demonstrated that the developmental stage of *E. angustifolia* plants has an impact on the correlation between root and shoot echinacoside levels in the greenhouse.

Table 3.1: Results for soil analyses of growth substrates collected at harvest time. Greenhouse and field samples consisted of 5 and 6 pooled sub-samples respectively.

	OM (%)	N (ppm)	P (ppm)	K (ppm)	S (ppm)	pH	EC (dS/m)
Greenhouse	54.4	78	130	230	4	5.1	1.64
Field	7.2	12	27	50	1	5.8	0.13

EC: electrical conductance; dS/m: deciSiemens per meter; OM: organic matter; ppm: parts per million

Table 3.2: Concentrations of *E. angustifolia* marker compounds in organs of plants grown under two different growing environments. Means are presented in mg·g^{-1} dry weight ± standard deviations with ranges indicated underneath in brackets. Different superscript annotations represent significantly different mean concentrations by organ and site for each marker compound separately. Greenhouse root and shoot n=32. Field root and shoot n=30, capitula n=15.

Compound	Environment	Root	Shoot	Capitula
Tetraenes	Greenhouse	5.16 ± 2.26^{b} (1.22 - 11.18)	0.14 ± 0.16^{cd} (0.00 - 0.59)	na
	Field	7.78 ± 3.43^{a} (2.36 – 15.40)	0.00 ± 0.01^{d} (0.00 - 0.07)	1.54 ± 1.24^{c} (0.16 - 3.81)
Cichoric acid	Greenhouse	0.14 ± 0.12^{c} (0.00 - 0.50)	0.41 ± 0.27^{c} (0.06 - 1.34)	na
	Field	0.26 ± 0.19^{c} (0.00 - 0.82)	1.27 ± 0.91^{b} (0.12 - 3.53)	2.00 ± 2.01^{a} (0.08 - 7.53)
Caftaric acid	Greenhouse	0.01 ± 0.03^{c} (0.00 - 0.14)	0.80 ± 0.52^{b} (0.13 - 2.78)	na
	Field	0.04 ± 0.07^{c} (0.00 - 0.27)	2.11 ± 0.56^{a} (1.15 - 3.28)	1.00 ± 0.70^{b} (0.08 - 2.58)
Chlorogenic acid	Greenhouse	0.24 ± 0.15^{b} (0.00 - 0.74)	0.40 ± 0.18^{b} (0.00 - 0.79)	na
	Field	1.56 ± 1.69^{a} (0.36 - 7.68)	0.33 ± 0.30^{b} (0.00 - 1.32)	1.50 ± 1.29^{a} (0.00 - 4.79)
Cynarin	Greenhouse	2.09 ± 0.91^{b} (0.56 - 4.70)	0.04 ± 0.07^{c} (0.00 - 0.28)	na
	Field	5.03 ± 2.50^{a} (0.13 -11.25)	0.46 ± 0.30^{c} (0.00 - 1.29)	0.20 ± 0.21^{c} (0.00 - 0.72)
Echinacoside	Greenhouse	8.06 ± 4.60^{b} (0.26 - 20.08)	0.58 ± 0.33^{c} (0.00 - 1.48)	na
	Field	23.98 ± 9.22^{a} (9.56 - 43.04)	0.49 ± 0.60^{c} (0.00 - 3.05)	1.63 ± 1.36^{c} (0.00 - 4.70)

Table 3.3: Spearman's rho correlation coefficients (*r*) for marker compound concentrations between root and shoot for the total population of greenhouse-grown *E. angustifolia* plants harvested after 7 months.

	Total population (n=32)	Flowering only (n=14)
Compound	*r*	*r*
Tetraenes	0.10 ^{NS}	0.12 ^{NS}
Cichoric acid	0.29 ^{NS}	0.06 ^{NS}
Echinacoside	0.53***	0.75***
Cynarin	0.54***	0.18 ^{NS}
Chlorogenic acid	0.20^{NS}	0.25 ^{NS}
Caftaric acid	na[a]	na[a]

[a]Caftaric acid was not detected in roots.
^{NS},*** Nonsignificant or significant at P<0.001, respectively.

Table 3.4: Spearman's rho correlation coefficients (*r*) for marker compound concentrations between root and shoot, and between root and capitula, in field-grown *E. angustifolia* plants harvested after 7 months of age.

	Root:Shoot (n=30)	Root:Capitula (n=15)
Compound	*r*	*r*
Tetraenes	na[z]	0.30[NS]
Cichoric acid	0.12[NS]	-0.48[MS]
Echinacoside	0.09[NS]	0.05[NS]
Cynarin	0.15[NS]	0.21[NS]
Chlorogenic acid	0.11[NS]	0.01[NS]
Caftaric acid[y]	-0.72*	-0.05[NS]

[z]Tetraenes were not present in shoots or were below the limit of quantification of 1 $\mu g \cdot mL^{-1}$.

[y] Correlations based only on samples with detectable levels of caftaric acid (n=9 for root:shoot correlation and n=6 for root:capitula correlation).

[NS], [MS], * marginally significant ($p<0.1$) or significant at $P<0.05$ respectively.

Figure 3.1: Relationship between concentrations of echinacoside in roots and shoots in individual greenhouse-grown *E. angustifolia* plants. Points indicated by stars represent flowering plants (n=32 plants).

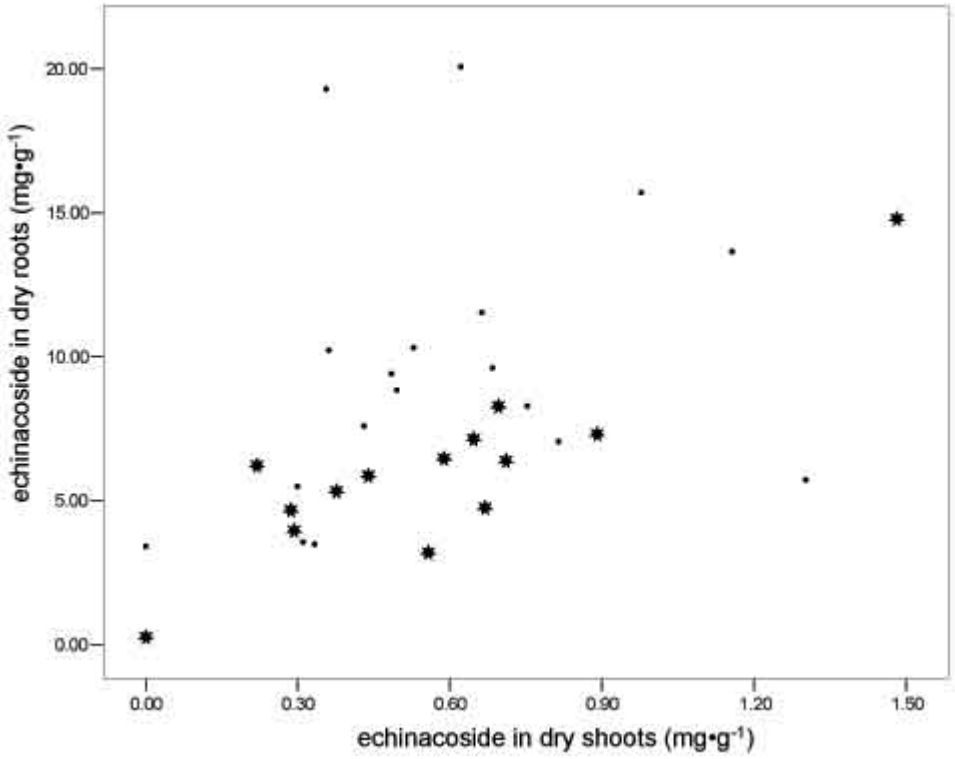


Figure 3.2: Relationship between concentrations of cichoric acid in roots and shoots in individual greenhouse-grown *E. angustifolia* plants. Points indicated by stars represent flowering plants (n=32 plants).

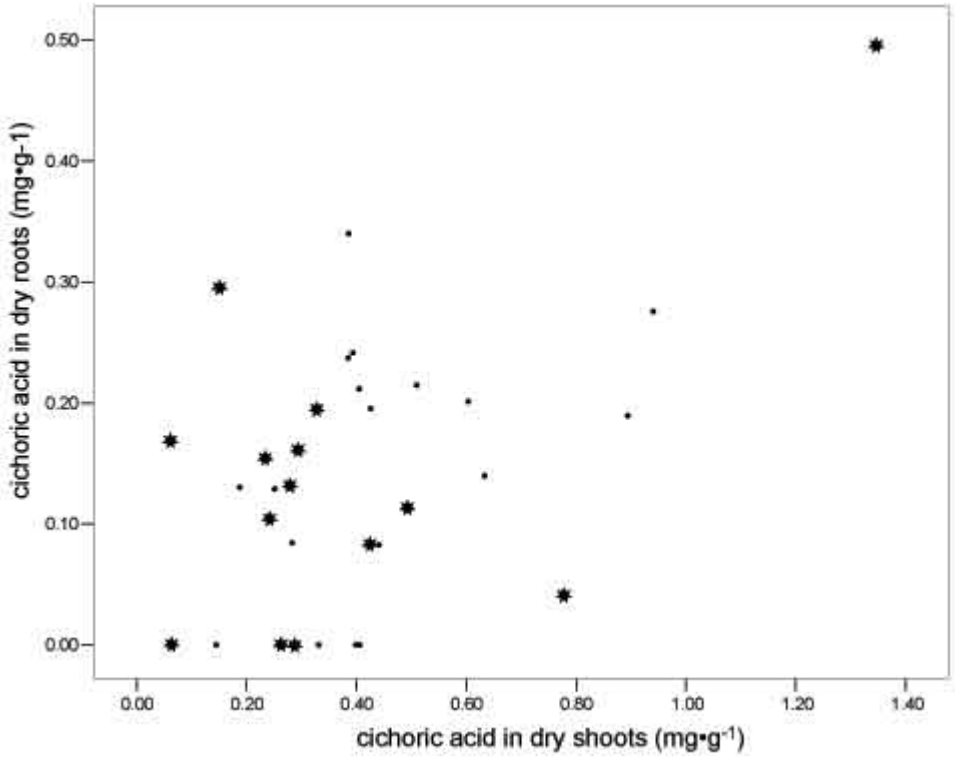


Figure 3.3: Relationship between concentrations of tetraenes in roots and shoots of individual greenhouse-grown *E. angustifolia* plants. Points indicated by stars represent flowering plants (n=32 plants).

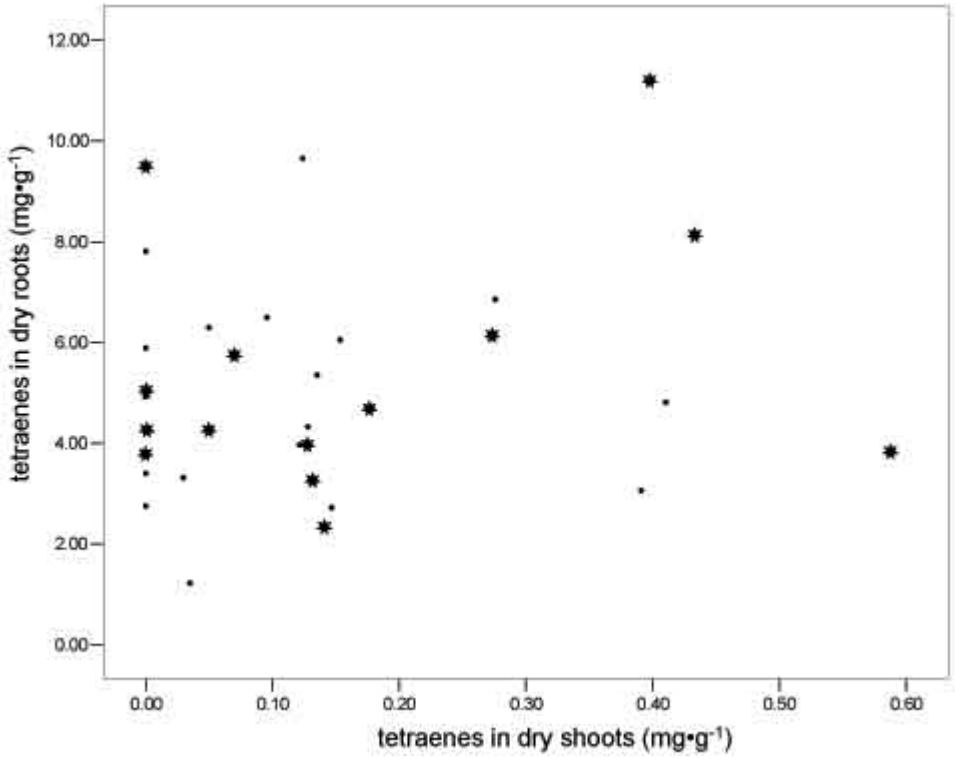
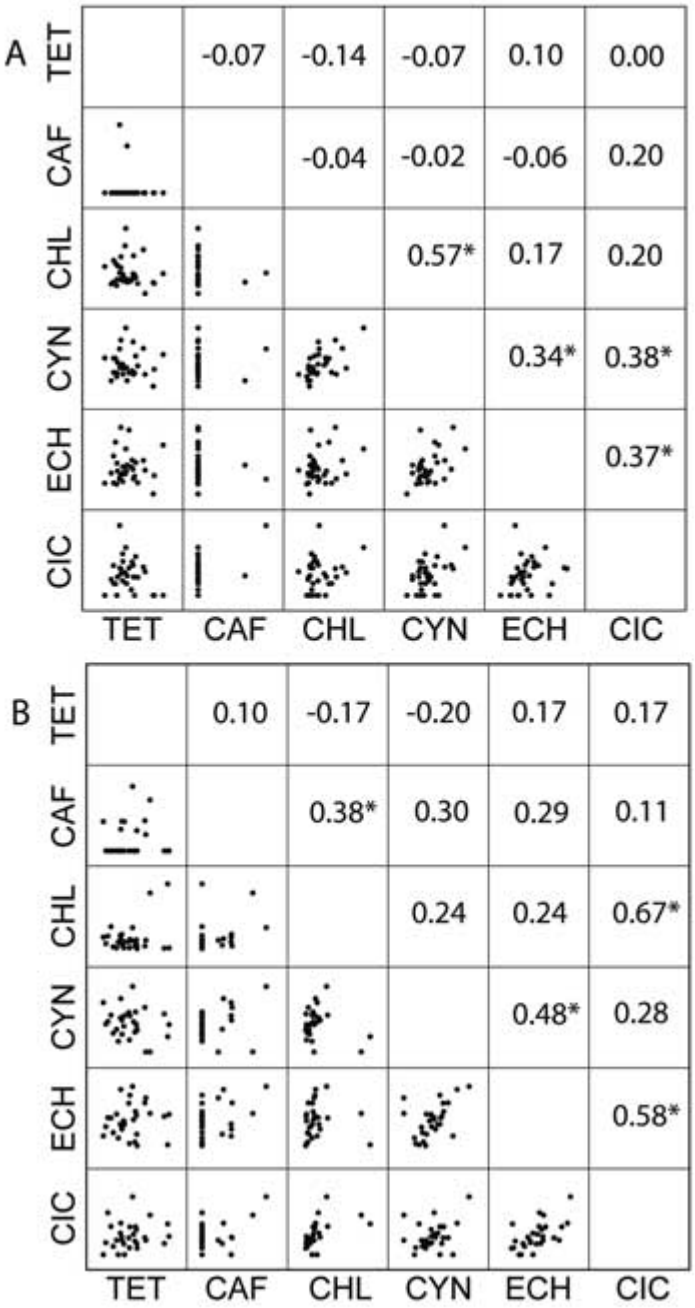


Figure 3.4: Within-root correlations of marker compound concentrations in (A) greenhouse (n=32) and (B) field grown plants (n=30). Spearman's rho correlation coefficients are presented in the upper right boxes of the matrix and correspond to their graphical representation in their mirror boxes in the lower left side. Coefficients indicated with stars have significant p-values (p<0.05). TET: tetraenes concentrations, CAF: caftaric acid concentrations, CHL: Chlorogenic acid, CYN: cynarin concentration, ECH: echinacoside, CIC: cichoric acid.



3.5 REFERENCES

Barnes, J., L. Anderson, S. Gibbons, J.D. Phillipson. 2005. *Echinacea* species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt.,*Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. *Journal of Pharmacy and Pharmacology*. 57:929-954.

Barrett, B. 2003. Medicinal properties of *Echinacea*: A critical review. *Phytomedicine*. 10:66-86.

Bauer, R. 1998. *Echinacea*: Biological effects and active principles. pp 140-157. In: L.D. Lawson and R. Bauer (eds.). Phytomedicines of Europe: Chemistry and Biological Activity. ACS Symposium Series 691; American Chemical Society. Washington, D.C.

Bauer, R., I.A. Khan, and H. Wagner. 1988. TLC and HPLC analysis of *Echinacea pallida* and *E. angustifolia* roots. *Planta Medica*, 54:426-430.

Bauer, R., P. Remiger. 1989. TLC and HPLC analysis of alkamides in *Echinacea* drugs. *Planta Medica*. 55:367- 371.

Bauer, R. and H. Wagner. 1991. *Echinacea* species as potential immunostimulatory drugs. pp.253-3321. In: Wagner and Farnsworth (eds). Economic and medicinal plant research, Volume 5. Academic Press, New York.

Bergeron, C., J.F. Livesey, D.V.C. Awang, J.T. Arnason, J. Rana, B.R. Baum, W. Letchamo. 2001. A Quantitative HPLC method for the quality assurance of *Echinacea* products on the North American market. *Phytochemical Analysis*. 11:207-215.

Bergvinson, D.J., J.T. Arnason, R.I. Hamilton, S. Tachibana, G.H.N. Towers. 1994. Putative role of photodimerized phenolic acids in maize resistance to *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Environmental Entomology*. 23:1516-1523.

Berti, M., R. Wilkens, S. Fischer, and F. Hevia. 2002. Effect of harvest season, nitrogen, phosphorus and potassium on root yield, echinacoside and alkylamides in *Echinacea angustifolia* L. in Chile. *Acta Horticutlurae*. 576:303-310.

Bilal, H.A., C.L. Tian, S. J. Murch, P.K. Saxena. 2007. Light-enhanced caffeic acid derivatives biosynthesis in hairy root cultures of *Echinacea purpurea*. *Plant Cell Reports*. 26:1367-1372.

Binns, S.E., I. Inparajah, B.R. Baum, J.T. Arnason. 2001. Methyl jasmonate increases reported alkamides and ketoalkene/ynes in *Echinacea pallida* (Asteracea). *Phytochemistry*. 57:417-420.

Binns, S.E., J.F. Livesey, J.T. Arnason, and B.R. Baum. 2002a. Phytochemical variation in *Echinacea* from roots and flowerheads of wild and cultivated populations. *Journal of Agricultural and Food Chemistry*. 50:3673-3687.

Binns, S.E., J.T. Arnason, B.R. Baum. 2002b. Phytochemical variation within populations of *Echinacea angustifolia* (Asteraceae). *Biochemical Systematics and Ecology*. 30:837-854.

Blumenthal, M., G.K.L. Ferrier, C. Cavaliere. 2006. Total sales of herbal supplements in United States show steady growth. *HerbalGram*. 71:64-66.

Dalby-Brown, L., H. Barsett, A.K. Landbo, A.S. Meyer, P. Molgaard. 2005. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on *in vitro* oxidation of human low-density lipoproteins. *Journal of Agricultural and Food Chemistry*. 53(24):9413-9423.

Dufault, R. J., J. Rushing, R. Hassell, B.M. Shepard, G. McCutcheon, B. Ward. 2003. Influence of fertilizer on growth and marker compound of field-grown *Echinacea* species and feverfew. *Scientia Horticulturae*. 98:61-69.

El-Gengaihi, S.E., A.S. Shalaby, E.A. Agina, and S.F. Hendawy. 1998. Alkylamides of *Echinacea purpurea* L. as influenced by plant ontogony [*sic*] and fertilization. *Journal of Herbs, Spices, and Medicinal Plants*. 5:35-41.

Gallais, A. 1983. Use of indirect selection in plant breeding. pp.45-59. In: W. Lange, A.C. Zeven, and N.G. Hogenboom (eds.). Efficiency in Plant Breeding; Proceedings of the 10th congress of the European association for research on plant breeding, EUCARPIA. Pudoc. Wageningen. Netherlands.

Gray, D.E., S.G. Pallardy, H.E. Garrett, G.E. Rottinghaus. 2003. Acute Drought Stress and Plant Age Effects on Alkamide and Phenolic Acid Content in Purple Coneflower Roots. *Planta Medica*. 69:50-55.

Institute for Neutraceutical Advancement. 2005. Phenolics in *Echinacea* by HPLC, INA method 106.000. Inst. for Neutr. Adv. Ann Arbor, Mich. July 2005 <http://www.nsf.org/business/ina/echinacea.asp?program=INA>.

Jansen, M.A.K., V. Gaba, and B.M. Greenburg. 1998. Higher plants and UV-B radiation: balancing damage, repair and acclimation. *Trends in Plant Science*. 3:131-135.

Krizek, D.T., Clark, D.H., and Mirecki, R.M. 2005. Spectral properties of selected UV-blocking and UV-transmitting covering materials with applications for production of high-value crops in high tunnels. *Photochemistry and Photobiology*. 81:1047-1051.

Letchamo, W., L.V. Polydeonny, N.O. Gladisheva, T.J. Arnason, J. Livesey, and D.V.C. Awang. 2002. Factors affecting *Echinacea* quality. Pp.514-521. In: J. Janick and A. Whipkey (eds.). Trends in New Crops and New Uses. ASHS Press, Alexandria, VA.

Matthias, A., R.S. Addison, K.G. Penman, R.G. Dickinson, K.M. Bone, and R. P. Lehmann. 2005. *Echinacea* alkamide disposition and pharmacokinetics in humans after tablet ingestion. *Life Sciences*. 77:2018-29.

Pellati, F., S. Benvenuti, L. Magro, M. Melegari, and F. Soragni. 2004. Analysis of phenolic compounds and radical scavenging activity of *Echinacea* spp. *Journal of Pharmaceutical and Biomedical Analysis*. 35: 289-301.

Perry, N., E.J. Burgess, and V.L. Glennie. 2001. *Echinacea* standardization: analytical methods for phenolic compounds and typical levels in medicinal species. *Journal of Agricultural and Food Chemistry*. 49:1702-1706.

Qu, L.P., Y. Chen, X.P. Wang, R. Scalzo, and J.M. Davis. 2005. Patterns of variation in alkamides and cichoric acid in roots and aboveground parts of *Echinacea purpurea* (L.) Moench. *HortScience* 40:1239-1242.

