Phytochemical localization and fungal inhabitants in the fruits of four Echinacea taxa

by

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Abstract

Relatively little is known about the fruits of Echinacea, as they are not