Rininger, J.A., S. Kickner, P. Chigurupati, A. McLean, and Z. Franck. 2000. Immunopharmacological activity of *Echinacea* preparations following simulated digestion on murine macrophages and human peripheral blood mononuclear cells. *Journal of Leukocyte Biology*. 68:503-510.

Schoop, R., P. Klein, A. Suter, S.L. Johnston. 2006. *Echinacea* in the prevention of induced rhinovirus colds: a meta-analysis. *Clinical Therapeutics*. 28:174-183.

Schulz, H., S. Pfeffer, R. Quilitzsch, B. Steuer, and K. Reif. 2002. Rapid and non-destructive determination of the echinacoside content in *Echinacea* roots by ATR-IR and NIR spectroscopy. *Planta Medica*. 68:926-929.

Shah, S.A., S. Sander, C.M. White, M. Rinaldi, C.I. Coleman. 2007. Evaluation of *Echinacea* for the prevention and treatment of the common cold: a meta-analysis. *The Lancet Infectious Diseases*. 7: 473-480.

Speroni, E., P. Govoni P, S. Guizzardi, C. Renzulli, M.C. Guerra. 2002. Anti-inflammatory and cicatrizing activity of *Echinacea pallida* Nutt. root extract. *Journal of Ethnopharmacology*. 79:265-272.

United States Pharmacopeia. 2004. *Echinacea angustifolia* monograph, p.2075-2083. In: USP 28- National Formulary 23. USP Convention: Rockville, Md.

Woelkart, K. and R. Bauer. 2007. The role of alkamides as an active principle of *Echinacea*. *Planta Medica*. 73: 615-623.

Woelkart, K., C. Koidl, A. Grisold, J.D. Gangemi, R.B. Turner, E. Marth, R. Bauer. 2005. Bioavailability and pharmacokinetics of alkamides from the roots of *Echinacea angustifolia in humans*. *Journal of Clinical Pharmacology*. 45:683-689.

Wu, L., J. Bae, G. Kraus, E.S. Wurtele. 2004. Diacetylenic isobutylamides of *Echinacea*: synthesis and natural distribution. *Phytochemistry*. 65:2477-84.

Chapter Four

GENOTYPE X ENVIRONMENT INTERACTIONS IN PHYTOCHEMICAL TRAITS OF *ECHINACEA ANGUSTIFOLIA* RESULT IN RE-RANKING OF GENOTYPES GROWN UNDER FIELD AND GREENHOUSE CONDITIONS[1]

4.1 INTRODUCTION

A key element in obtaining high quality standardized material for the formulation of natural health products is efficient and effective selections of superior genotypes within the context of a larger breeding program. *Echinacea angustifolia* presents a particular challenge in this regard as many of the plant's reported therapeutic benefits have not been convincingly attributed to any specific metabolic constituents (Barrett 2003). In fact, similarly to other herbal products, the biological activity of *Echinacea* appears to stem from complimentary, if not synergistic, effects from a suite of compounds (Dalby-Brown et al. 2005, Barrett 2003). This makes the choice of useful phytochemical traits as selection criteria somewhat precarious given the current state of knowledge.

[1] A version of this chapter will be submitted for publication. Boucher, A.Y. and S.E. Cowan. Genotype x Environment Interactions in Phytochemical Traits of *Echinacea angustifolia* Result in Re-ranking of Genotypes Grown Under Field and Greenhouse Conditions.

Despite disagreement over their relative importance to the reported immunological activity of *Echinacea* (reviewed in Barrett 2003), the marker compounds that are currently used as quality and taxonomic indicators of *E. angustifolia* products (Awang 1999, Bergeron et al. 2000) have been the primary focus of phytochemical breeding efforts (Letchamo et al. 2002). Stemming from two distinct chemical classes, i.e. caffeic acid derivatives and fatty acid derived alkylamides, these marker compounds are synthesized via unrelated metabolic pathways. Consequently, their synthesis and accumulation are likely influenced differentially by environmental and genetic factors. A considerable amount of research has been focused on investigating the isolated effects of environmental (Zheng et al. 2006, Dufault et al. 2003, Gray et al. 2003, El-Gengaihi et al. 1998) and genotypic (Binns et al. 2002) factors on the expression of these traits in *E. angustifolia* and other *Echinacea* species.

Changes in a genotype's response to environmental factors, or phenotypic plasticity, can be represented by its norm of reaction, a profile of its phenotypic response across different environments (Schmalhausen 1949). Variation in phenotypic plasticity within a group of genotype leads to genotype by environment interaction (GE) effects. Given the potential for differential genotypic response in varying environments, GE has long been regarded either as an impediment to genetic gain from artificial selection (Robertson 1959, James 1961, Dickerson 1962), or as an exploitable attribute of genetically varied populations to maximize site-specific yield from specialized cultivars (Ceccarelli 1994). Much of the research examining GE related questions, in the context of plant improvement, reports its effects on yield-related traits with considerably less focus on the expression of secondary metabolites.

Whereas only the magnitude of the differences between genotype response across environments is affected by quantitative GE interactions, qualitative GE interactions, referred to as crossover type GE (Gail & Simon 1985), lead to changes in their direction (i.e. genotypic re-ranking across environments). In theory, if traits of interest show significant crossover GE, two separate selection strategies may be considered. The first is to focus selection efforts on high yielding genotypes with stable expression across a range of target environments. The second, GE interactions can be exploited by selecting genotypes directly in the target environment or in a similar selection environment, assuming information about the causes of GE is known in order to replicate the relevant target conditions. Although the latter approach would result in highly desirable environmentally specialized cultivars specifically adapted for cultivation in experimentally determined mega-environments, it would undoubtedly require considerably more resources than the former. It has been argued that site-specific selection is in fact necessary when breeding for marginal environments (Simmonds 1991, Ceccarelli 1994).

There is a complete absence in the literature, at this point, of direct information pertaining to GE effects in either field- or greenhouse-grown *E. angustifolia*. A superficial examination of recent GE interaction research suffices to show that the presence of significant GE effects on plant traits, including secondary metabolites, appears to be more commonly reported than its absence. Whether this is a reflection of GE ubiquity or a publication bias towards significant findings is unclear. Bradshaw (1965) and others, have suggested that phenotypic plasticity for a given trait is a selectable trait in and of itself, probably under the control of plasticity regulating genes

(Schlichting & Pigliucci 1993). Selection could therefore act on phenotypic plasticity independently from mean trait values. There is strong evidence that phenotypic plasticity in cultivated plant varieties is higher than in their undomesticated counterparts (Schlichting & Levin 1988), a likely result of indirect selection of high yielding genotypes in favourable growing environments (Falconer 1981). With these considerations in mind, it may be hypothesized that levels of phenotypic plasticity for *E. angustifolia* genotypes are low due to its very recent introduction into cultivation and the effective absence of selected cultivars in this species. Wild *E. angustifolia* populations have characteristically high levels of genetic variation (Baskauf et al. 1994, Kapteyn et al. 2001), which may translate into high variability in environmental response among genotypes. This variability in turn might increase the likelihood of significant GE effects on traits of interest. Previous research has suggested that, as competing strategies for survival in different environments, phenotypic plasticity and genetic variation may be negatively correlated between populations (Bradshaw 1965; Marshall & Jain 1968).

A selection environment different from the target cultivation environment may be desirable for various practical reasons. For example, greenhouse selections of a field crop could increase the number of selection cycles in a year. The expression of a trait measured in two different environments however should in fact be considered as genetically correlated, yet separate traits (Falconer 1981). Hence any selection in an environment other than the intended growing environment is a form of indirect selection whose efficacy is dependant on the assumption of genotype stability, or the absence GE on the selected trait. A number of recent examples of cross-environmental genotypic stability of secondary metabolite-related traits have been reported. For instance, in

studies conducted on six raspberry cultivars harvested in three different locations in the Pacific Northwest of the US and Canada, an absence of GE effect was reported for concentrations of different volatile aroma compounds (Moore et al. 2002), anthocyanins and other fruit quality-related traits (Burrows & Moore 2002). O'Reilly-Wapstra et al. (2005) reported an absence of GE interaction on a range of secondary metabolites, including total phenolics in leaves of seedlings from two distinct populations of *Eucalyptus globulus* that differed in herbivore resistance and grown under high or low fertilizer treatments. GE effects on concentrations of various phenolic acids, including caffeic acid, in six hard spring wheat cultivars grown at four locations across Western Canada were also undetectable (Mpofu et al. 2006).

The objectives of this study were, firstly, to assess variation attributable to GE in phytochemical traits and root biomass allocation of clonally propagated *E. angustifolia* genotypes grown under greenhouse and field conditions, and secondly, to compare and evaluate different representative environments for the selection of *E. angustifolia* genotypes using GGE biplot analyses (Yan et al. 2000). This study is the first to directly address the question of GE effects on *E. angustifolia* phytochemistry and the potential for genotypic re-ranking across different environments.

4.2 MATERIALS AND METHODS

Propagation of selected plants

Five individual plants (hereafter referred to as genotypes) were selected from an initial population of 230 *E. angustifolia* plants based on their responsiveness to vegetative propagation by root cuttings, observably healthy appearance and differing

morphologies (eg. height, branching pattern of flowering stem, leaf surface hairiness, root structure). The plants had been grown under greenhouse conditions from achenes obtained from a phytochemically-certified cultivated *E. angustifolia* population in Savonah, British Columbia. Source germplasm for this population was identified to species in November 2002 by the Saskatchewan Herb Research Program at the University of Saskatchewan. Clonal populations of the genotypes were generated by root cuttings collected in March 2005. Whole root systems were washed under tap water to remove soil, bleached for 30 minutes in a 1% NaOCl solution and rinsed under tap water. The roots were cut into 3cm long segments varying between ca. 7-10 mm in diameter. Individual cuttings were planted up-right just below the surface of a 1:1 mixture of perlite and vermiculite in 20 cm long plastic cones. The cuttings were kept in a mist chamber until leaves were observed emerging from the substrate. Six weeks after root segments were initially harvested, cuttings with sprouted leaves were transferred to 72-plug plastic tray inserts (Kord, Brampton, Ont., Canada) filled with greenhouse growing medium to allow for fine root development before planting out in the appropriate growing site. The greenhouse growing medium consisted of a mixture of 8 parts Sunshine Mix #1 (Sun Gro Horticulture, Vancouver, B.C., Canada) : 1 part steam pasteurized mineral soil : 1 part Perlite (by volume) and was amended with 5.9 kg m^{-3} Osmocote 13-13-13 (Scotts, Marysville, Ohio) for slow nutrient release.

Experimental design and planting layout

Ten plants per genotype were planted in two rows with 30 cm inter-plant spacing and 150 cm inter-row spacing, except in the greenhouse where the inter-row spacing was

reduced to 30 cm to accommodate all of the plants on a single flood bench. In the case of the field sites, two border plants were planted at both ends of the two rows. The plants were arranged in a complete randomized block design with the two rows being divided into 5 blocks and individual plants from the different genotypes being randomly assigned within the blocks. The total sample size was 150 plants.

Growing environments

Clonal populations were planted out in three distinctively different environments, one greenhouse and 2 field sites. The field sites are located in two distinct ecoregions (Demarchi 1996). The Totem Field (TO) site (49˚15'26"N 123˚14'59"W, elevation: 81m) is located within the Lower Mainland ecoregion whereas the Flying-Two (FT) site (49˚48'54"N 119˚26'48"W; elevation: 507m) is located within the Thompson-Okanagan Plateau ecoregion. At both field sites, the soil texture was loamy sand. Soil and growing medium samples at each site were collected at time of planting and were analysed by Norwest Soil Research Ltd. (Surrey, Canada). For each field site, the final sample was a composite of 6 sub-samples collected at evenly spaced locations along the field plots at a depth of 30 cm. The sub-samples were pooled and mixed before analyses. Since greenhouse growing media had been well mixed prior to planting, it was assumed to be reasonably heterogeneous and, for this reason, only one representative sample was used in the analysis. Results of all soil analyses are given in Table 4.1.

To characterize climatic conditions at the two field sites during the growing season, daily data was obtained from the Environment Canada weather stations nearest to each site: Kelowna automated weather station (49˚57'36"N 119 22'49" elevation:

423m) for FT and Vancouver International A weather station (49 12' N 123 10' W elevation 4m) for Totem Field (TF). The linear distance between weather stations and field sites was approximately 16 km and 8 km for FT and TO respectively. Monthly mean environmental data on temperature, precipitation and growing degree days (GDD) is presented in Table 4.2.

Plant harvesting and measured root-yield parameters

All of the plants were harvested between the 15th and the 24th of October, 2005. Plant height and number of capitula were recorded before being dug up, with observations recorded for any signs of disease or damage. The plants were washed with tap water to remove soil on site and then left to dry whole in darkness in an open room (≈26 ºC, RH ≈30%) for up to 15 days or until any reduction in mass due to moisture loss was no longer detectable. Total dry mass of whole individual plants were weighed on a Sartorius L420D laboratory scale (Goettinburg, Germany). Root systems were then cut and weighed separately to obtain root biomass and root:shoot biomass ratios.

Root extraction

The whole dried root system of each plant was chopped up in an Osterizer blender fitted with a 250 mL glass jar. Afterwards, the root material was ground to a fine powder with a mortar and pestle and sieved through a 35 mesh (US Standard) sieve and kept at –20C until time of extraction. Storage time did not exceed 7 days. Powdered root material from each plant was extracted separately using the following protocol based on Bergeron et al. (2000). In 15 mL Falcon ™ tubes (Becton Dickinson Labware, Franklin Lakes, NJ), ca. 0.5 g of powder was weighed out precisely on a Sartorius 1602 MP8

analytical scale (Goettinburg, Germany). Ten millilitres of 70% EtOH was added to the powder and sonicated for 5 minutes in a Branson Model B-220 Ultrasonic Cleaner (Danbury, Conn.). The tubes were placed in a Sorvall GLC-1 benchtop centrifuge and spun for 5 min at 2500 rpm. The supernatant was poured off into a 50 mL Falcon™ tubes. The extraction process was repeated 2 more times and the pooled supernatants were brought up to exactly 30 mL. The extracts were all stored at –20ºC until HPLC analysis.

RP-HPLC analysis for quantification of CADs and alkamides in *E. angustifolia* ethanolic extracts

Aliquots of the extracts were filtered through 0.22um PVC filters before injection into an HPLC instrument. Phenolic compounds were analyzed using RP-HPLC according to the validated INA method 106.000 'Phenolics in *Echinacea* by HPLC' (INA 2005). The stationary phase was a Cosmosil 5C18-AR-II (4.6 mm x 150 mm, 5 um) column (Nacalai Tesque, Kyoto, Japan). The mobile phase consisted of a changing gradient of the following two solvents: 0.1% phosphoric acid in nanopure water (A) and acetonitrile (B). The mobile phase gradient was as follows: initial conditions at 90% A were gradually reduced to 78% over 12 minutes, then further reduced to 60% over 2 min. This proportion was held for 0.5 minutes followed by a 4 min post run time to allow the system to return to initial conditions. The flow rate was 1.5mL/min and detection was performed at 330nm. The injection volume was 5.00uL and the column was maintained at 35.0C in a column oven equipped with a thermostat. Further HPLC instrument details are provided below. For the analysis of alkamide compounds, the HPLC method used

was based on the validated method for determination of the concentrations of dodecatetraenoic acid isobutylamides in *E. angustifolia* described in USP-28 NF-23 (USP 2004). A Prodigy 5u ODS3 100A (4.6 mm x 250 mm, 5 um) column (Phenomenex, Torrance, CA) was used. The elution was a 40 min isocratic run of a mobile phase consisting of nanopure water and acetonitrile (55:45). The flow rate was 1.5mL/min and detection was made at 254nm. Injection volume was 23.0uL and the column thermostat was set to 30C.

Both phenolic and alkamide determinations were performed on an Agilent 1100 series instrument equipped with a photodiode array detector controlled by the Chemstation Chromatography Data system software (Agilent Technologies, Santa Clara, CA). Peak identification and quantification was determined from relative retention times and 6 point external calibration curves for each standard of the analysed compounds. The lower limit of quantification for the methods used was 1 $\mu g \cdot mL^{-1}$. Standards for the CADs (chlorogenic acid, cynarin, and echinacoside), as well as for the alkamides (dodeca-2-(E),4(E),8(Z),10-(Z)-tetraenoic acid, dodeca-2(E),4(E)-dienoic acid [alk11] and undec-2-ENE-8,10-diynoic acid [alk12]) were all purchased from Chromadex (Santa Ana, CA). Compound identification was further confirmed by comparisons to published chromatograms and UV spectra (Bauer et al. 1988). Tetraene concentrations are reported herein as the sum of concentrations of the two major isomers. Quantification of the second tetraene isomer, dodeca-2-(E),4(E),8(Z),10-(E)-tetraenoic acid was extrapolated from the response factor and standard curve obtained from the available 10(Z) isomer.

Data analysis

Analysis of variance

Overall statistical significance was determined by 2-way analysis of variance with an alpha level of 0.05. None of the assumptions for analysis of variance were determined to be violated and as such, data transformation was not required. Post-hoc multiple comparisons were done using a Tukey pairwise comparison. All statistical analyses were done using SPSS 13.0 software (SPSS Inc., Chicago, Ill.). 'Genotype' and 'Environment' were considered as random factors for the purpose of analysis of variance, since the factor levels used in this study were intended to be representative of a larger population of potential genotypes and growing environments and did not constitute the full range of all possible levels of interest (Little et al. 2002). Type III sum of squares obtained in the analyses of variance was used to determine the percentage of total variation attributable to 'Genotype', 'Environment' and 'Genotype X Environment' or 'GE' effects.

GGE biplot analysis

Phytochemical and morphological trait responses to genotype by environment effects were analysed using GGE biplot analysis software (Yan 2001). GGE biplot analysis facilitates GE data visualisation by providing clear and informative graphical representation of genotypic (G) and GE effects on genotype response to different growing environments and the relationships among genotypes and environments. Since G and GE effects are the only two contributors to variance that are of relevance to genotype evaluation, the first step in the GGE biplot analysis procedure is to center the original

data about the environment (Yan et al. 2000). The biplots are produced using the first two principal components obtained from singular value decomposition (SVD) of the environment-centered multi-environment data. A more detailed and in-depth description of the GGE biplot analysis and its underlying model can be found in Yan et al. (2000), however, the following paragraphs offer an overview of GGE biplot properties and their interpretation.

Overview GGE biplot properties and interpretations

Two GGE biplots are presented for each of the traits. Environment-metric preserving representations of the biplots, found in the upper part of Figures 4.1 to 4.3, are appropriate for comparing environments and analysing relationships among them. The genotype-metric preserving representations, which are presented in the lower part of Figures 4.1 to 4.3, are useful in comparing genotypic performance and their interaction with environments.

In an environment-metric preserving GGE biplot, the length of the environment vectors, which are represented in the graphs by lines connecting the origin and the individual environment coordinates, is an approximation of the environments standard deviation and, as a result, can be interpreted as a measure of an environment's ability to discriminate among the tested genotypes (Yan et al. 2002). Additionally, sites with environment vectors of similar length have similar discriminatory ability. The cosine of the angle formed by two environment vectors is an indicator of how correlated overall trait response is in these two environments. Therefore, environments with vectors forming right angles are not correlated, those forming acute angles are positively

correlated, and those forming obtuse angles are negatively correlated. High negative correlations are usually an indicator of important cross-over GE effects.

An ideal genotype is defined here as one that will offer both above average performance for a given trait and high stability across various target environments (wide adaptability). The ideal genotypes for each of the traits measured in this study are clearly demonstrated in relation to the growing sites in the genotype-metric preserving GGE biplots (lower panels of Figures 4.1 to 4.3). The superimposed axes represent the average environment coordination (AEC), which consists of the average environment abscise (AEA), the single arrowed axis, and the average environment ordinate (AEO), the double-arrowed axis (Yan 2002). Genotypes located further along the AEA, in the direction of the arrow, have increasingly higher levels of the trait in question. The distance between a genotype and the origin, in either direction along the AEO, is inversely proportional to the stability of its performance across the tested environments. Thus, the centre of the concentric circles indicates the coordinate where the ideal genotype would be expected, so that genotypes closest to this point are considered the best performers of the trial.

4.3 RESULTS

Comparison of growing environments (soil and climate)

The three different growing environments had a number of noteworthy differences in regards to soil and climatic conditions (Tables 4.1 and 4.2). All of the growth substrates had pH levels equal or slightly below the lower end of the optimal range for *Echinacea* cultivation as recommended in Li (2000). Along with the lowest

pH, the substrate used in the greenhouse pots had much higher levels of OM and EC than those found in either of the two field sites. Whereas the TO site had low nitrate and potassium concentrations compared to the other sites, the highest levels of phosphate were found in the greenhouse substrate.

Environmental data from the two field sites show differences in temperature and precipitation (Table 4.2). Larger differences between the monthly mean maximum and minimum temperatures were characteristic of the FT site. Cumulative GDD for the growing season were however similar at both sites. Although cumulative precipitation recorded at the end of the season near the TO site was almost double that at the FT site, the large difference was mainly accounted for by heavy rainfall at TO in October.

GE effects on phytochemical and root yield traits

GE effects were significant for all of the measured phytochemical and morphological traits. Variation attributed to GE effects among the markers were highest for cynarin (34%) and tetraenes (28%) (Tables 4.3 and 4.4). Growing environment also had a clearly significant effect on all of the phytochemical markers studied (Table 4.3), however, not on root mass nor on the root:shoot ratio of plant biomass. Overall, the highest percentage of variation explained by a GE effect was observed for root:shoot ratio (40%).

GGE plots and norms of reaction

The first two principal components generated in the GGE biplot analyses were highly representative of the original data matrices. This is reflected by high percentages

of data approximation for all of the studied traits with summed values ranging from 86.4%, for tetraenes, to 99.9% for chlorogenic acid.

Genotype performance discrimination in different growing sites (Environment-metric preserving GGE biplots)

Based on the properties of a GGE biplot presented in the methods section, we see in the case of the phytochemical traits that the TO site was either the most, or comparable to the most effective site for differentiating phytochemical performance of the different genotypes (Figures 4.1 & 4.2). The FT site, on the other hand, was least effective at discriminating genotypes for the compounds in the alkamide class (Figure 4.2). The GH site had relatively low discriminatory ability compared to TO for all of the CADs, especially in the case of chlorogenic acid where very low concentrations of this marker compound were produced in all of the genotypes (Figure 4.1a). In regards to morphological traits, the best discrimination of genotypes based on root mass was observed at the FT site (Figure 4.3a) whereas the GH site was most discriminating of genotypes based on root:shoot ratio (Figure 4.3b).

According to the angles formed between the environment vectors, it is clear that correlations of genotype response between the different environments are inconsistent across the different measured traits. In fact, these inconsistencies in environment correlations are observed even within chemical classes (i.e. alkamides and CADs). For instance, whereas FT and TO appear to be highly correlated for tetraenes (Figure 4.2 a), FT is highly correlated with GH and not TO in the case of alk12 response (Figure 4.2 c).

Similarly, inconsistencies in environmental response is apparent for CADs, especially between chlorogenic acid and echinacoside (Figure 4.1 a & c).

Among the genotypes considered in this trial, genotype C appears to be the best performer for the majority of the different phytochemical traits with the exceptions of cynarin and alk11 for which genotype A had higher than average concentrations and the highest stability across environments (Figure 4.1 & 4.2). Genotype B on the other hand is closest to the ideal genotype for both root dry mass and root:shoot ratio (Figure 4.3).

As mentioned in the methods section, obtuse angles between environment vectors in GGE biplots are reflective of important crossover GE interactions. Patterns of genotypic crossover can be directly seen in the reaction norm graphs (Figure 4.4 to 4.6) showing mean values of the different traits for each genotype-environment combination. Even though overall GE effects were determined to be significant for all of the measured traits (Tables 4.3 & 4.4), few to no crossover interactions (i.e. crossing reaction norms) are observed in environmental comparisons for some of the phytochemical (Figure 4.4 & 4.5) and morphological traits (Figure 4.6). For instance, genotypic rankings based on chlorogenic acid production are consistent across the different sites with a single exception in the comparison between FT and TO where reaction norms for genotypes D and E crossover. Large crossover interactions in genotypic response are apparent for cynarin, echinacoside and tetraenes in their norm of reaction graphs comparing either field sites with the greenhouse site. This observation is further supported by the corresponding environment vectors with angles greater than 90º (Figure 4.1 & 4.2).

As reflected in the analysis of variance by the absence of main genotype and environment effects (Table 4.4), root:shoot ratios were similar among environments and

genotypes. This trait also showed relatively constant rankings for genotypic performance across environments despite a significant GE effect, with the exception of genotype D, which is discussed later.

4.4 DISCUSSION

With the emergent interest in natural products research and the importance of breeding therapeutically superior cultivars, a better understanding of the plasticity of phytochemical phenotypes across different target environments is essential. Relevant information can be gleaned from an important body of ecological research examining GE effects on secondary metabolism of plants, often in the context of plant defense and resource allocation (eg. Baldwin 1999). Given the significant GE effects on all of the phytochemical and morphological traits of interest in *E. angustifolia* measured in this study (Table 4.3 to 4.5), it is apparent that serious consideration will have to be given to the potential disruptive effects that genotypic re-ranking would impose on selection efficacy. The study of GE effects in cultivated plants has focused primarily on yield-related traits. Relatively less work has dealt with GE effects on the secondary metabolism of cultivated plants, with the majority of examples focusing on major commercial crops (eg. zum Felde et al. 2006, Mpofu et al. 2006).

My results show clear differences among the growing sites in their ability to discriminate among genotypes on the basis of phytochemical traits (Figure 4.1 & 4.2). Whereas selection environments that effectively discriminate among genotypes are desirable, those which are consistently unable to do so are counterproductive and should be eliminated from future selection trials. The ideal selection environment for *E.*

angustifolia breeding is one with a relatively high ability to discriminate among genotypes for all of the traits of commercial interest that would be used as selection criteria. Further, highly correlated trait responses in different sites (eg. TO and FT for tetraenes) are easily visualized on the GGE biplots and are reflective of low GE interaction (Yan et al. 2000). As previously stated, low or non-significant GE effects are desirable when evaluating possible selection environments that differ from target cultivation environments. However, in the context of genotype-testing environments, highly correlated environments with low GE are redundant and should be avoided for the sake of resource management. Truly redundant environments produce similar responses over different years and therefore, any conclusion regarding testing environment redundancy must be based on evaluations conducted over a number of growing seasons for a given trait, or suite of traits of interest (Yan et al. 2000).

The potential for complications to arise in choosing selection and testing environments is apparent given the inconsistencies uncovered in this study among the relative discriminating abilities and environmental similarities of the tested environments across the different traits (eg. tetraenes vs. chlorogenic acid). Certainly a better understanding of the specific environmental and physiological factors affecting phytochemical expression in *E. angustifolia* would be useful in this matter. For instance, isoforms of phenylalanine ammonia-lyase (PAL), the catalyzing enzyme of the first committed step in the shikimic acid pathway (Jones & Hartley 1999) from which CADs are ultimately synthesised, have been shown to respond differentially to environmental cues (Liang et al. 1989). PAL may be responsible, in part, for the GE effects in CAD traits measured in this study. However, it is only one of many enzymatic steps, most of

which are currently unknown, that have the potential to influence the biosynthesis of CADs and alkamides in *E. angustifolia*. This could form the basis of a future investigation into the secondary metabolic response to environmental cues in *E. angustifolia* and related taxa.

The interest in *E. angustifolia* as a crop is directly dependant on its bioactivity. Much remains to be elucidated about the activity of *Echinacea* extracts and how it correlates with the various metabolites it contains. Therefore, it would only be speculative at this point to suggest that the observed GE effects of marker compounds in this trial would lead to similar variability in the bioactivity of cultivated *Echinacea*. However, this study shows that the response of *Echinacea* phytochemical markers to GE effects is not uniform. Therefore, future investigations should focus first on elucidating biological mechanism of action and more delineated therapeutic relevance of the marker compounds in this *Echinacea* variety.

It is interesting to note from the cross-genotype marginal means for the three growing sites that the CADs were invariably lower in the greenhouse sites compared to the two field sites (Figure 4.7). The same observation has been previously reported for unselected, seed propagated plants grown in the same environments in different years (Chapter 3). As previously examined abiotic factors, including nutrient availability, have been shown to have minimal to insignificant effects on the production of secondary metabolites in *Echinacea* roots (Zheng et al. 2006, Dufault et al. 2003, El-Gengaihi et al. 1998), it seems that the observed differences may be attributable to as of yet untested factors. The soil analyses data support the idea that macronutrient availability from substrate is not a strong determinant of caffeic acid derivative production (Table 4.1 and

Figure 4.7). For instance, the TO field site was almost twenty-fold and more than six-fold lower for N than the greenhouse and other field site, respectively, (Table 4.1). The inverse relationship between N availability and production of carbon-based secondary metabolites such as CADs, especially visible in the case of echinacoside (Figure 4.7), is wholly consistent with the predictions advanced by the carbon/nutrient balance hypothesis (Bryant et al. 1983). It is also in agreement with earlier findings by del Moral (1972) that showed a 5-fold increase in chlorogenic acid root concentrations in plants from another Asteraceae species, *Helianthus annuus*, that were subjected to nitrogen deficiency.

Zheng et al. (2006) clearly highlight some of the benefits of greenhouse *Echinacea* culture and provide valuable information for its optimisation. However, the highest reported root CAD level (echinacoside ≈ 6.75mg*g^{-1}) in their study was almost identical to the average GH echinacoside level (6.19 mg*g^{-1}) observed in the present study (Figure 4.7). Perhaps also pertinent to my results, the del Moral (1972) study showed a 2.5-fold increase of the root chlorogenic acid following plant exposure to UV radiation. Although not specifically measured in this experiment, nor reported in Zheng et al. (2006), UV levels are generally much lower in greenhouses compared to those measured outside because of the UVB filtering properties of standard greenhouse glass (Krizek et al. 2005). A physiological explanation for the above observations may well be related to the known stimulatory effects of environmental factors such as light (Zucker 1972), and nutrient limitations (Tan 1980) on the activity of PAL. The focus of this point in the discussion on potential UV effects is speculative and should not be taken to

discount other potential factors specific to greenhouse culture such as reduced volume for root development compared to field conditions.

Alkamide expression, on the other hand, does not appear to be disadvantaged by greenhouse growth. In fact, alkamide levels were higher in greenhouse plants than those grown at the FT field site (Figure 4.7). The results of FT and GH alkamide yields (Fig 4.7) are consistent with the carbon-nutrient balance (CNB) hypothesis (Bryant et al. 1983), given that alkamides are N-containing secondary metabolites and that markedly lower levels of N and P were measured in FT soil (Tab 4.1). However, of all the sites, the highest levels of alkamides were observed in TO (Fig 4.7). This is contrary to the CNB prediction that lower nutrient availability (Tab 4.1) would lead to lower N-based metabolite accumulation. Another explanation for the unexpectedly low N-based marker levels in TO (independent of nutrient availability, or interacting with nutrients) may be induction in response to biotic pressures.

Alkamides, which have demonstrated insecticidal (Clifford et al. 2002, Jacobson 1967) and anti-fungal activity (Binns et al. 2000), have been shown to be inducible in *Echinacea* roots by the defence-related molecular signal methyl jasmonate (Binns et al. 2001). Given the continuous moisture of the greenhouse growing medium and the higher levels of precipitation in TO than in FT (Table 4.2), especially in the last month when the plants were harvested, the sites with the highest reported levels of alkamides likely presented conditions favouring soil-borne pathogenic fungal pressure. The notable exception to the rank-order stability for root:shoot ratios in Table 4.4 observed for genotype D is further support for the idea of higher disease pressue in the greenhouse. This genotype had an extremely high mean root:shoot ratio in the greenhouse site

compared to its relatively lower values in both field sites. Genotype D appeared especially susceptible to crown rot in the greenhouse given the rapid die-back of a large proportion of shoot biomass, a characteristic symptom of this disease. Hence the high root:shoot ratios for genotype D were a result of low shoot mass as opposed to extraordinarily large root biomass (Figure 4.6).

The results and conclusions from this study are based on plants harvested after a single growing season. As *Echinacea* is a multi-year crop (typically 2-4 years), the findings presented here should be further extended in future trials looking at older *E. angustifolia* plants.

Conclusion

This study presents critical and novel data for the *Echinacea* phytopharmaceutical industry and crop improvement, namely evidence of ranking rearrangement of selected *E. angustifolia* genotypes. The next logical step is to focus on specific factors that may be responsible for the observed trait instability in different environments. Until we have a better understanding of such factors and their impact on phytochemical and agronomical trait expressions in *E. angustifolia*, genotype selection must be made in environments closely resembling the target growing environment in order to maximize selection efficiency in the near term.

Table 4.1: Results of soil analyses from samples[a] taken at 3 different sites at time of planting.

Sites	N(ppm)	P(ppm)	K(ppm)	S(ppm)	pH	EC(dS/m)[b]	OM(%)
GH	78	130	230	4	5.1	1.64	54.4
FT	25	32	250	2	6	0.25	2.2
TO	4	36	80	1	5.7	0.11	7.8

[a]For each field site, the final sample was a composite of 6 subsamples collected at evenly spaced locations along the field plots at a depth of 30 cm. The subsamples were pooled and mixed before analyses.
GH: greenhouse; FT: Flying-Two Ranch; TO: Totem Field;
EC: electrical conductance; dS/m: deciSiemens per meter; OM: organic matter; ppm: parts per million

Table 4.2: Meteorological data for Totem Field and Flying Two field sites during growing season (May-October, 2005).

Site	Month	Mean temperature (ºC)	Mean max. temperature (ºC)	Mean min. temperature (ºC)	Precipitation (mm)	Cumulative Precipitation (mm)	Monthly GDD	Cumulative GDD
Totem (Vancouver)	May	16.2	20.9	11.5	12.6	12.6	49.6	49.7
	June	15.6	18.9	12.3	49.6	62.2	167.2	216.9
	July	18.1	22.3	13.9	43.6	105.8	251.2	468.1
	Aug.	19.0	23.4	14.4	28.6	134.4	277.3	645.3
	Sep.	14.7	18.8	10.5	53.6	188.0	139.2	884.6
	Oct.	11.9	14.7	9.1	155.4	343.4	32.0	916.6
Flying Two (Kelowna)	May	15.4	26.5	4.1	7.0	7.0	42.7	42.7
	June	15.3	22.3	8.4	67.0	74.0	159.2	201.9
	July	18.7	27.4	10.0	25.0	99.0	268.8	470.7
	Aug.	18.3	28.7	7.9	33.0	132.0	257.5	728.2
	Sep.	12.3	20.3	4.4	21.5	153.5	83.3	811.5
	Oct.	8.6	14.3	2.8	20.5	174.0	0.6	812.1

Table 4.3: The effects of genotype (G), growing environments (E) and their interaction (GE) on phytochemical traits in roots of *E. angustifolia* plants.

Compound	Effect	Degrees of freedom	Type III sum of squares	p-value	Contribution to explained variance
Chlorogenic acid	E	2	35214422.33	0.01**	38%
	G	4	38810693.78	0.04*	42%
	GE	**8**	**18499857.43**	**0.00****	**20%**
Cynarin	E	2	27049435.10	0.03*	50%
	G	4	8922431.65	0.48	16%
	GE	**8**	**18700809.27**	**0.00****	**34%**
Echinacoside	E	2	2926214742.00	0.00**	69%
	G	4	659634825.00	0.18	16%
	GE	**8**	**654239745.10**	**0.00****	**15%**
Tetraenes	E	2	102756620.60	0.01**	58%
	G	4	25241005.83	0.45	14%
	GE	**8**	**49797533.02**	**0.00****	**28%**
Alk.11	E	2	2732641.17	0.01**	23%
	G	4	7848112.91	0.00**	67%
	GE	**8**	**1132437.42**	**0.00****	**10%**
Alk.12	E	2	2471313.94	0.01**	51%
	G	4	1440166.07	0.08	30%
	GE	**8**	**918426.90**	**0.00****	**19%**

*$p \leq 0.05$, **$p \leq 0.01$

Table 4.4: The effects of genotype (G), growing environments (E) and their interaction (GE) on root yield traits of *E. angustifolia* plants.

Compound	Effect	Degrees of freedom	Type III sum of squares	p-value	% contribution to explained variation
Root Dry Mass	E	2	441.23	0.07	15%
	G	4	1929.08	0.01**	68%
	GE	**8**	**483.72**	**0.02***	**17%**
Root:Shoot ratio	E	2	37.57	0.11	30%
	G	4	37.51	0.29	30%
	GE	**8**	**50.23**	**0.00****	**40%**

*p≤0.05, **p≤0.01

Figure 4.1: GGE biplots based on root concentrations of the three measured caffeic acid derivative traits (a. chlorogenic acid; b. cynarin; c. echinacoside) in 5 *E. angustifolia* genotypes grown in three environments (GH: UBC horticultural greenhouse; FT: Flying-Two Ranch; TO: Totem Field). The data for each trait is represented by 2 biplots. The upper graph, suitable for comparing growing sites, is environment-metric preserving (as defined in Yan et al. 2002). The bottom graph is genotype-metric preserving (Yan et al. 2002), useful for comparisons between genotypes and their correlation with growing sites. Genotype markers have been omitted from environment-metric preserving biplots for clarity.

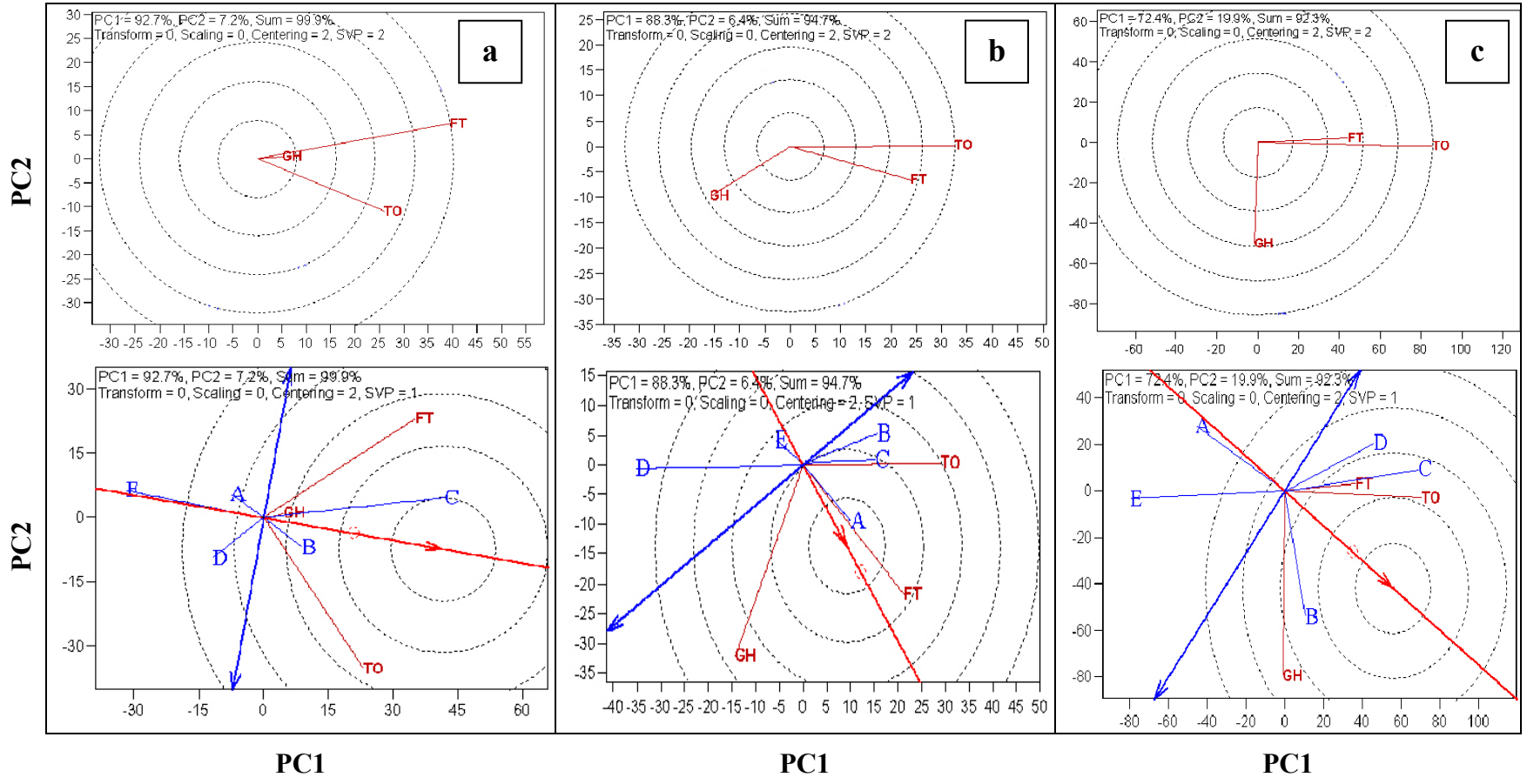


Figure 4.2: GGE biplots based on root concentrations of the three measured alkamide markers (a. tetraenes; b. alk11; c. alk12) in 5 *E. angustifolia* genotypes grown in three environments (GH: UBC horticultural greenhouse; FT: Flying-Two Ranch; TO: Totem Field). The data for each trait is represented by 2 biplots. The data for each trait is represented by 2 biplots. The upper graph, suitable for comparing growing sites, is environment-metric preserving (as defined in Yan et al. 2002). The bottom graph is genotype-metric preserving (Yan et al. 2002), useful for comparisons between genotypes and their correlation with growing sites. Genotype markers have been omitted from environment-metric preserving biplots for clarity.

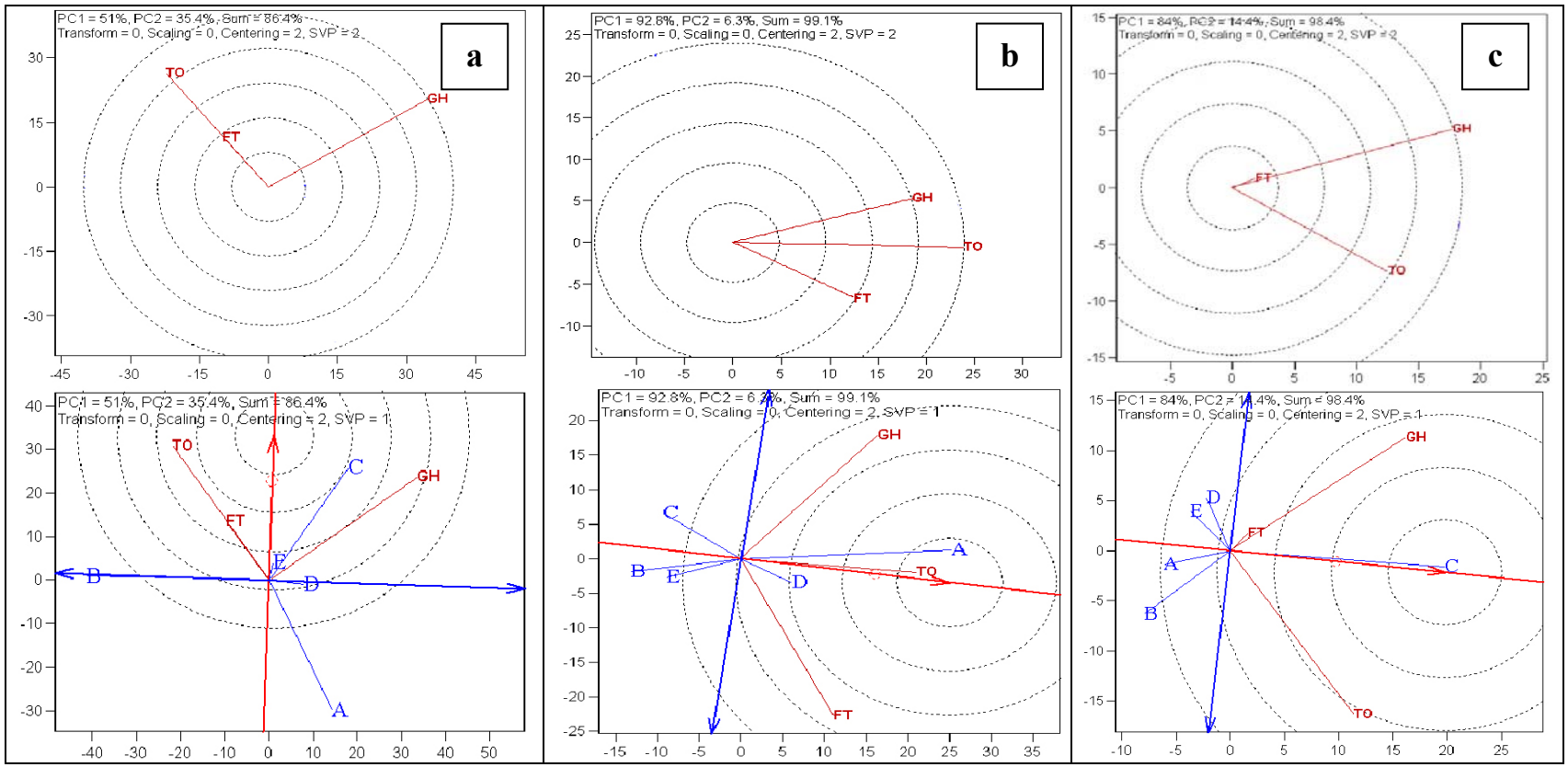


Figure 4.3: GGE biplots based on root yield-related traits (a. root dry mass; b. root:shoot dry mass ratio) in 5 *E. angustifolia* genotypes grown in three environments (GH: UBC horticultural greenhouse; FT: Flying-Two Ranch; TO: Totem Field). The data for each trait is represented by 2 biplots. The upper graph, suitable for comparing growing sites, is environment-metric preserving (as defined in Yan et al. 2002). The bottom graph is genotype-metric preserving (Yan et al. 2002), useful for comparisons between genotypes and their correlation with growing sites. Genotype markers have been omitted from environment-metric preserving biplots for clarity.

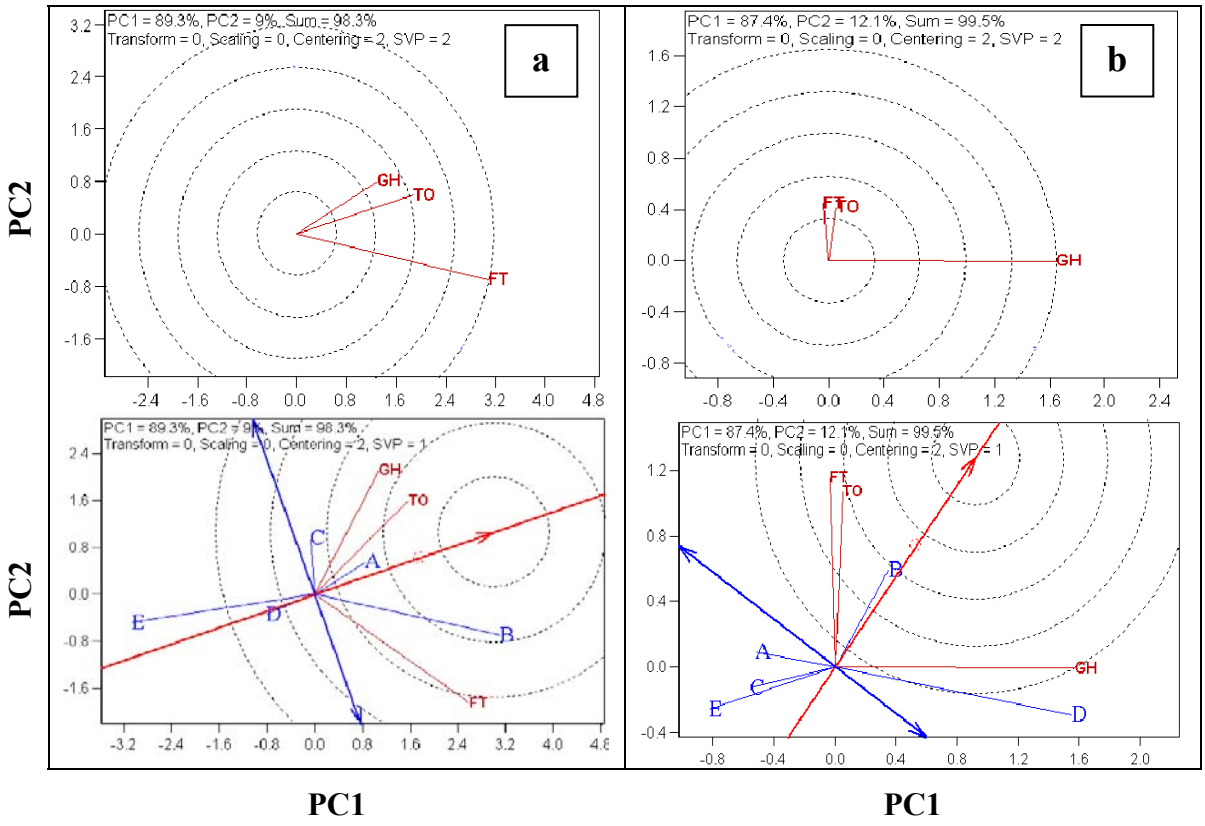


Figure 4.4: Norm of reaction plots for root concentrations of caffeic acid derivative phytochemical markers in *E. angustifolia* roots grown in three growing environments; Flying Two Ranch (ft), Totem field (to), UBC horticultural greenhouse (gh). Chlorogenic acid concentrations are depicted in panel a, cynarin concentrations in panel b, and echinacoside concentrations in panel c.

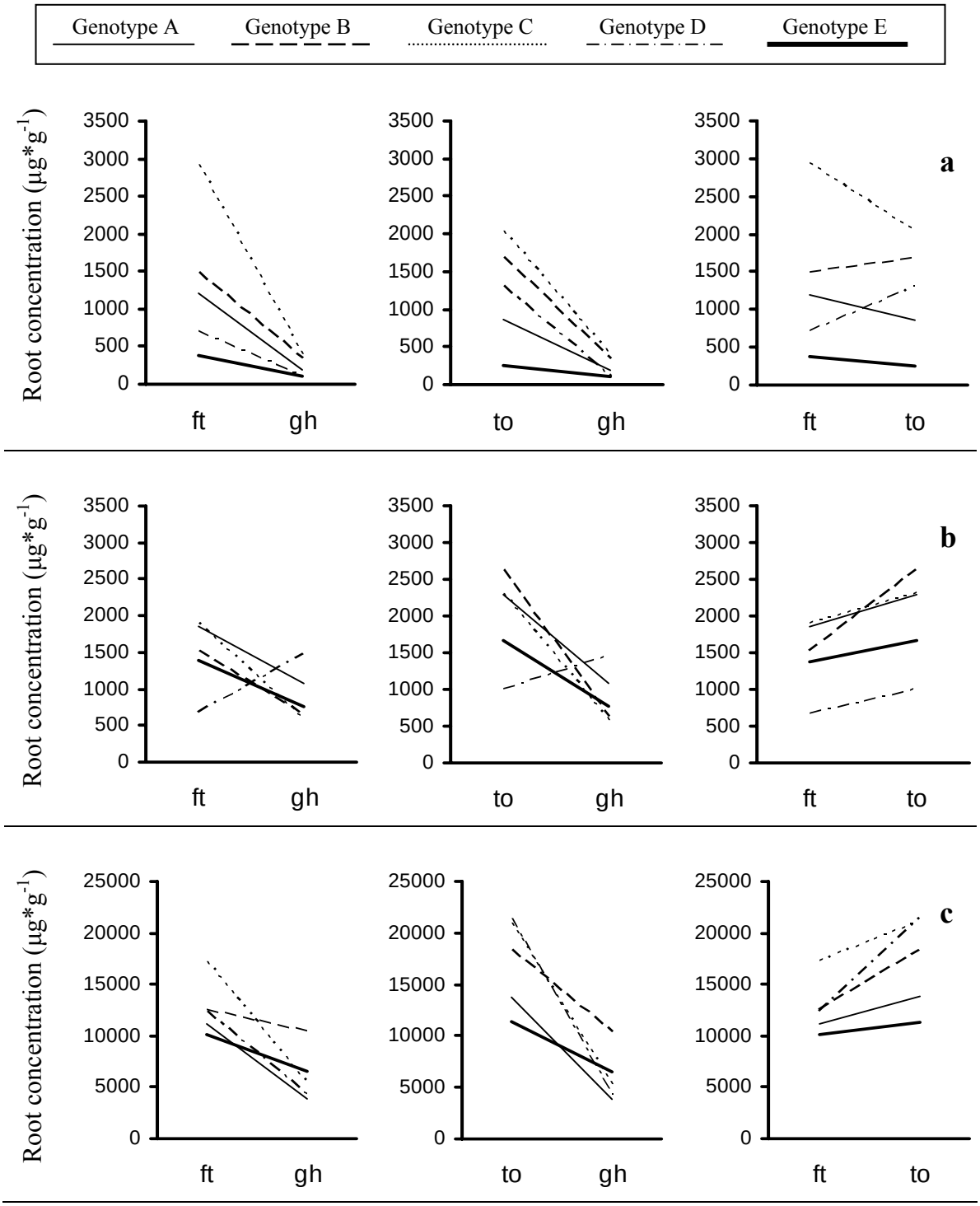


Figure 4.5: Norm of reaction plots for root concentrations of alkamide markers in *E. angustifolia* roots grown in three growing environments; Flying Two Ranch (ft), Totem field (to), UBC horticultural greenhouse (gh). Tetraene concentrations are depicted in panel A, alkamide 11 in panel B, and alkamide 12 in panel C.

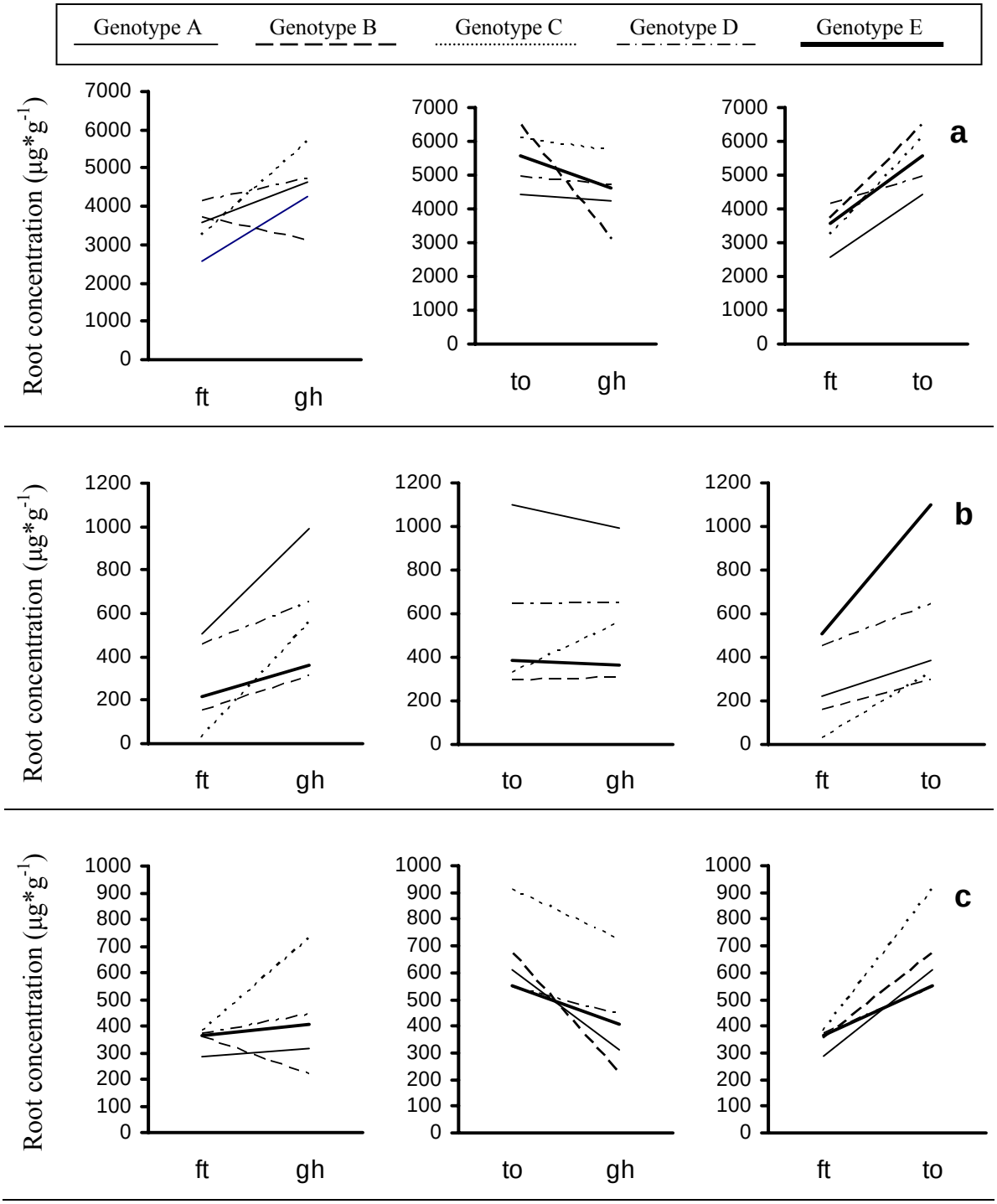


Figure 4.6: Norm of reaction plots for morphological traits associated with *E. angustifolia* roots grown in three growing environments; Flying Two Ranch (ft), Totem field (to), UBC horticultural greenhouse (gh). Root dry mass reaction norms are depicted in panel A, and root:shoot ratios in panel B.

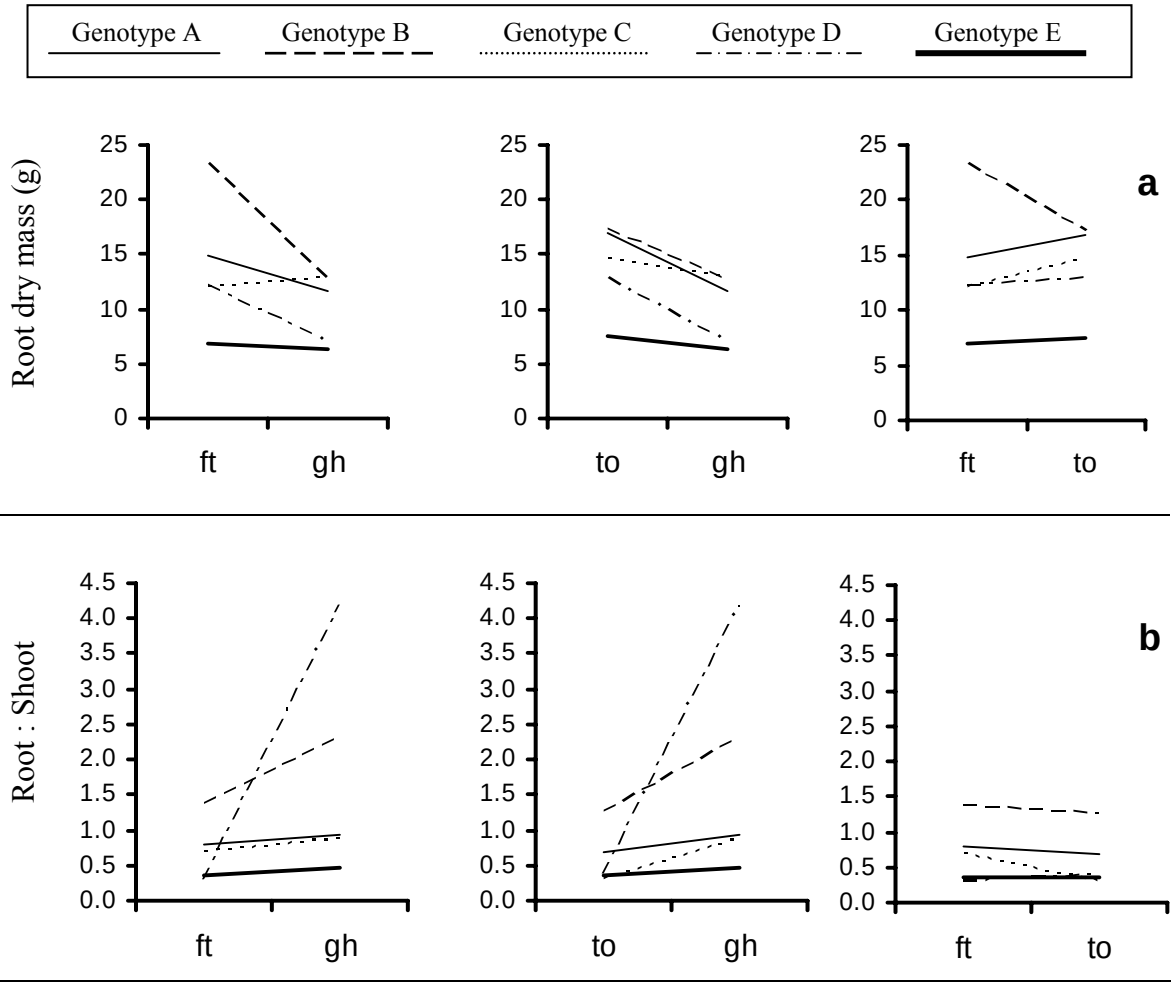
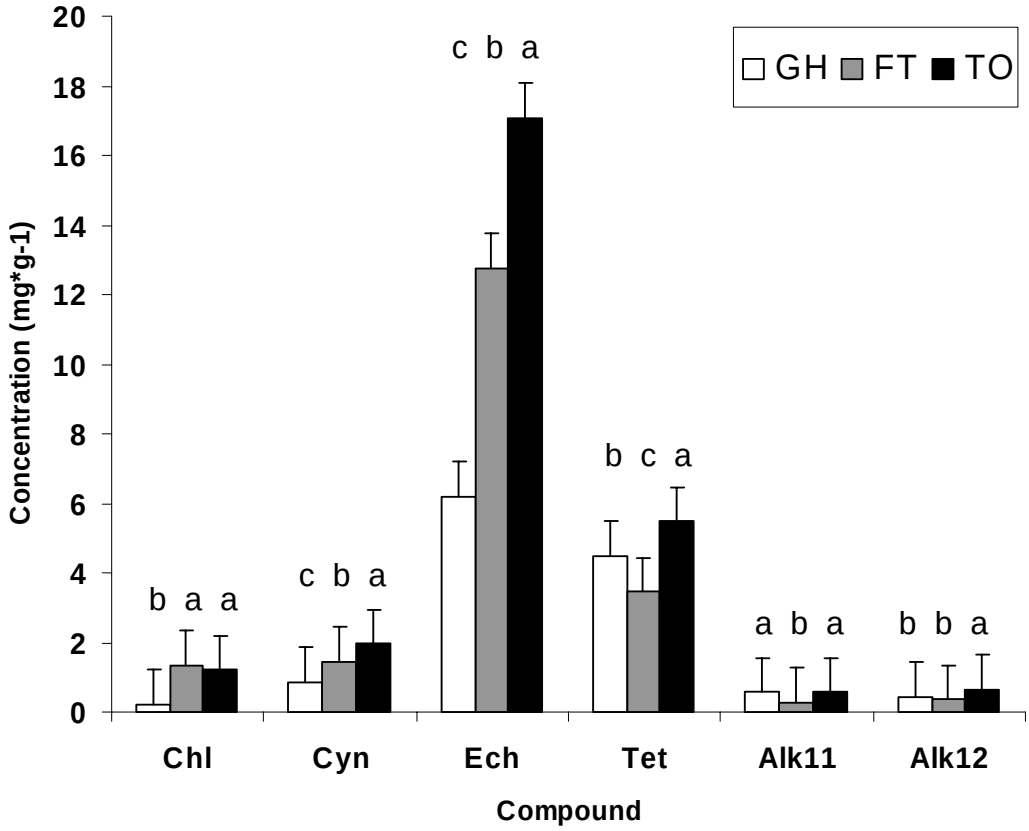


Figure 4.7: Cross-genotypic marginal means of phytochemical root concentrations in *E. angustifolia* plants grown at three different sites; greenhouse (GH), Flying-Two Ranch (FT), and Totem Field (TO). Error bars represent one standard deviation. Letter annotations above sets of bars are representative of statistical difference at the 5% level as determined by analysis of variance and Tukey's pairwise comparison.



4.5 REFERENCES

Awang D.V.C. 1999. Immune Stimulants and Antiviral Botanicals: *Echinacea* and Ginseng. In: J. Janick (ed.), Perspectives on new crops and new uses. ASHS Press, Alexandria, VA. pp.450-456.

Baldwin, I.T. 1999. Inducible nicotine production in native *Nicotiana* as an example of adaptive phenotypic plasticity. *Journal of Chemical Ecology*. 25:3-30.

Barrett B. 2003. Medicinal properties of *Echinacea*: A critical review. *Phytomedicine*. 10:66-86.

Baskauf, C.J., D.E. McCauley, W.G. Eickmeier. 1994. Genetic analysis of a rare and a widespread species of *Echinacea* (Asteraceae). *Evolution*. 48: 180-188.

Bauer, R., A. Khan, H. Wagner. 1988. TLC and HPLC analysis of *Echinacea pallida* and *E. angustifolia* roots. *Planta Medica*. 54: 426-430.

Bergeron, C., J.F. Livesey, D.V.C. Awang, J.T. Arnason, J. Rana, B.R. Baum, W. Letchamo. 2000. A Quantitative HPLC method for the quality assurance of *Echinacea* products on the North American market. *Phytochemical Analysis*. 11:207-215.

Binns S.E., B. Purgina, C. Bergeron, M.L. Smith, L. Ball., Baum, B.R., and Arnason, J.T. 2000. Light-mediated antifungal activity of *Echinacea* extracts. *Planta Medica*. 66:241-244.

Binns S.E., Inparajah, I., Baum, B.R., and Arnason, J.T. 2001. Methyl jasmonate increases reported alkamides and ketoalkene/ynes in *Echinacea pallida* (Asteraceae). *Phytochemistry*. 57: 417-420.

Binns S.E., J.T. Arnason, and B.R. Baum. 2002. Phytochemical variation within populations of *Echinacea angustifolia* (Asteraceae). *Biochemical Systematics and Ecology*. 30:837-854.

Bradshaw, A.D. 1965. Evolutionary significance of phenotypic plasticity in plants. *Advances in Genetics*. 13:115-155.

Bryant J.P., F. S. Chapin, D.R. Klein. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos*. 40: 357-368.

Burrows C. and P. P. Moore. 2002. Genotype x Environment Effects on Raspberry Fruit Quality. *Acta Horticulturae 585: Proceedings of the Eighth International Rubus and Ribes Symposium*. 2: 467-473.

Ceccarelli, S. 1994. Specific adaptation and breeding for marginal conditions. *Euphytica*. 77: 205-219.

Clifford, L.J., M.G. Nair, J. Rana, D.L. Dewitt. 2002. Bioactivity of alkamides isolated from *Echinacea purpurea* (L.) Moench. *Phytomedicine*. 9:249-253.

Dalby-Brown, L., H. Barsett, A.K. Landbo, A.S. Meyer, P. Molgaard. 2005. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on *in vitro* oxidation of human low-density lipoproteins. *Journal of Agricultural and Food Chemistry*. 53(24):9413-9423.

del Moral, R. 1972. On the variability of chlorogenic acid concentration. *Oecologia*. 9: 289-300.

Demarchi, D.A. 1996. An introduction to the ecoregions of British Columbia. Wild life branch, Ministry of Environment, Lands and Parks. Victoria, B.C. 47 pp.

Dickerson, G.E. 1962. Implications of genetic-environmental interaction in animal breeding. *Animal Production*. 4:47-64.

Dufault, R. J., J. Rushing, R. Hassell, B.M. Shepard, G. McCutcheon, B. Ward. 2003. Influence of fertilizer on growth and marker compound of field-grown *Echinacea* species and feverfew. *Scientia Horticulturae*. 98:61-69.

El-Gengaihi, S.E., A.S. Shalaby, E.A. Agina, and S.F. Hendawy. 1998. Alkylamides of *Echinacea purpurea* L. as influenced by plant ontogony [*sic*] and fertilization. *Journal of Herbs, Spices, and Medicinal Plants*. 5:35-41.

Falconer, D.S. 1981. Introduction to quantitative genetics. 2nd ed. Longman. New York.

Gail, M. and R. Simon. 1985. Testing for qualitative interactions between treatment effects and patient subsets. *Biometrics*. 41:361-372.

Gray, D.E., S.G. Pallardy, H.E. Garrett, G.E. Rottinghaus. 2003. Acute Drought Stress and Plant Age Effects on Alkamide and Phenolic Acid Content in Purple Coneflower Roots. *Planta Medica*. 69:50-55.

Institute for Neutraceutical Advancement. 2005. Phenolics in *Echinacea* by HPLC, INA method 106.000. Institute. for Neutraceutical Advancement. Ann Arbor, Mich. July 2005 <http://www.nsf.org/business/ina/echinacea.asp?program=INA>.

Jacobson, M. 1967. The structure of Echinacein, the insecticidal component of American coneflower roots. *The journal of organic chemistry*. 32:1646.

James, J.W. 1961. Selection in two environments. *Heredity*. 16:145-152.

Jones, C.G., S.E. Hartley. 1999. A protein competition model of phenolic allocation. *Oikos*. 86: 27-44.

Kapteyn J., P.B. Goldsbrough, J.E. Simon. 2002. Genetic relationships and diversity of commercially relevant *Echinacea* species. *Theoretical and Applied Genetics.* 105:369-376.

Krizek, D.T., D.H. Clark, and R.M. Mirecki, 2005. Spectral properties of selected UV-blocking and UV-transmitting covering materials with applications for production of high-value crops in high tunnels. *Photochemistry and Photobiology*. 81:1047-1051.

Letchamo, W., Polydeonny, L.V., Gladisheva, N.O., Arnason, T.J., Livesey, J., and Awang, D.V.C. 2002. Factors affecting *Echinacea* quality. In: Jannick and Wipckey (ed.) Trends in New Crops and New Uses. ASHS Press, Alexandria, VA. pp.514-521.

Li, T.S.C. 2000. Medicinal plants: culture, utilization and phytopharmacology. Technomic, Lancaster, PA.

Liang, X., M. Dron, C.C. Cramer, R.A. Dixon, and C.J. Lamb. 1989. Differential regulation of phenylalanine ammonia-lyase genes during plant development and by environmental cues. *The Journal of Biological Chemistry*. 264:14486-14492.

Littell R.C., G.A. Milliken, W.W. Stroup, R.D. Wolfinger. 2002. SAS System for Mixed Models. Cary, NC: SAS Institute Inc.

Marshall D.R., S.K. Jain. 1968. Phenotypic plasticity of *Avena fatua* and *A. barbata*. *American Naturalist*. 102: 457-467.

Moore P.P., C. Burrows, J. Fellman, and D.S. Mattinson. 2002. Genotype x environment variation in raspberry fruit aroma volatiles. *Acta Horticulturae 585: Proceedings of the Eighth International Rubus and Ribes Symposium*. 2: 511-516.

Mpofu A., H.D. Saperstein, and T. Beta. 2006. Genotype and Environmental Variation in Phenolic Content, Phenolic Acid Composition, and Antioxidant Activity of Hard Spring Wheat. *Journal of Agricultural and Food Chemistry*. 54: 1265-1270.

O'Reilly-Wapstra, J.M., B.M. Potts, C. MacArthur, N.W. Davies. 2005. Effects of nutrient variability on the genetic-based resistance of *Eucalyptus globulus* to a mammalian herbivore and on plant defensive chemistry. *Oecologia*. 142: 597-605.

Robertson, A. 1959. The sampling variance of the genetic correlation coefficient. *Biometrics*. 15:469-485.

Schlichting C.D., and D.A. Levin. 1988. Phenotypic plasticity in Phlox. I. Wild and cultivated populations of *P. drummondii*. *American Journal of Botany*. 75: 161-169.

Schlichting, C.D. and M. Pigliucci. 1993. Control of phenotypic plasticity via regulatory genes. *The American Naturalist*. 142: 366-370.

Schmalhausen, I.I. 1949. Factors of evolution: the theory of stabilizing selection. Blackiston, Philadelphia, PA.

Simmonds N.W. 1991. Selection for local adaptation in a plant breeding programme. *Theoretical and Applied Genetics*. 82: 363-367.

Tan S.C. 1980. Phenylalanine ammonia-lyase and the phenylalanine ammonia-lyase inactivating system: effects of light, temperature and mineral deficiencies. *Australian Journal of Plant Physiology*. 7:159-167.

United States Pharmacopeia. 2004. *Echinacea angustifolia* monograph, p.2075-2083. In: USP 28- National Formulary 23. USP Convention: Rockville, Md.

Yan, W., L.A. Hunt, Q. Sheng, and Z. Szlavnics. 2000. Cultivar evaluation and mega-environment investigation based on the GGE biplot. *Crop Science*. 40:597-605.

Yan, W. 2001. GGE Biplot- A Windows application for graphical analysis of multivenvironment trial data and other types of two-way data. *Agronomy Journal*. 93:1111-1118.

Yan, W. 2002. Singular-Value Partitioning in Biplot Analysis of Multienvironment Trial Data. *Agronomy Journal*. 94:990-996.

Zheng, Y., M. Dixon, P.K. Saxena. 2006. Growing Environment and Nutrient Availability Affect the Content of Some Phenolic Compounds in *Echinacea purpurea* and *Echinacea angustifolia*. *Planta Medica*. 72: 1407-1414.

Zucker M. 1972. Light and enzymes. *Annual Review of Plant Physiology*. 23:133-156.

zum Felde, T., H.C. Becker, and C. Möllers. 2006. Genotype X environment interactions, heritability, and trait correlations of sinapate ester content in winter rapeseed (*Brassica napus* L.). *Crop Science*. 46: 2195-2199.

Chapter Five

ANTI-INFLAMMATORY BIOASSAYS TO GUIDE SELECTION OF THERAPEUTICALLY DESIRABLE *ECHINACEA ANGUSTIFOLIA* GENOTYPES[1]

5.1 INTRODUCTION

Plants from the North American genus *Echinacea* are commercially important sources of medicinal natural products employed in the treatment of various common ailments (Blumenthal et al. 2003) with a long history of traditional use by First Nations societies (Borchers et al. 2000). Its present-day use is primarily in mitigating symptoms of upper respiratory tract infections (URTI) caused by viruses (e.g. rhinovirus and influenza virus). This indication is supported by an increasing body of scientific and clinical evidence (Goel et al. 2004, Goel et al. 2005, Schoop et al. 2006, Shah et al. 2007). There is mounting evidence that various biological activities attributed to *Echinacea* (Bauer & Wagner 1991), other than immunostimulation (Freier et al. 2003, Miller 2005, Morazzoni et al. 2005), may also be important to its efficacy in treating URTIs (Binns et al. 2002, Hudson et al. 2005). Specifically, the anti-inflammatory action

[1] A version of this chapter will be submitted for publication. Boucher, A.Y., S.E. Cowan, S. Katz, and R. Harris. Anti-inflammatory bioassays to guide selection of therapeutically desirable *Echinacea angustifolia* genotypes.

of *Echinacea* extracts, which has been demonstrated in a number of *in vitro* and *in vivo* models (Speroni et al. 2002, Sharma et al. 2006, Lalone et al. 2007) is believed to be a significant factor in mediating URTI symptoms.

The phytochemical markers used to confirm identification and quality in *Echinacea* products include alkamides and caffeic acid derivatives (CADs are responsible, at least in part, for the biological activities attributed to *Echinacea* (Bauer & Wagner 1991, Woelkart & Bauer 2007). Current research efforts are aimed at uncovering their relative importance to the therapeutic properties of *Echinacea* along with likely synergies among them (Dalby-Brown et al. 2005, Thygesen et al. 2007). Of these *Echinacea* marker compounds, *E. angustifolia* root products are characterized by two phenolics - echinacoside and cynarin, as well as high concentrations of the isomeric mixture of the two main alkamides commonly referred to as the tetraenes. Studies have demonstrated genotypic (Murch et al. 2006) and environmental (Zheng et al. 2006) effects on the accumulation of some of these compounds in other species of *Echinacea*. However, there are no reported assessments of how the marker phytochemicals commonly used to assess *E. angustifolia* quality might correspond to differences in biological activity of whole root extracts.

There has been considerable advancement lately in elucidating the mechanisms underlying the anti-inflammatory properties of *Echinacea*. *E. angustifolia* extracts and isolated alkamides have been shown recently to reduce the production of the inflammatory vaso- and bronchodialating molecule prostaglandin(PG)E2 (Woelkart et al. 2006, Hinz et al. 2007, Lalone et al. 2007). Hinz et al. (2007) specify that PGE2 suppression by *Echinacea* extracts likely results from the ability of alkamides to interfere

with cyclooxygenase (COX)-2 activity rather than inhibiting its expression. Another demonstrated mechanism involves alkamides as cannabimimetic agonists binding to cannabinoid receptor CB2 (Gertsch et al. 2004, Raduner et al. 2006), which are known to be involved in the inflammatory response (Klein 2005). CADs, which are major elements of the phenolic fraction in *E. angustifolia* extracts, also have demonstrated anti-inflammatory properties *in vivo* (dos Santos et al. 2006, Speroni et al. 2002) and *in vitro* (da Cunha et al. 2004, Calixto et al. 2004) with some observed effects on cytokine and chemokine production in cell cultures (Krakauer 2002). Although strong evidence to support an underlying mechanism is still lacking, their effect on the inflammatory response may be related to their well established antioxidative properties (da Cunha et al. 2004) and their ability to scavenge nitric oxide radicals (Xiong et al. 2000).

Inflammation is characterized by a signaling cascade mediated by key molecules, including cytokines. Paradoxical effects of *Echinacea* extracts and their isolated constituents on cytokine production have repeatedly been demonstrated in different cell models. Pro-inflammatory cytokine suppression is generally observed in cells having been stimulated with inflammatory agents (e.g. virus and endotoxin) and then treated with *Echinacea* extracts (Krakauer 2002, Gertsch et al. 2004, Sasagawa et al. 2006, Senchina et al. 2006a, Sharma et al. 2006). Conversely unstimulated cells have shown increased levels of certain cytokine proteins (Hwang et al. 2004, Raduner et al. 2006, Senchina et al. 2006b, Sharma et al. 2006) and/or cytokine mRNA (Randolph et al. 2003, Brovelli et al. 2005) in response to *Echinacea* treatment.

Assays measuring pro-inflammatory cytokines like interleukin (IL)-6 and IL-8 in cell models are valuable tools in assessing the anti-inflammatory potential of isolated

compounds and plant extracts (Calixto et al. 2004). Rhinovirus infection of human bronchial epithelial cells (HBEC) has been shown to trigger an inflammatory response marked by significant increases in pro-inflammatory cytokine levels using the immortalized BEAS-2B cell line (Sharma et al. 2006). HBEC are pertinent models to evaluate the effect of *E. angustifolia* on inflammation given their proximity to the primary indication of *Echinacea* preparations and the fact that they have been shown to express functional CB2 proteins (Gkoumassi et al. 2007), which, as previously stated, appear to be important in the alkamides' mode of action.

To date, breeding efforts aimed at improving medicinal *Echinacea* crop quality have focused primarily on phytochemical evaluations of selected genotypes (Letchamo et al. 2002). Using therapeutic activity as a selectable trait in genotypic selection is a novel approach to breeding medicinal *Echinacea* with only scarce published reports describing any genotypic or phenological effects on the therapeutic qualities of the plant. Brovelli et al. (2005) used immune system gene expression assays to demonstrate the effect of plant developmental stage on the immunomodulating properties of *E. purpurea* herb extracts. Other papers have compared genetic effects on an inter-specific level showing clear differences on biological activities ascribed to *Echinacea* such as human cytokine response (Randolph et al. 2003, Senchina et al. 2005, Senchina et al. 2006b), antioxidant activity (Hu & Kitts 2000), and phagocytosis (Bauer & Wagner 1991).

This study was designed to determine whether genotypic and environmental differences in phytochemical concentrations of *E. angustifolia* root extracts would translate into significant differences in their anti-inflammatory potential, measured as relative interleukin secretion in BEAS-2B cells following rhinovirus challenge. To my

knowledge, this is the first study comparing differences among phytochemically-distinct genotypes selected within the species *E. angustifolia*. In addition, the two marker compounds found at the highest concentrations in ethanolic extracts of *E. angustifolia* roots, namely echinacoside and dodeca-2(E),4(E),8(Z),10(Z)-tetraenoic acid isobutylamide were used in isolation to verify their anti-inflammatory activity in the experimental cell model.

5.2 MATERIALS AND METHODS

Plant material

Plants were grown in a greenhouse from *E. angustifolia* achenes (taxonomically-identified in November 2002 by the Saskatchewan Herb Research Program, U. Sask.) obtained from a cultivated population in Savonah, British Columbia. Five different plants, representing 5 different genotypes, were selected from 230 in the greenhouse population, on the basis of morphological differences, healthy appearance, and responsiveness to vegetative propagation. Ten clonal plantlets were produced per genotype through vegetative propagation by root cuttings (as described in Chapter 4), and were transplanted to 3 different growing environments: 1 greenhouse (UBC horticultural greenhouse) and 2 field sites (Totem field at UBC and Flying Two Herb Farm). Clones were planted in two rows with 30 cm inter-plant spacing and 150 cm inter-row spacing, except in the greenhouse where the inter-row spacing was reduced to 30 cm to accommodate all of the plants on a single bench. The plants were arranged in a complete randomized block design with the two rows being divided into 5 blocks and individual plants from the different genotypes being randomly assigned within the blocks. The total

sample size was 150 plants (50 per site). All of the plants were harvested between the 15th and the 24th of October, 2005 and left to dry whole in darkness in an open room (≈26 ºC, RH ≈30%) for up to 15 days or until reduction in mass due to moisture loss was no longer recordable. Further details on growing sites can be found in Chapter 4.

Root extracts

The whole dried root system of each plant was chopped up in an Osterizer blender fitted with a 250 mL glass jar. Afterwards, the root material was ground to a fine powder with a mortar and pestle and sieved through a 40 mesh (US Standard) sieve. Powdered root material from each plant was extracted separately using the following protocol based on Bergeron et al. (2000). In 15 mL Falcon ™ tubes (Becton Dickinson Labware, Franklin Lakes, NJ), ca. 0.5 g of powder was weighed out precisely on a Sartorius 1602 MP8 analytical scale (Goettinburg, Germany). Ten millilitres of 70% EtOH was added to the powder and sonicated for 5 minutes in a Branson Model B-220 Ultrasonic Cleaner (Danbury, Conn.). The tubes were placed in a Sorvall GLC-1 benchtop centrifuge and spun for 5 min at 2500 rpm. The supernatant was poured off into a 50 mL Falcon™ tubes. The extraction process was repeated 2 more times and the pooled supernatants were brought up to exactly 30 mL. The extracts were all stored at –20ºC until they were used for the cell assays.

HPLC analysis of phenolics and alkylamides in E. angustifolia ethanolic extracts

Aliquots of the extracts were filtered through 0.22 μm PVC filters before injection into the HPLC instrument. Phenolic compounds were analyzed using HPLC according to

a method validated by the Institute for Neutraceutical Advancement (INA 2005). The stationary phase consisted of a Cosmosil 5C18-AR-II (4.6 mm x 150 mm, 5 μm) column (Nacalai Tesque, Kyoto, Japan). The mobile phase consisted of 0.1% phosphoric acid in nanopure water (A) and acetonitrile (B) in a changing gradient as follows: initial conditions at 90% A were gradually reduced to 78% over 12 min, then further reduced to 60% over 2 min. This proportion was held for 0.5 min followed by a 4 min post run time to allow system to return to initial conditions. The flow rate was 1.5 mL/min and detection was done at 330 nm. The injection volume was 5.00 μL and the column was maintained at 35.0ºC in a column oven equipped with a thermostat. Further HPLC instrument details are provided below.

For the analysis of alkamide compounds, the HPLC method used was based on the United States Pharmacopeia method for determination of the concentrations of dodecatetraenoic acid isobutylamides in *E. angustifolia* described in USP-28 NF-23 (USP 2004). A Prodigy 5u ODS3 100A (4.6 mm x 250 mm, 5 μm) column (Phenomenex, Torrance, CA) was used. The elution was a 40 min isocratic run of a mobile phase consisting of nanopure water and acetonitrile (55:45). The flow rate was 1.5 mL/min and detection was made at 254 nm. Injection volume was 23.0 μL and the column thermostat was set to 30ºC.

Both phenolic and alkamide determinations were performed on an Agilent 1100 series instrument equipped with a photodiode array detector controlled by the Chemstation Chromatography Data system software (Agilent Technologies, Santa Clara, CA). Peak identification was based on retention times and UV spectra comparisons with isolated standard compounds obtained from Chromadex (Santa Ana, CA). The standards

used for alkamide measurements were the following: undec-2-ENE-8,10-diynoic acid (Alk12), dodec-2-ENE-8,10-diynoic acid (Alk14), dodeca-2-(E),4(E),8(Z),10(Z)-tetraenoic acid (tetraene), and dodeca-2(E),4(E)-dienoic acid (Alk11). Total measured alkamide (AlkTo) concentrations were taken as the sum of concentrations of all alkamides for which external standards, as previously listed, were obtained. Alk11 and Alk14 were not individually reported as the majority of extracts had only trace to undetectable concentrations of these two alkamides. For measurements of phenolics, the following standards were used: chlorogenic acid, cynarin, and echinacoside.

Cell culture and virus

The assays were performed on BEAS-2B cells, a line of human bronchial epithelial cells immortalized by infection with Ad12-SV40 virus (Reddel et al. 1988). The cells were originally obtained from American Type Culture Collection (ATCC, Manassas, VA) and had been cultured in a full media consisting of 1:1 DMEM/F-12 (v/v) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, Burlington, ON) and kept in a 5% CO^2-95% air atmosphere at 37ºC. They were passaged on a weekly basis, when confluent, and fed twice a week. The bioassays were set-up by passaging confluent cells from their regular culture flasks into non-pyrogenic sterile 6-well tissue culture plates (Sarstedt, Newton, NC). Three ml of cell suspension in full media was added to each well. Cells used for the *Echinacea* ethanolic extract assays were from passage numbers 23 and 24. Those used in the pure compound assays were from passage number 48. The human rhinovirus type 1A (RV 1A) was originally obtained from

ATCC. The virus was propagated at 34ºC in H1HeLa human cervical cells, also from ATCC, and assayed according to published methods (Sharma et al. 2006).

Rhinovirus infection and treatment of cells

Supernatant culture media was aspirated 48 hr after passage when cells had reached 90-100% confluence and replaced with DMEM/F12 (0% FBS) media mixed with the required quantity of Rv 1A stock to obtain an moi = 1.0 pfu/cell. Media without RV 1A stock was added to rhinovirus-free control wells. Cells were incubated for 1 hr at 37ºC and swirled gently every 15 minutes. The supernatant was removed and 3 ml of full culture media was added along with 50 µl of *E. angustifolia* extracts or 70% ethanol for the vehicle controls. Single compound assays were done with the two marker compounds commonly found in highest concentrations in *E. angustifolia* roots; echinacoside and dodeca-2(E),4(E), tetraenoic acid isobutylamide (tetraene). Both pure compounds were obtained from Chromadex (Santa Ana, CA). The standards were diluted in 70% ethanol to give final treatment concentrations of 2, 10, and 100 µg/ml for echinacoside and 0.35, 0.7, 1.4, 2.8, 5.6, and 28.2 µg/ml for tetraene. For each of these concentrations, 60 µl of the appropriate stock solution was added in each of the wells with the culture media. Final EtOH concentration in culture media for all of the treatments was below 2% of total volume. The cells were incubated in darkness for 48 hr at 37ºC and 5% CO_2 with occasional gentle swirling by hand. Following incubation with *E. angustifolia* extracts, pure standards, or 70% ethanol only, the supernatant culture media was collected, spun for 10 min at 2000 rpm to eliminate cell debris and transferred to clean 1.5 ml microtubes in 0.5 ml aliquots and stored at –70ºC until ELISA analysis.

Cytokine measurements by ELISA

Secreted IL-6 and IL-8 protein concentrations in culture media were determined by ELISA according to manufacturer's instructions. BD OptEIA™ Human IL-6 and Human IL-8 kits were obtained from BD Biosciences (San Diego, CA). Each sample was measured in duplicate. The plates were read at a wavelength of 450 nm on a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical analyses

All of the statistical analyses described below were performed with the SPSS statistical software package (Release 13.0, SPSS Inc. Chicago, IL). Significance of effect of main factors (genotype and growing site) and interactions, on phytochemical traits and interleukin responses were determined by two-way analysis of variance (ANOVA). All of the independent variables were considered as fixed. When overall effects were found to be significant, differences between individual treatment levels were determined by Tukey HSD multiple comparisons. Alpha levels for significance were preset at 0.05. Principal component analysis was done on the phytochemical traits data using the 'Factor Analysis' procedure in SPSS with 'Principal components' as the extraction method. To facilitate the interpretation of the loadings matrix, the orthogonal rotation procedure varimax was applied. Stepwise multiple linear regressions were performed to determine relationships between interleukin responses and principal component scores. F-statistic probabilities of 0.05 and 0.10 were used for entry and removal, respectively, of individual predictors in the stepping method. As recommended in Graham (2003), all of the principal components on which at least one of the phytochemical traits had a high loading

were used in the multiple regression analyses even though some factors had eigenvalues below unity . The significance of effect of pure marker compound treatments on interleukin response was determined by one-way ANOVA followed by Tukey HSD multiple comparisons with the same alpha levels as stated above.

5.3 RESULTS

Phytochemical variations in extracts and their effect on relative interleukin levels

Genotype, growing environment and the interaction of these two main factors had significant effects on the production of *E. angustifolia* marker compounds with two exceptions (Table 5.1): Neither cynarin nor AlkTo concentrations were significantly affected by genotype across the three growing environments. Given the significant GXE interactions, the data do not support any single genotype(s) having superior performance across the 3 different environments. However, a comparison of the marginal concentration means for the 5 genotypes (i.e. averaged across the three sites) reveals that genotype A was lower for all of the reported alkamides. Similarly, the marginal means for the 3 sites show that all of the CADs were lowest in the treatments obtained from greenhouse-grown plants and that the highest levels of all alkamides were obtained from the Totem Field site. In fact, the Totem Field site produced the highest overall levels for all of the markers with the exception of chlorogenic acid.

Cytokine response was not significantly affected by either of the main factors, nor by their interaction (Table 5.2). The following trends, however, were notable. Firstly, the two measured interleukins appear to be reacting differently to *E. angustifolia* extracts.

In the case of IL-8, more than half of the treatments (9 of 15) have upper limits of their mean's 95% CI below a level of 1 (relative to vehicle control). Most treatments, in the case of IL-6, have 95% CI overlapping the relative response of 1 (Figure 5.1). These results suggest that *E. angustifolia* treatment in this *in vitro* model tend towards inhibition of IL-8 production whereas IL-6 secretion is not affected or slightly stimulated. Secondly, upper limits of 95% CI for mean IL-8 secretion (relative to vehicle control) were consistently below the relative level of 1 in cell response to treatment with Genotype C extracts (Figure 5.1).

PCA on phytochemical markers and their relationship with interleukin secretion

The PCA revealed latent variables represented by a number of factors (Table 5.3). Only the first two principal components had Eigenvalues over 1 and accounted for 77.4% of the variance (PC1=50.3%, PC2=27.1%). Based on loading factors for the different phytochemical markers, PC1 is representative of the variance contributed by alkamides, specifically Alk8, Alk12 and AlkTo. Alk9 had the highest loading in PC2 (0.909), indicating that this principal component, like PC1, is also strongly related to alkamide variance. The phenolic markers, namely echinacoside, chlorogenic acid and cynarin, individually had the highest loading in the next 3 principle components: PC4, PC5, and PC3, respectively (Table 5.3). PC6 and PC7 accounted for less than 1.3% of variance and none of the phytochemical markers had loading factors higher than 0.313. For this reason, these components were omitted from Table 5.3 and subsequent analyses.

Multiple regressions were done to investigate possible relationships between relative interleukin secretion and principal component scores that reflect variance in

marker compound concentrations in the treatments. Based on the recommendation from Graham (2003) the first 5 principle components were included as possible predictors, even though only the first two had eigenvalues superior to 1. Multiple regressions on all of the pooled data points showed that both IL-6 and IL-8 secretion were significantly and negatively related to PC1, the principal component that represented alkamide-related variance PC1 (Table 5.4). In the case of IL-6, PC3, the component related to cynarin was a significant and negative predictor of relative cytokine secretion. On the other hand, IL-8 was negatively affected by two different components, one related to chlorogenic acid variance (PC5) and the other related to the second isomeric tetraene Alk9 (PC2). However, coefficients of determination show that the individual explanatory variables as well as the full models explain very little of the observed variability in the response variables ($R^2 < 10\%$ for both interleukins

Treatment of challenged cells with two isolated, pure E. angustifolia marker phytochemicals

Significant reductions in secretion of both interleukins were observed with various concentrations of tetraenes and echinacoside (Figure 5.2). The effect of tetraenes however was much more pronounced where, at the highest treatment concentration (28.2µg/mL), IL-6 and IL-8 levels were reduced to levels comparable to un-challenged vehicle controls (Figures 5.2A-1&2). Although the range of concentrations used in these experiments did not allow for the determination of reliable dose-dependent relationships, there is a clear downward trend in IL-6 and IL-8 levels with increasing concentrations of tetraene. A similar trend is observed for echinacoside, albeit with an apparently lesser

rate of decline. The highest concentration (100µg/mL) however did not significantly reduce secretion in the case of IL-8 (Figure 5.2B-2).

5.4 DISCUSSION

This study clearly demonstrates that phytochemical distinctiveness of an *E. angustifolia* extract is not necessarily reflected in its therapeutic potential, at least based on anti-inflammatory activity. My findings do offer some support to the value of genotype selections based on currently used *E. angustifolia* phytochemical markers, given the demonstrated activity of the isolated compounds (Figure 5.2) along with the significant relationships demonstrated between interleukin response and concentrations of alkamides and certain phenolic markers (i.e. cynarin and chlorogenic acid) in whole extracts (Table 5.4).

In my experiments, the difference observed between the response to *E. angustifolia* extracts by IL-6, a key inflammatory molecular signal, and by IL-8, a potent chemoattractant of immune cells, is similar to findings obtained in other experiments. Using LPS-stimulated human whole blood cells, Raduner et al. (2006) showed reductions of IL-8 expression of around 20% relative to control cells with treatments of Alk9 (at a concentration of 5000nM) and Alk12 (at 5 & 50nM). IL-6 response was mostly unchanged or tended towards a slight increase when cells were treated with Alk9, Alk11, and Alk12.

The results in Figure 5.2 suggest that a stronger response may have been observed in the experiments where whole extracts were used (i.e. Figure 5.1) had the treatment concentrations been higher (i.e. a 10 fold increase). The *E. angustifolia* extract

concentrations were chosen with two main considerations in mind. Firstly, they were intended to reflect previously reported non-cytotoxic active concentrations in a similar *in vitro* model (Sharma et al. 2006). Secondly, the choice of concentration was intended to approximate a range of biologically relevant levels of alkamides. Matthias et al. (2005) found mean and maximum peak concentrations (Cmax) for total alkamides of 0.34μg/ml and 1.30μg/ml respectively in plasma from human subjects following a single oral administration of 4 tablets equivalent to a dose of 43.68mg of total alkamides. From their results, they estimated a minimum percent uptake of 1.9% based on calculated total plasma volumes. Similarly, Woelkart et al. (2005) observed a mean plasma Cmax value of 0.02μg/ml for total alkamides after 30 minutes following oral administration of an *E. angustifolia* root ethanolic extract containing 4.6mg of the major alkamides. These findings demonstrate that the alkamide concentrations obtained through whole extract treatment were well within the range or higher than those obtained in bioavailability studies.

Echinacea CADs, on the other hand, have very little to no expected bioavailability following ingestion according to some studies (Matthias et al. 2004, Matthias et al. 2005). For this reason, CAD concentrations present in the whole extract treatments are likely much higher than one would expect following normal oral administration. Based on the previously published reports cited above, the higher levels of phenolic compounds needed to achieve the strongest level of cytokine inhibition in my experiments (Figure 5.2) would be difficult to attain *in vivo* following oral administration.

Despite the apparent lack of phenolic marker bioavailability in human subjects, the importance of their effectiveness in reducing key inflammatory signals (Table 5.4 &

Figure 5.2) cannot be discounted for *Echinacea* preparations intended for topical application or even intravenous administration. Conclusions drawn from the ethanolic extract experiments pertaining to any causative effects of CADs on cytokine inhibition must, however, be interpreted cautiously. As higher levels of certain secondary metabolites may be a reflection of the source plant's physiological state, significant relationships between measured CADs and cytokine suppression may have resulted from correlations with other unmeasured bioactive metabolites that respond similarly to environmental and/or physiological cues. It is, however, important to note that other studies have also attributed anti-inflammatory properties to various CADs (da Cunha et al. 2004), including those found in *E. angustifolia* roots such as echinacoside (Xiong et al. 2000, Speroni et al. 2002) and chlorogenic acid (dos Santos et al. 2006).

Mechanisms involved in alkamide mediation of cytokine release

My results add to a rapidly increasing body of evidence supporting the role of *Echinacea* alkamides in the modulation of immune-related processes including inflammation. Inverse relationships were observed between alkamide concentrations and inflammatory interleukin secretion in both whole ethanolic extract treatments and isolated tetraene treatments. Alkamide modulation of cytokine secretion is reportedly regulated by various different proposed mechanisms, among which, selective binding of alkamides to the CB2 receptor has been convincingly demonstrated in non-activated cell models (Gertsch et al. 2004, Raduner et al. 2006). The expression of CB2 receptors has been shown in human bronchial epithelial cells along with their ability, when stimulated, to diminish TNF-α induced release of IL-8 (Gkoumassi et al. 2007).

Nevertheless, it appears that interleukin inhibition as observed in my study is likely regulated by other mechanisms. Raduner et al. (2006) argue that, contrary to the cytokine-stimulating activity of alkamides observed in non-stimulated cells, CB2 independent mechanisms must play a role in the cytokine-inhibitory activity because Alk12, which was not one of the cannabinomimetic alkamides, inhibited LPS-induced cytokine release at levels similar to other alkamides that have selective affinity to cannabinoid receptors. Other hypothesized modes of action explaining the anti-inflammatory properties of *Echinacea* extracts and their constituents include interference with COX-2 activity (Müller-Jakic et al. 1994, Hinz et al. 2007) and 5-lipoxygenase inhibition (Müller-Jakic et al. 1994, Merali et al. 2003). These two inducible enzymes, critical to the inflammatory response, have demonstrated interactions with the production of IL-6 (Thivierge & Rola-Pleszczynski 1994, Ho et al. 2004) and IL-8 (Aoki et al. 1998, Singh et al. 2006) in different cell models

Conclusion

This study provides additional support for genotypic selections of *E. angustifolia* based on high alkamide production. However, these results do not support the assumption that significantly higher phytochemical marker concentrations lead to significant differences in anti-inflammatory activities of ethanolic extracts of *E. angustifolia.* Given the enduring uncertainty surrounding the relative importance of marker compounds to the biological activity of *E. angustifolia*, a bioassay-based approach to genotype selection for the improvement of *E. angustifolia* therapeutic qualities is essential. Further work must be focused on developing refined bioassays that

are able to reliably estimate the influence of genotype and growing environment on the various reported biological activities of *Echinacea*.

Table 5.1: Environmental (E) and genotypic (G) effects on E. angustifolia marker compound concentrations in ethanolic extracts as reflected by differences among mean concentrations in treatments. Letters below mean concentrations indicate statistical differences among genotype-field combinations as determined by Tukey HSD multiple comparison tests.

	Mean marker concentration in treatment (µg/mL)															p-values for sources of variation		
Marker	Genotype A			Genotype B			Genotype C			Genotype D			Genotype E					
	To	Ft	Gh	To	Ft	Gh	To	Ft	Gh	To	Ft	Gh	To	Ft	Gh	G	E	GxE
n	9	9	8	10	10	11	12	11	8	10	11	5	7	8	8			
Alk 8	1.14 bcde	0.64 f	1.14 bcde	1.82 a	0.96 cdef	0.81 def	1.26 bcd	0.75 ef	1.20 bcde	1.36 bc	1.12 bcde	1.16 bcde	1.52 ab	0.93 cdef	1.17 bcde	0.005	0.000	0.000
Alk 9	0.14 bcd	0.08 d	0.13 bcd	0.18 bc	0.10 cd	0.08 d	0.36 a	0.20 b	0.33 a	0.13 bcd	0.12 bcd	0.13 bcd	0.16 bcd	0.09 cd	0.13 bcd	0.000	0.000	0.000
Alk 12	0.18 abc	0.08 d	0.09 cd	0.22 a	0.09 cd	0.07 d	0.24 a	0.11 bcd	0.19 ab	0.16 abcd	0.13 bcd	0.12 bcd	0.17 abc	0.10 cd	0.12 bcd	0.000	0.000	0.005
Alk tot	1.81 abc	0.96 d	1.67 abcd	2.35 a	1.22 cd	1.05 d	2.06 ab	1.09 d	1.91 abc	1.86 abc	1.59 bcd	1.56 bcd	2.05 ab	1.23 cd	1.58 bcd	0.340	0.000	0.000
Chl	0.25 bcde	0.34 bcd	0.05 e	0.41 b	0.41 b	0.1 cde	0.48 b	0.83 a	0.2 bcde	0.36 bc	0.21 bcde	0.03 e	0.06 de	0.1 cde	0.03 e	0.000	0.000	0.000
Cyn	0.66 a	0.51 ab	0.29 ab	0.67 a	0.42 ab	0.18 b	0.53 ab	0.55 ab	0.2 b	0.29 ab	0.17 b	0.52 ab	0.45 ab	0.36 ab	0.22 b	0.141	0.000	0.003
Ech	3.92 bcd	3.28 cdef	1.05 h	5.09 ab	3.63 bcde	2.91 defg	5.24 ab	4.99 abc	1.74 fgh	5.76 a	3.71 bcde	1.32 gh	3.26 cdef	2.74 defgh	1.97 efgh	0.000	0.000	0.000

Table 5.2: Interleukin response to phytochemically characterized extracts of *E. angustifolia* does not vary according to growing environment or genotype of plants; ANOVA table of treatment effects on measured interleukin concentrations

Response	Source of variation	df	Mean Square	F	p
IL-6	Genotype	4	0.040	0.512	0.727
	Site	2	0.184	2.373	0.097
	Genotype X Site	8	0.055	0.707	0.685
	Total	135			
IL-8	Genotype	4	0.069	1.198	0.315
	Site	2	0.055	0.961	0.385
	Genotype X Site	8	0.085	1.536	0.152
	Total	135	0.057		

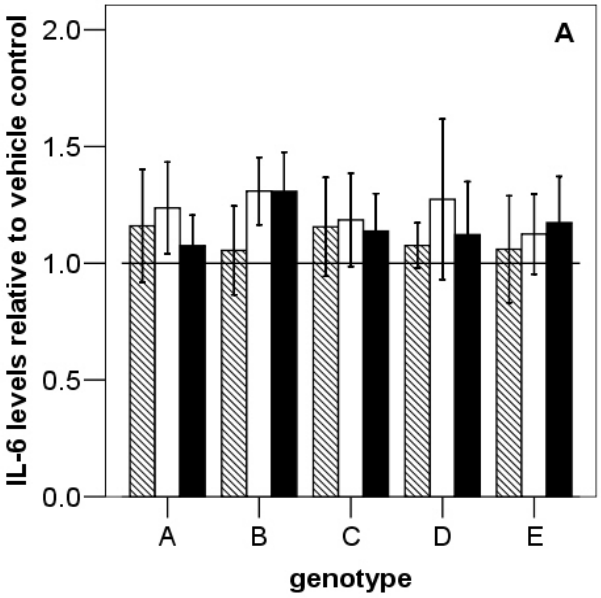
Table 5.3: Principal component analysis loadings matrix of *E. angustifolia* phytochemical trait concentrations in extracts used for treating cells. Eigenvalues and percentages of variance explained by each principle component are indicated in parentheses. High loading factors are in bold.

	Principal loading factors				
	PC1 (3.52; 50.3%)	PC2 (1.89; 27.1%)	PC3 (0.71; 10.2%)	PC4 (0.54; 7.7%)	PC5 (0.23; 3.3%)
Alk 8	**0.980**	0.127	0.031	0.091	-0.070
Alk 9	0.384	**0.909**	0.006	0.064	0.147
Alk 12	**0.746**	0.534	0.071	0.236	0.019
Alk tot	**0.933**	0.329	0.044	0.068	-0.059
ECH	0.179	0.086	0.189	**0.905**	0.323
CHL	-0.120	0.138	0.252	0.339	**0.888**
CYN	0.060	0.012	**0.965**	0.160	0.200

Table 5.4: Multiple regression models demonstrating relationships between relative IL-6 and IL-8 secretion in BEAS-2B cells and principle components of measured *E. angustifolia* phytochemical markers (n=137).

Response	Predictor	β	SE	p	r^2 (R^2)
IL-6	PC 1	-0.064	0.023	0.006	0.052
	PC 3	-0.060	0.023	0.010	0.046
	Total	…	…	0.001	0.099
IL-8	PC 1	-0.047	0.021	0.023	0.036
	PC 5	-0.045	0.020	0.029	0.027
	PC 2	-0.041	0.020	0.047	0.033
	Total	…	…	0.004	0.098

Figure 5.1: Relative interleukin levels from cells treated with extracts from genetically distinct plants grown in different environments. Error bars represent 95% confidence intervals. Hatched and white bars represent field grown plants from Totem Field and Flying-Two respectively. Black bars represent greenhouse grown plants.



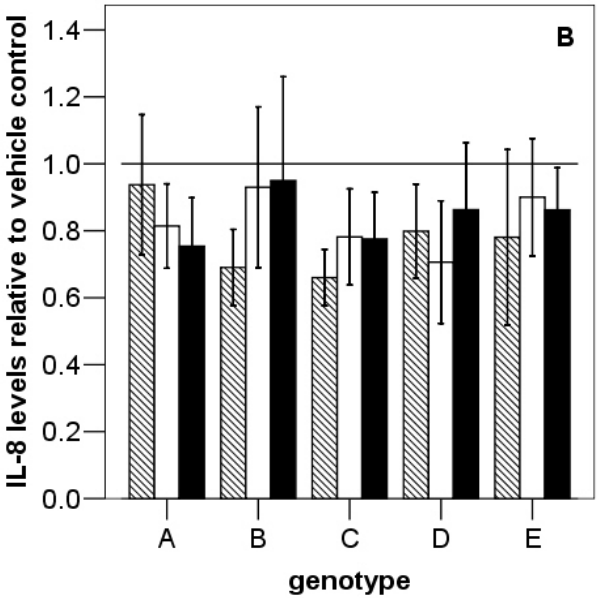
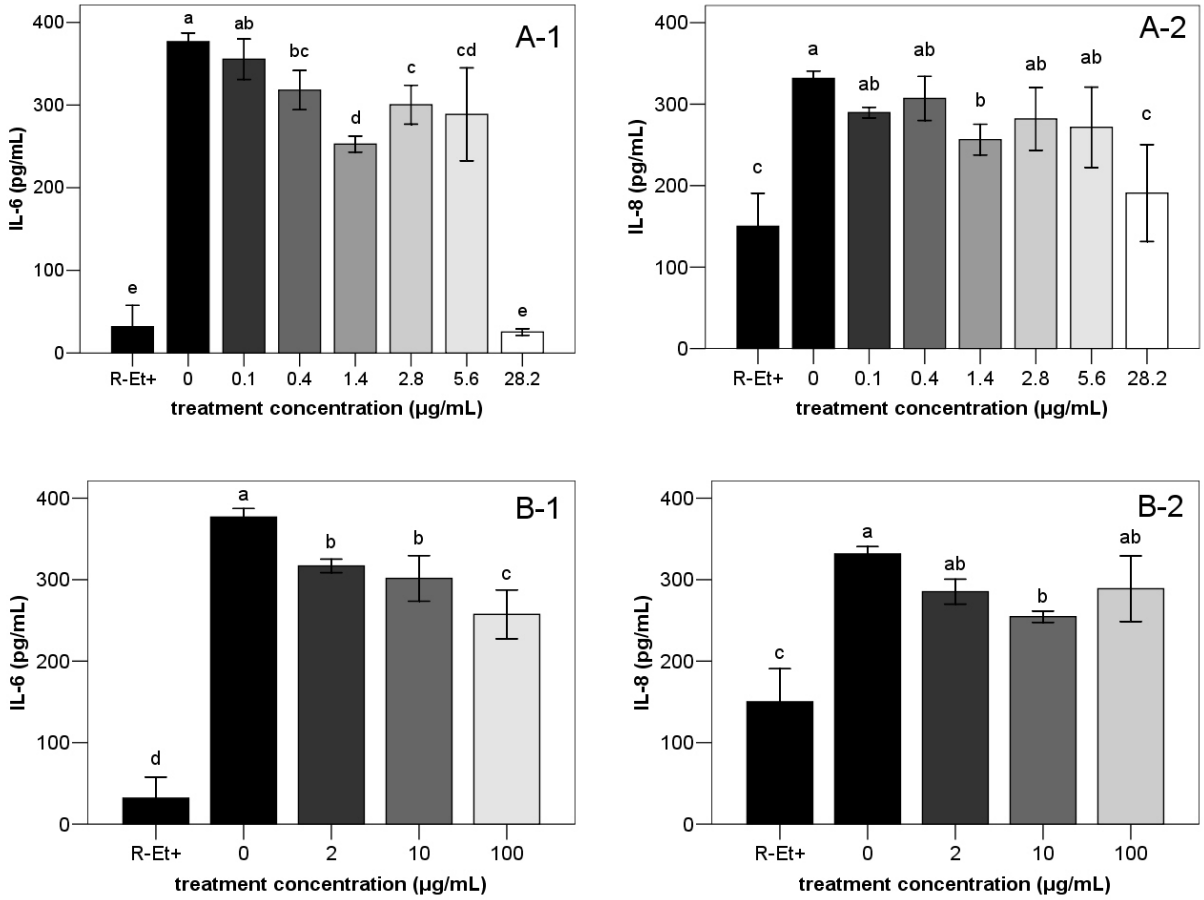


Figure 5.2: Major *E. angustifolia* marker compounds have inhibitory effects on interleukin secretion by human epithelial cells challenged with rhinovirus. Effect of tetraene on IL-6 and IL-8 secretion (A-1 and A-2 respectively); effect of echinacoside on IL-6 and IL-8 secretion (B-1 and B-2 respectively). Error bars represent standard deviations.



5.5 REFERENCES

Aoki, Y., D. Qiu, G.H. Zhao, and P.N. Kao. 1998. Leukotriene B4 mediates histamine induction of NF-kB and IL-8 in human bronchial epithelial cells. *American Journal of Physiology – Lung Cellular and Molecular Physiology*. 274: 1030-1039.

Bauer, R. and H. Wagner. 1991. *Echinacea* species as potential immunostimulatory drugs. pp.253-3321. In: H. Wagner and N.R. Farnsworth (eds). Economic and medicinal plant research, Volume 5. Academic Press, New York.

Bergeron, C., J.F. Livesey, D.V.C. Awang, J.T. Arnason, J. Rana, B.R. Baum, W. Letchamo. 2001. A Quantitative HPLC method for the quality assurance of *Echinacea* products on the North American market. *Phytochemical Analysis*. 11:207-215.

Binns S.E., J. Hudson, S. Merali, J.T. Arnason. 2002. Antiviral activity of characterized extracts from *Echinacea* spp. (Heliantheae: Asteraceae) against *Herpes simplex* virus (HSV-I). *Planta Medica*. 68: 780-783.

Blumenthal, M., J. Brinkman, and B. Wollschlaeger. 2003. *Echinacea* monograph. In: Blumenthal (ed.), The ABC Clinical Guide to Herbs. American Botanical Council. Austin TX. pp. 122-123.

Borchers A.T., C.L. Keen, J.S. Stern, and M.E. Gershwin. 2000. Inflammation and Native American medicine: the role of botanicals. *The American Journal of Clinical Nutrition*. 73:339-347.

Brovelli, E.A., D. Rua, H. Roh-Schmidt, and A. Chandra. 2005. Human gene expression as a tool to determine horticultural maturity in a bioactive plant (*Echinacea purpurea* L. Moench). *Journal of Agricultural and Food Chemistry*. 53: 8156-8161.

Calixto, J.B., M.M. Campos, M.F. Otuki, and A.R.S. Santos. 2004. Anti-inflammatory compounds of plant origin. Part II. Modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. *Planta Medica*. 70:93-103.

da Cunha, F.M., D. Duma, J. Assreuy, F.C. Buzzi, R. Niero, M.M. Campos, and J.B. Calixto. 2004. Caffeic acid derivatives: *In vitro* and *in vivo* anti-inflammatory properties. *Free Radical Research*. 38: 1241-1253.

Dalby-Brown, L., H. Barsett, A.K. Landbo, A.S. Meyer, P. Molgaard. 2005. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on *in vitro* oxidation of human low-density lipoproteins *Journal of Agricultural and Food Chemistry*. 53(24):9413-9423.

dos Santos, M.D., M.C. Almeida, N.P. Lopes, G.E.P. de Souza. 2006. Evaluation of the anti-inflammatory, analgesic and antipyretic activities of the natural polyphenol chlorogenic acid. *Biological and Pharmaceutical Bulletin*. 29: 2236-2240.

Freier, D.O., K. Wright, K. Klein, D. Voll, K. Dabiri, K. Cosulich, and R. George. 2003. Enhancement of the humoral immune response by *Echinacea purpurea* in female swiss mice. *Immunopharmacology and immunotoxicology*. 25: 551–560.

Gertsch, J., R. Schoop, U. Kuenzlea, A. Suter. 2004. *Echinacea* alkylamides modulate TNF-α gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. *FEBS Letters*. 577: 563–569.

Gkoumassi, E., B.G.J. Dekkers, M.J. Droege, C.R.S. Elzinga, M. Schmidt, H. Meurs, J. Zaagsma, and S.A. Nelemans. 2007. Virodhamine and CP55,940 modulate cAMP production and IL-8 release in human bronchial epithelial cells. *British Journal of Pharmacology*. 151:1041-1048.

Goel V., R. Lovlin, C. Chang, J.V. Slama, R. Barton, R. Gahler, R. Bauer, L. Goonewardene, and T.K. Basu. 2005. A proprietary extract from the *Echinacea* plant (*Echinacea purpurea*) enhances systemic immune response during a common cold. *Phytotherapy Research*. 19: 689–694.

Graham, M.H. 2003. Confronting multicollinearity in ecological multiple regression. *Ecology*. 84:2809-2815.

Hinz, B., K. Woelkart, R. Bauer. 2007. Alkamides from *Echinacea* inhibit cyclooxygenase-2 activity in human neuroglioma cells. *Biochemical and Biophysical Research Communications*. 360: 441-446.

Ho, J.C., G. Tipoe, L. Zheng, T.M. Leung, K.W.T. Tsang, D.K.Y. Shum, C.S. Lau, J.C.W. Mak, W.K. Lam, M.S.M. Ip. 2004. *In vitro* study of regulation of IL-6 production in bronchiectasis. *Respiratory Medicine*. 98: 334-341.

Hu C. and D.D. Kitts 2000. Studies on the antioxidant activity of *Echinacea* root extract. *Journal of Agricultural and Food Chemistry*. 48:1466-1472.

Hudson J., S. Vitnalanathan, L. Kang, V. Treyvaud Amiguet, J. Livesey, and J.T. Arnason. 2005. Characterization of antiviral activities in *Echinacea* root preparations. *Pharmaceutical Biology*. 43: 790-796.

Hwang S.A., A. Dasgupta and J.K. Actor. 2004. Cytokine production by non-adherent mouse splenocyte cultures to *Echinacea* extracts. *Clinica Chimica Acta*. 343: 161-166.

Institute for Neutraceutical Advancement. 2005. Phenolics in *Echinacea* by HPLC, INA method 106.000. Inst. for Neutr. Adv. Ann Arbor, Mich. July 2005 <http://www.nsf.org/business/ina/echinacea.asp?program=INA>.

Klein, T.W. 2005. Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nature Reviews Immunology*. 5: 400-411.

Krakauer, T. 2002. The polyphenol chlorogenic acid inhibits staphylococcal exotoxin-induced inflammatory cytokines and chemokines. *Immunopharmacology and Immunotoxicology*. 24:113-119.

LaLone, C.A., K.D.P. Hammer, L. Wu, J. Bae, N. Leyva, Y. Liu, A.K.S. Solco, G.A. Kraus, P.A. Murphy, E.S. Wurtele, O. Kim, K.I. Seo, M.P. Widrlechner, and D.F. Birt. 2007. *Echinacea* species and alkamides inhibit prostaglandin E2 production in RAW264.7 mouse macrophage cells. *Journal of Agricultural and Food Chemistry* 55: 7314-7322.

Letchamo, W., L.V. Polydeonny, N.O. Gladisheva, T.J. Arnason, J. Livesey, and D.V.C. Awang. 2002. Factors affecting *Echinacea* quality. Pp.514-521. In: J. Janick and A. Whipkey (eds.). Trends in New Crops and New Uses. ASHS Press, Alexandria, VA.

Matthias, A., J.T. Blanchfield, K.G. Penman, I. Toth, C.S. Lang, J.J. De Voss, and R.P. Lehmann. 2004. Permeability studies of alkylamides and caffeic acid conjugates from *Echinacea* using a Caco-2 cell monolayer model. *Journal of Clinical Pharmacy and Therapeutics*. 29: 7–13.

Matthias, A., R.S. Addison, K.G. Penman, R.G. Dickinson, K.M. Bone, and R. P. Lehmann. 2005. *Echinacea* alkamide disposition and pharmacokinetics in humans after tablet ingestion. *Life Sciences*. 77:2018-29.

Merali, S., S. Binns, M. Paulin-Levasseur, C. Ficker, M. Smith, B. Baum, E. Brovelli, and J.T. Arnason. 2003. Antifungal and anti-inflammatory activity of the genus *Echinacea*. *Pharmaceutical Biology*. 41: 412-420.

Miller. S.C. 2005. *Echinacea*: a miracle herb against aging and cancer? Evidence *in vivo* in mice. *Evidence-based Complementary and Alternative Medicine*. 2: 309-314.

Morazzonia, P., A. Cristonia, F. Di Pierrob, C. Avanzinic, D. Ravarinod, S. Stornellod, M. Zuccac, and T. Musso. 2005. *In vitro* and *in vivo* immune stimulating effects of a new standardized *Echinacea angustifolia* root extract (Polinacea). *Fitoterapia*. 76: 401– 411.

Müller-Jakic, B, W. Breu, A. Probstle, K. Redl, H. Greger, and R. Bauer. 1994. *In vitro* inhibition of cyclooxygenase and 5-lipoxygenase by alkamides from *Echinacea* and *Achillea* species. *Planta Medica*. 60: 37-40.

Murch S.J., S.E. Peiris, W.L. Shi, S.M.A. Zobayed, P.K. Saxena. 2006. Genetic diversity in seed populations of *Echinacea purpurea* controls the capacity for regeneration, route of morphogenesis and phytochemical composition. *Plant Cell Report*. 25: 522–532.

Raduner, S., A. Majewska, J. Chen, X. Xie, J. Hamon, B. Faller, K. Altmann, and J. Gertsch. 2006. Alkylamides from *Echinacea* are a new class of cannabinomimetics. *Journal of Biological Chemistry*. 281:14192-14206.

Randolph, R.K., K. Gellenbeck, K. Stonebrook, E. Brovelli, Y. Qian, D. Bankaitis-Davis, and J. Cheronis. 2003. Regulation of human immune gene expression as influenced by a commercial blended *Echinacea* product: preliminary studies. *Experimental Biology and Medicine*. 228: 1051-1056.

Reddel, R.R., Y. Ke, B.I. Gerwin, M.G. McMenamin, J.F. Lechner, R.T. Su., D.E. Brash, J. Park, J.S. Rhim, C.C. Harris. 1988. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Research*. 48: 1904-1909.

Sasagawa, M., N.B. Cech, D.E. Gray, G.W. Elmer, C.A. Wenner. 2006. *Echinacea* alkylamides inhibit interleukin-2 production by Jurkat T cells. *International Immunopharmacology*. 6: 1214–1221.

Senchina D.S., D.A. McCann, J.M. Asp, J.A. Johnson, J.E. Cunnick, M.S. Kaisere, M.L. Kohut. 2005. Changes in immunomodulatory properties of *Echinacea* spp. root infusions and tinctures stored at 4ºC for four days. *Clinica Chimica Acta* 355 67–82.

Senchina, D.S., L.E. Flagel, J.F. Wendel, and M.L. Kohut. 2006a. Phenetic comparison of seven *Echinacea* species based on immunomodulatory characteristics. *Economic Botany*. 60: 205-211.

Senchina, D.S., L. Wu, G.N. Flinn, D.N. Konopka, J.A. McCoy, M.P. Widrlechner, E.S. Wurtele, and M.L. Kohut. 2006b. Year-and-a-half old, dried *Echinacea* spp. roots retain cytokine-modulating capabilities in an *in vitro* human older adult model of influenza vaccination. *Planta Medica*. 72: 1207-1215.

Shah, S.A., S. Sander, C.M. White, M. Rinaldi, C.I. Coleman. 2007. Evaluation of *Echinacea* for the prevention and treatment of the common cold: a meta-analysis. *The Lancet Infectious Diseases*. 7: 473-480.

Sharma M., J.T. Arnason, A. Burt, and J.B. Hudson. 2006 *Echinacea* extracts modulate the pattern of chemokine and cytokine secretion in rhinovirus-infected and uninfected epithelial cells. *Phytotherapy Research*. 20, 147–152.

Schoop, R., P. Klein, A. Suter, S.L. Johnston. 2006. *Echinacea* in the prevention of induced rhinovirus colds: a meta-analysis. *Clinical Therapeutics*. 28:174-183.

Singh, B., J.A. Berry, L.E. Vincent, and A. Lucci. 2006. Involvement of IL-8 in COX-2-mediated bone metastases from breast cancer. *Journal of Surgical Research*. 134: 44-51.

Speroni, E., P. Govoni, S. Guizzardi, C. Renzulli, M.C. Guerra. 2002. Anti-inflammatory and cicatrizing activity of *Echinacea pallida* Nutt. root extract. *Journal of Ethnopharmacology*. 79:265-272.

United States Pharmacopeia. 2004. *Echinacea angustifolia* monograph, p.2075-2083. In: USP 28- National Formulary 23. USP Convention: Rockville, Md.

Thivierge, M., M. Rola-Pleszczynski. 1994. Involvement of both cyclooxygenase and lipoxygenase pathways in platelet activating factor-induced interleukin-6 production by alveolar macrophages. *Annals of the New York Academy of Science*. 725: 213-222.

Thygesen L., J. Thulin, A. Mortensen, L.H. Skibsted, P. Molgaard. 2007. Antioxidant activity of cichoric acid and alkamides from *Echinacea purpurea*, alone and in combination. *Food Chemistry* 101: 74–81.

Woelkart, K. and R. Bauer. 2007. The role of alkamides as an active principle of *Echinacea*. *Planta Medica*. 73: 615-623.

Woelkart, K., C. Koidl, A. Grisold, J.D. Gangemi, R.B. Turner, E. Marth, R. Bauer. 2005. Bioavailability and pharmacokinetics of alkamides from the roots of *Echinacea angustifolia in humans*. *Journal of Clinical Pharmacology*. 45:683-689.

Woelkart, K., R. Bauer, B. Hinz. 2006. Alkamide from *Echinacea angustifolia* roots inhibit cyclooxygenase-2-dependent prostaglandin synthesis in human neuroglioma cells. *Planta Medica*. 324: 621-626.

Xiong Q., Y. Tezuka, T. Kaneko, H. Li, L.Q. Tran, K. Hase, T. Namba, S. Kadota. 2000. Inhibition of nitric oxide by phenylethanoids in activated macrophages. *European Journal of Pharmacology*. 400: 137–144.

Zheng, Y., M. Dixon, and P.K. Saxena. 2006. Growing Environment and Nutrient Availability Affect the Content of Some Phenolic Compounds in *Echinacea purpurea* and *Echinacea angustifolia*. *Planta Medica*. 72: 1407-1414.

Chapter Six

GENERAL DISCUSSION AND CONCLUSIONS

The novel information provided in this study will offer valuable insight that will help guide key decisions in the development of efficient selection strategies aimed at improving *E. angustifolia* medicinal crops. This chapter summarizes the major findings obtained from the thesis objectives along with a critical look at the strengths and limitations of the underlying trials and experiments. At the end of this chapter, I propose ideas for future avenues of investigation in the field of *Echinacea* research that I believe, based on the outcomes of the current project, offer the greatest potential to advance our basic understanding of *E. angustifolia* chemical ecology and how it relates to the plant's reported pharmacological effects.

6.1 SUMMARY OF CONCLUSIONS AND IMPORTANT FINDINGS

The results presented in Chapter 2 provided no significant evidence of differences among cultivated populations from British Columbia and the state of Washington in regards to traits of commercial and potentially therapeutic value. Further, I tested the hypothesis that cultivated populations, when compared to wild populations, benefited from higher levels of phytochemical root concentrations as a result of either unintentional or unreported intentional selection by growers. Samples from natural populations within

the native range of *E. angustifolia* were grown alongside the cultivated populations in the common greenhouse environment. This hypothesis was not supported by the results of Chapter 2 as no overall clear distinction was observed between the measured phytochemical traits of plants from wild and cultivated populations. In fact, the most significant difference observed was that for echinacoside between two wild populations. In essence, these findings support the idea that selection efforts intended for phytochemical improvement need not be focused on any of the surveyed cultivated populations with demonstrated suitability to cultivation outside of their native range.

In another *Echinacea* species, Qu et al. (2005) presents evidence of selection in commercially cultivated *E. purpurea* populations in the form of reduced seed dormancy compared to wild populations. The results presented in Chapter 2 do not justify extending their hypothesis to *E. angustifolia* populations from British Columbia, at least not based on the phytochemical traits that I have selected to study. This is perhaps not surprising in light of the fact that *E. purpurea* has been under cultivation in various parts of the world for at least 80 years and has been developed as an important ornamental species whereas *E. angustifolia*, which is essentially insignificant in the horticultural industry, has only recently been introduced into cultivation (Letchamo et al. 2002).

Chapter 3 demonstrates that shoot concentrations of phytochemical markers are not very strong predictors of root marker concentrations. The relatively low phenotypic correlations between root and shoot concentrations in plants grown under field and greenhouse conditions, along with their inconsistency in the two environments, highlight the importance of direct selections based on phytochemical evaluations of root material to maximize expected genetic yield. Further, Chapter 3 adds to the developing picture of

phenological effects on *Echinacea* phytochemistry (Binns et al. 2002a, Berti et al. 2002, Letchamo et al. 1999, Seidler-Lozykowska & Dabrowska 1999) with the demonstration that the correlation between root and shoot concentrations of echinacoside is effected by flowering stage.

With the aim to characterize effects of genotype x environment interaction on phytochemical traits of vegetatively propagated *E. angustifolia*, clonal populations of 5 genotypes that were selected based on greenhouse performance and observable morphological differences were grown in three different sites representative of possible growing conditions in British Columbia. Presented in Chapter 4, this study revealed differences among the examined phytochemical traits in their response to environments as well as clear evidence of inconsistent genotypic ranking across the various sites. This study represents one of only two reported instances where clonally propagated genotypes of *E. angustifolia* were used to address fundamental questions regarding genotypic and environmental effects on phytochemical performance (see also Letchamo et al. 2002).

The commercial value of the phytochemical markers studied in this project is undeniable given their importance in product certification and the premiums offered for higher concentrations. Their therapeutic value on the other hand is less certain, with considerable debate in the literature surrounding their relative contributions to the various benefits attributed to *E. angustifolia* products (Shah et al. 2007, Turner et al. 2005, Barrett 2003). When breeding medicinal plants, the therapeutic efficacy of a genotype should be regarded as a selectable trait and, ideally, evaluated with the same rigour as agronomic and yield-related traits.

Sensitive and effective bioassays not only offer a potentially powerful and direct means to evaluate genotypic performance of medicinal plants on the basis of biological activity, but can also serve as an invaluable tool to investigate environmental effects on the therapeutic quality of plant material. With these considerations in mind, the study presented in Chapter 5 was conducted to develop a bioassay-guided selection strategy for *E. angustifolia*. The bioassay proposed in this chapter was based on the documented anti-inflammatory activity of *E. angustifolia* root extracts (Borchers et al. 2000) and, more specifically, on their modulating effects on pro-inflammatory interleukin secretion from human bronchial epithelial cells in response to rhinovirus infection (Sharma et al. 2006). Regression analyses from Chapter 5 offer support for genotypic selection based on alkamide concentration to increase the anti-inflammatory activity of *Echinacea* extracts. However, significant genotypic and environmental effects on phytochemical root concentrations (Chapter 4) did not translate into significant effects on interleukin inhibition, as determined by analysis of variance.

The logical order in which I have chosen to present the chapters in this dissertation corresponds closely to the chronological sequence in which their underlying experiments were conducted. This final chapter is written with the benefit of hindsight on all of the results from this project. Such a perspective affords me with the valuable opportunity to review experimental designs and findings from earlier chapters in light of results and conclusions from later ones.

For instance, the choice of growing environment might be reconsidered for future genotypic and population evaluations similar to the one presented in Chapter 2. This recommendation is based on the lower ability of the greenhouse environment to

discriminate genotypes based on CAD concentrations compared to that of the Totem Field site (Chapter 4). On the other hand, the findings in Chapter 4 suggest that the greenhouse environment was in fact the most likely of the three tested growing sites to reveal genetic differences in root tetraene concentrations among the populations because of its higher discriminating ability for alkamide concentrations.

It is also worth highlighting that the occurrence of lower CAD concentrations in greenhouse conditions compared to field conditions was directly observed in both genetically mixed (Chapter 3) and homogeneous (Chapter 4) populations. The conclusion that growth in greenhouse lead to a reduction in CAD levels based on direct comparisons between measurements in field versus greenhouse material (Chapter 3) may be viewed as tenuous given that different analytical methods were used for the two types of material. However, the fact that the same differences appeared in the results from Chapter 4, where material from all three sites were analysed in a random order using identical analytical methods is convincing evidence that the findings in Chapter 3 are based on true biological effects rather than protocol artefact. Further evidence for this point, although indirect, is that the CAD levels observed in Chapter 2 are much closer to the ranges observed in the greenhouse material from Chapters 3 and 4 than those in the field grown material.

6.2 Critical Evaluation of Strengths and Limitations of the Thesis Research

The results and conclusions reported throughout this work provide novel information that will be valuable in the establishment of guidelines for the future

development of effective breeding programs intended to increase phytochemical yield in selected cultivars of *E. angustifolia*. Even though this research has focused particularly on the cultivation of *E. angustifolia* in British Columbia, the implications of most of the results obtained are not necessarily geographically restricted to this region.

The surprising disconnect among the numerous disciplines involved in natural health product research has at times lead to shortcomings in experimental protocols and, as a result, weaker conclusions. As an example, one can refer to published clinical trials using *Echinacea* products for which little to no phytochemical information for the source material is provided and/or available (eg. Grimm & Müller 1999). The objectives in this project were intended to answer fundamental questions by using approaches that stem from various relevant disciplines (e.g. horticulture, phytochemistry, and pharmacology), thereby stimulating new questions of broad interest and hopefully encouraging future collaborative efforts in this field of research.

Some of the specific limitations of this research that have been discussed in the various corresponding chapters bear repeating in this section. For instance, due to time and funding constraints, all of the material used for measuring phytochemical concentrations and biomass yield was derived from plants harvested after a single growing season. Since *Echinacea* crops are generally harvested after 2 to 4 growing seasons, extending my findings to current field production requires the assumption that the results of my experiments would be similar in older *E. angustifolia* plants. Even though there is currently no available evidence to discount the validity of this assumption, it will be necessary to verify it experimentally in future work.

The research presented in this dissertation focused on questions pertaining primarily to the phytochemical accumulation of two biologically active classes of marker compounds, CADs and alkamides. A number of reports have also linked the polysaccharide/glycoprotein fraction to the immunological properties of *Echinacea* (Blaschek et al. 2006, Classen et al. 2006, Alban et al. 2002, Luettig et al. 1989). These two classes of compounds were not considered in this research mainly because of the dearth of validated analytical methods, which has persisted until very recently (Bergeron & Gafner 2007), and limitations in available technical resources that precluded the quantitative assessment of polysaccharide and glycoprotein in my experimental material. Also, the extraction procedures in my experiments, which were intended to reflect those used in the preparation of the major North American *E. angustifolia* product, namely root ethanolic extracts, result in low levels of polysaccharides and glycoprotein (eg. Altamirano-Dimas et al. 2007), thus lessening their importance to the underlying theme of this project.

The clonal propagation of genotypes required for the experiments proved to be very challenging. Despite numerous publications reporting successful *Echinacea* micropropagation (reviewed in Abbasi et al. 2007), a minority of these deal specifically with *E. angustifolia*. By far, the majority of this tissue culture research has focused on the species *E. purpurea*. My preliminary work suggested that *E. angustifolia* plants were generally recalcitrant to the published *E. purpurea* protocols, and had inadequate response to rooting protocols as previously reported (Harbage 2001), and only a very small proportion of genotypes responded to shoot initiation.

In light of these difficulties, the plants were vegetatively propagated using the generic root cutting techniques described in Chapter 4. The efficacy of this approach was also highly genotype dependent, albeit less than that of the tested *in vitro* micropropagation protocols. In addition, the root cuttings and the plantlets sprouting from them were prone to disease, putatively of a fungal nature, as evidenced by their sudden and rapid deterioration, despite rigorous root sterilising procedures. Because of these limitations, only a relatively small number of plants were available for the genotype x environment study, which in turn reduced the number of genotypes, environments and growing seasons available for the analyses in Chapter 4. The absence of data from multi-year trials in the tested environments is a limiting factor in their characterization. Future research comparing year to year performance of selected genotypes grown in environments representative of BC ecoregions would be helpful in confirming their status as distinct macro-environments for *E. angustifolia* cultivation.

6.3 Relevance of Research to Current Knowledge and Operations in the Field of Medicinal *E. angustifolia* Cropping

The findings of significant GE effects highlight the caution required when extending conclusions of phytochemical differences (or similarities) among populations of *E. angustifolia* to environments other than those specifically tested. This is an important point that must be considered in interpreting results, past and future, in the field of *Echinacea* research. Many of the papers reporting phytochemical differences in secondary metabolites of *Echinacea* populations assumed to be genetically distinct have been based on evaluations of plant material grown in a single environment, often under

greenhouse conditions (eg. Murch et al. 2006, Binns et al. 2002b, Letchamo et al. 2002). This note of caution should also apply when extending the findings from Chapter 2 to field conditions. Further, assuming that common markers found in different *Echinacea* species share similar pathways and regulation, significant GE should likely also be assumed when drawing conclusions from interspecific phytochemical studies of the *Echinacea* genus as a whole (Binns et al. 2002a).

As previously mentioned, this study includes one of the only reports of phytochemical examination of *E. angustifolia* relying on cloned plants from various genotypes in order to isolate environmental effects on the accumulation of secondary metabolites. Information derived from studies on genetically homogenous populations is essential to efforts towards *E. angustifolia* crop improvement. There are limitations in using clones when addressing questions regarding the genetic effects on traits of interest since additive variance cannot be estimated separately from other genetic sources of variance, i.e. dominance and epistasis (Falconer, 1981). Specific measures of additive variance are required to estimate narrow sense trait heritability, which is of primary importance to predict genetic advancement of seed propagated crops. Nevertheless, given the strict self-incompatibility that characterises the *E. angustifolia* mating system (Wagenius 2006), vegetative propagation could be a viable means to fix traits in selected genotypes rapidly, in which case, a better estimate of expected genetic gain would be clonal repeatability as defined in Falconer (1981). The major disadvantage of vegetatively propagated crops is the added expense of transplanting plantlets instead of directly sowing easily transported and manipulated seeds. However, current recommendations for establishing *E. angustifolia* crops in Canada already include

transplanting bare roots or plugs instead of direct seeding (CSIDC 2000) because of erratic achene germination (Qu et al. 2004). Therefore, the additional cost of working with transplants has likely already been accounted for in many *Echinacea* growing operations.

6.4 RECOMMENDATIONS ARISING FROM RESEARCH FINDINGS

Based on the findings derived from this research, the following recommendations should be considered in the planning of *E. angustifolia* genotype selection work for cultivation in British Columbia:

a) Since none of the tested cultivated populations yielded significantly higher levels of marker compounds when grown in a common environment, undue attention should not be placed specifically on any one of these populations.

b) Even though non-destructive measurements of phytochemical concentrations in aerial parts would increase the speed and reduce the resources needed for genotype evaluation, direct evaluation of root phytochemical yield is required given the low phenotypic correlation in marker compounds between different plant parts.

c) Genotypic assessment of CAD yield should be carried out under field conditions to maximize differentiation between tested genotypes.

d) In light of significant crossover GE effects on phytochemical traits of *E. angustifolia* plants, selections and evaluations should be made, ideally, in the target growing environments. Greenhouse-assessed genotypic performance should not be assumed to be a reliable predictor of field performance.

6.5 COMMENTS ON FUTURE RESEARCH

Some of the ideas for future avenues of research arising from this project have been presented throughout the preceding chapters. Those that are likely to have the most impact on further refining breeding and cultivation guidelines of *E. angustifolia* crops are revisited and further discussed here.

Specific causes of GE interactions of *E. angustifolia* phytochemical traits

Now that significant GE effects have been documented for the phytochemical traits of therapeutic and commercial interest in the production of *E. angustifolia*, research should be aimed at identifying the specific causes of GE interactions and the significance of genotype by year interactions on these traits. With a better understanding of the sources of GE interactions in *E. angustifolia*, breeders would be in a position to replicate relevant conditions from target growing environments in their selection environments. This approach could, on one hand, reduce the detrimental effects of GE interaction on genetic advancement of selected populations or, on the other hand, limit the costly requirements of site-specific breeding.

Causes of reduced CAD root concentrations in greenhouse-grown plants

Setting the debate over relative activity of *Echinacea* secondary metabolites aside, the cause of reduced phenolic levels observed in greenhouse grown material compared to field material (Chapters 3 & 4) warrants closer investigation, not only from a production and commercialization standpoint, but also for the purpose of advancing our understanding of the biochemical pathways involved in CAD metabolism. If the

observed reduction turns out to be caused by a single factor, such as light quality for instance, remedial measures could easily be applied to maximize levels of bioactive compounds in greenhouse-grown material. This research avenue would be instrumental in taking advantage of the suggested benefits from controlled environments that greenhouse production offers (Zheng et al. 2006). A logical start-off point for this research would be an investigation on the effect of ultraviolet radiation on root levels of CADs in *E. angustifolia* and its related commercial species for the reasons discussed in Chapter 4, which include the demonstrated effect of UV-B on the phenylpropanoid metabolic pathway and its key enzyme, PAL (Tevini and Teramura 1989). Other factors relevant to greenhouse production that require investigation given their demonstrated influence on phenylpropanoid metabolism in other species, but not in *Echinacea* include wounding or feeding by herbivores, low temperature, and deficiencies in micronutrients such as iron and boron (Dixon and Paiva 1995; Blevins and Lukaszewski 1998).

Development and refinement of genotype assessment bioassays (GABs)

Future research should be aimed at refining genotype screening methods based on therapeutically relevant biological activity. Without an effective means of assessing genotype performance based directly on biological activity, the efficacy of medicinal plant selection efforts based on a few secondary metabolites is unavoidably weakened by the indirect nature of such an approach. This is especially true in the case of *Echinacea* given the apparent complexity of its effects on animal physiology. This idea is evidenced in my research by the absence of genotypic effect on interleukin secretion despite statistically significant genotypic differences in the levels of the putative active

compounds in the extracts (Chapter 5). The concept of using bioassays to evaluate genotypes with the aim of breeding higher levels of complex biological activity has already been advanced for other cultivated species. For instance, a wide range of screening methods to evaluate allelopathic potential of rice plants have been adopted in efforts to breed cultivars able to suppress competing weeds (Khanh et al. 2007).

Admittedly, selections of *E. angustifolia* genotypes based on GABs would also involve a certain degree of extrapolation. For this reason, the ideal GABs must meet the following criteria to be useful: a) relevance to the desired therapeutic effect, b) quantitative response to *Echinacea* treatments, c) and relatively fast and inexpensive execution. A number of papers have been published since the start of this project that report the successful use of *in vitro* models to measure various key molecular signals and/or genes related to animal immune response and to compare the effects of various *Echinacea*-related treatments on their regulation (Table 6.1). These molecules may serve as potentially useful biomarkers in the further development of GABs. DNA microarrays have recently been used to compare immune-related gene expression profiles in human bronchial epithelial cells treated with different *Echinacea* preparations (Altamirano-Dimas et al. 2007). Despite being a highly informative and powerful approach, DNA microarray analyses come at a higher cost and would not be appropriate for assessing large numbers of samples on a routine basis, thereby limiting their potential for use in genotype selection. However, the same mircroarray study revealed that many of the genes affected by *Echinacea* extracts in human airway cells were interconnected through one major node, the transcription factor C/EBPβ. This biomarker or other

similarly responsive nodes in physiological pathways could potentially be excellent target candidates on which to base GABs for *Echinacea* selection.

There is an underlying scepticism about the efficacy of *Echinacea* and even plant derived health products in general. This has been, and continues to be, a significant obstacle in natural health products research. It is often fuelled, rightly or wrongly, by reports of treatment inefficacy in various models and clinical trials. What is perhaps most surprising is the level of dogmatism involved at times on both sides of the scientific debate over herbal medicines and an apparent reliance on confirmation bias. The biological activity of *Echinacea* is indisputable given the ample evidence supporting its various effects on physiological functions in animals. However, the question regarding its biological activity, unfortunately, is often confused with the question of whether or not it "works" against colds and flu symptoms. It is crucially important, from a research perspective, to keep these questions separate and focus on developing useful applications and the best delivery method for high quality *Echinacea* products. This will only be accomplished through a concerted multi-disciplinary effort that effectively integrates both supporting and refuting findings from the various fields involved in *Echinacea* research.

Table 6.1: Recent examples of published quantitative in vitro assays relevant to *Echinacea* extracts with demonstrated dose dependence or preparation-dependant differences in effects on biomarker regulation (abbreviations and symbols are explained in footnotes).

Biomarker	Model (type of challenge)	Treatment	Observed effect	Study
IL-1, IL-8, IL-10, TNF-α, ICAM, (mRNA)	THP-1 human monocyte cells (none)	Extracts from EAN and EPU	↑ (expression)	Randolph et al. 2003
TNF-α	PBMC (LPS)	Echinaforce™ (EPU tincture)	↓ (short term)	Gertsch et al. 2004
Immunomodulating cytokines (mRNA)	THP-1 human monocyte cells (none)	EPU extracts at various stages of maturity	↑ (extract dependent)	Brovelli et al. 2005
NO	Raw 264.7 murine macrophages (LPS)	Alkamides	↓ (dose dependant)	Chen et al. 2005
IL-2	Jurkat E6.1 T cells (PHA/PMA)	Alkamides, CADs, and EPU extracts	↓ (dose dependant for extracts and alkamides only)	Sasagawa et al. 2006
IL-1β, TNF-α	PBMC (none)	Extracts from 7 ECH species	↑ IL-1β and TNF-α (species dependant)	Senchina et al. 2006a

(Table continued on next page)

Table 6.1: continued

Biomarker	Model (type of challenge)	Treatment	Observed effect	Study
IL-10, IL-2, IFN-γ	PBMC (influenza A virus)	Ethanolic extracts of 7 ECH species	↑ IL-10; ↓ IL-2, IFN-γ (species dependant)	Senchina et al. 2006b
IL-1β, IL-12, IL-6, IL-8, IL-10, TNF-α	Whole blood (none; LPS; αCD3/PMA)	ECH alkamides	m (challenge dependant)	Raduner et al. 2006
Immune response gene array	BEAS-2B bronchial epithelial cells (rhinovirus)	EPU extracts	m	Altamirano-Dimas et al. 2007
COX-2, PGE_2	H4 Human neuroglioma cells	EAN CO_2 extracts and alkamides	↓ of PGE_2 and COX-2 (activity)	Hinz et al. 2007
PGE_2	Raw 264.7 murine macrophages (none, LPS)	Extracts of 6 ECH species and alkamides	↓ by alkamides and by 4 species	LaLone et al. 2007
NFκ-B, TNF-α, NO	Raw 264.7 murine macrophages (LPS)	EAN and EPU extracts, cichoric acid, alkamides	m	Matthias et al .2007

Biomarkesr: **COX**: cyclooxygenase; **TNF**: tumor necrosis factor **ICAM**: intercellular adhesion molecule; **IFN**: interferon; **IL**: interleukin ; **NF**: nuclear factor; **NO**: nitric oxide; **PGE_2**: prostaglandin E_2.

Model / Challenge: **αCD3**: anti-CD3 antibody; **LPS**: lipopolysaccharide; **PBMC**: peripheral blood mononuclear cells; **PHA**: phytohaemaglutinin; **PMA**: phorbol ester (12-tetradecanoylphorbol-13 acetate).

Treatment: **CAD**: caffeic acid derivatives; **EAN**: *E. angustifolia*; **ECH**: *Echinacea*; **EPU**: *E. purpurea*.

Effects: ↑: stimulation; ↓: inhibition **m**: Modulation, referring to instances of significant effects not all in the same direction (ie. inhibition and stimulation).

6.6 REFERENCES

Abbasi, B.H., P.K. Saxena, S.J. Murch, and C.Z. Liu. 2007. *Echinacea* biotechnology: Challenges and opportunities. *In vitro Cellular and Developmental Biology*. 43:481-492.

Alban, S., B. Classen, G. Brunner, W. Blaschek. 2002. Differentiation between the complement modulating effects of an arabinogalactan-protein from *Echinacea purpurea* and heparin. *Planta Medica* 68: 1118-1124.

Altamirano-Dimas, M., J.B. Hudson, D. Cochrane, C. Nelson, and J.T. Arnason. 2007. Modulation of immune response gene expression by *Echinacea* extracts : results of a gene array analysis. *Canadian Journal of Physiology and Pharmacology*. 85: 1091-1098.

Barrett, B. 2003. Medicinal properties of *Echinacea*: A critical review. *Phytomedicine*. 10:66-86

Bergeron, C. and S. Gafner. 2007. Quantitative analysis of the polysaccharide and glycoprotein fractions in *Echinacea angustifolia* by HPLC-ELSD for quality control of raw material. *Pharmaceutical Biology*. 45: 98-105.

Berti, M., R. Wilckens, S. Fischer, and F. Hevia. 2002. Effect of harvest season, nitrogen, phosphorus and potassium on root yield, echinacoside and alkylamides in *Echinacea angustifolia* L. in Chile. *Acta Horticutlurae*. 576: 303-310.

Binns, S.E., J.F. Livesey, J.T. Arnason, and B.R. Baum. 2002a. Phytochemical variation in *Echinacea* from roots and flowerheads of wild and cultivated populations. *Journal of Agricultural and Food Chemistry*. 50:3673-3687.

Binns, S.E., J.T. Arnason, B.R. Baum. 2002b. Phytochemical variation within populations of *Echinacea angustifolia* (Asteraceae). *Biochemical Systematics and Ecology*. 30:837-854.

Blaschek, W., A. Zager, B. Classen, A.J. Ulmer. 2006. Stimulation of LAL-test by LPS-free arabinogalactan-protein preparations from *Echinacea purpurea*. *Planta Medica*. 72:P_233.

Blevins, D.G. and K.M. Lukaszewski. 1998. Boron in plant structure and function. *Annual Review of Plant Physiology and Plant Molecular Biology*. 49:481-500.

Borchers A.T., C.L. Keen, J.S. Stern, and M.E. Gershwin. 2000. Inflammation and Native American medicine: the role of botanicals. *The American Journal of Clinical Nutrition*. 73:339-347.

Brovelli, E.A., D. Rua, H. Roh-Schmidt, and A. Chandra. 2005. Human gene expression as a tool to determine horticultural maturity in a bioactive plant (*Echinacea purpurea* L. Moench). *Journal of Agricultural and Food Chemistry*. 53: 8156-8161.

Canada-Saskatchewan Irrigation Diversification Centre. 2000. Production practices for *Echinacea angustifolia*. Accessed February 25th 2008 <http://www.agr.gc.ca/pfra/csidc/echinacea_e.pdf>.

Chen, Y., T. Fu, T. Tao, J. Yang, Y. Chang, M. Wang, L. Kim, L. Qu, J. Cassady, R. Scalzo, and X. Wang. 2005. Macrophage activating effects of new alkamides from the roots of *Echinacea* species. *Journal of Natural Products*. 68: 773-776.

Classen, B., S. Thude, W. Blaschek, M. Wacka, and C. Bodinet. 2006. Immunomodulatory effects of arabinogalactan-proteins from *Baptisia* and *Echinacea*. *Phytomedicine*. 13: 688-694.

Dixon, R.A. and N.L. Paiva. 1995. Stress-lnduced phenylpropanoid metabolism. *The Plant Cell*. 7: 1085-1097.

Falconer, D.S. 1981. Introduction to quantitative genetics. 2nd ed. Longmans Green. London/New York.

Gertsch, J., R. Schoop, U. Kuenzlea, A. Suter. 2004. *Echinacea* alkylamides modulate TNF-α gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. *FEBS Letters*. 577: 563–569.

Grimm, W. and H.H. Müller. 1999. A randomized controlled trial of the effect of fluid extract of *Echinacea purpurea* on the incidence and severity of colds and respiratory infections. *The American Journal of Medicine*. 106: 138-143.

Harbage J.F. 2001. Micropropagation of *Echinacea angustifolia*, *E. pallida*, and *E. purpurea* from stem and seed explants. *HortScience*. 36: 360–364.

Hinz, B., K. Woelkart, R. Bauer. 2007. Alkamides from *Echinacea* inhibit cyclooxygenase-2 activity in human neuroglioma cells. *Biochemical. and Biophysical Research Communications*. 360: 441-446.

Khanh, T.D., T.D. Xuan, and I.M. Chung. 2007. Rice allelopathy and the possibility for weed management. *Annals of Applied Biology*. 151: 325-339.

LaLone, C.A., K.D.P. Hammer, L. Wu, J. Bae, N. Leyva, Y. Liu, A.K.S. Solco, G.A. Kraus, P.A. Murphy, E.S. Wurtele, O. Kim, K.I. Seo, M.P. Widrlechner, and D.F. Birt. 2007. *Echinacea* species and alkamides inhibit prostaglandin E2 production in RAW264.7 mouse macrophage cells. *Journal of Agricultural and Food Chemistry* 55: 7314-7322.

Letchamo, W., J. Livesey, T.J. Arnason, C. Bergeron, and V.S. Krutilina. 1999. Cichoric acid and isobutylamide content in *Echinacea purpurea* as influenced by flower developmental stages. In: Jannick (ed.). *Perspectives on new crops and new uses*. ASHS Press, Alexandria VA. pp.494-498.

Letchamo, W., Polydeonny, L.V., Gladisheva, N.O., Arnason, T.J., Livesey, J., and Awang, D.V.C. 2002. Factors affecting *Echinacea* quality. In: Jannick and Wipckey (ed.) *Trends in New Crops and New Uses*. ASHS Press, Alexandria VA. pp.514-521.

Luettig, B., C. Steinmuller, G. Gifford, H. Wagner, and M. Lohmann-Matthes. 1989. Macrophage activation by the polysaccharide arabinogalactan from the plant cell cultures of *Echinacea purpurea*. *Journal of the National Cancer Institute*. 81: 669-675.

Matthias, A., L. Banbury, L.M. Stevenson, K.M. Bone, D.N. Leach, and R.P. Lehmann. 2007. Alkylamides from *Echinacea* modulate induced immune responses in macrophages, *Immunological Investigations*. 36: 117 – 130.

Murch S.J., S.E. Peiris, W.L. Shi, S.M.A. Zobayed, P.K. Saxena. 2006. Genetic diversity in seed populations of *Echinacea purpurea* controls the capacity for regeneration, route of morphogenesis and phytochemical composition. *Plant Cell Report*. 25: 522–532.

Qu, L., X. Wang, E. Hood, and R. Scalzo. 2004. Ethephon promotes germination of *Echinacea angustifolia* and *E.pallida* in darkness. *HortScience*. 39: 1101-1103.

Qu, L., X. Wang, Y. Chen, R. Scalzo, M.P. Widrlechner, J.M. Davis, J.F. Hancock. 2005. Commercial seed lots exhibit reduced seed dormancy in comparison to wild seed lots of *Echinacea purpurea*. *HortScience*. 40: 1843–1845.

Raduner, S., A. Majewska, J. Chen, X. Xie, J. Hamon, B. Faller, K. Altmann, and J. Gertsch. 2006. Alkylamides from *Echinacea* are a new class of cannabinomimetics. *Journal of Biological Chemistry*. 281:14192-14206.

Randolph, R.K., K. Gellenbeck, K. Stonebrook, E. Brovelli, Y. Qian, D. Bankaitis-Davis, and J. Cheronis. 2003. Regulation of human immune gene expression as influenced by a commercial blended *Echinacea* product: preliminary studies. *Experimental Biology and Medicine*. 228: 1051-1056.

Sasagawa, M., N.B. Cech, D.E. Gray, G.W. Elmer, C.A. Wenner. 2006. *Echinacea* alkylamides inhibit interleukin-2 production by Jurkat T cells. *International Immunopharmacology*. 6: 1214–1221.

Seidler-Lozykowska, K. and J. Dabrowska. 2003. Yield and polyphenolic acids content in purple coneflower (*Echinacea purpurea* Moench.) at different growth stages. *Journal of Herbs, Spices & Medicinal Plants*. 10: 7-12.

Senchina, D.S., L.E. Flagel, J.F. Wendel, and M.L. Kohut. 2006a. Phenetic comparison of seven *Echinacea* species based on immunomodulatory characteristics. *Economic Botany*. 60: 205-211.

Senchina, D.S., L. Wu, G.N. Flinn, D.N. Konopka, J.A. McCoy, M.P. Widrlechner, E.S. Wurtele, and M.L. Kohut. 2006b. Year-and-a-half old, dried *Echinacea* spp. roots retain cytokine-modulating capabilities in an *in vitro* human older adult model of influenza vaccination. *Planta Medica*. 72: 1207-1215.

Shah, S., S. Sander, C.M. White, M. Rinaldi, and C. Coleman. 2007. Evaluation of *Echinacea* for the prevention and treatment of the common cold: a meta-analysis. *The Lancet Infectious Diseases*. 7: 473-480.

Sharma M., J.T. Arnason, A. Burt, and J.B. Hudson. 2006 *Echinacea* extracts modulate the pattern of chemokine and cytokine secretion in rhinovirus-infected and uninfected epithelial cells. *Phytotherapy Research*. 20, 147–152.

Tevini, M. and A.H. Teramura. 1989. UV-B effects on terrestrial plants. *Phytochemistry and Photobiology*. 50:479-487.

Turner R.B., R. Bauer, K. Woelkart, T.C. Hulsey, and J.D. Gangemi. 2005. An evaluation of *Echinacea angustifolia* in experimental rhinovirus infections. *The New England Journal of Medicine*. 353: 341-348.

Wagenius, S. 2006. Scale dependence of reproductive failure in fragmented *Echinacea* populations. *Ecology*. 87:931-941.

Zheng, Y., M. Dixon, and P.K. Saxena. 2006. Growing Environment and Nutrient Availability Affect the Content of Some Phenolic Compounds in *Echinacea purpurea* and *Echinacea angustifolia*. *Planta Medica*. 72: 1407-1414.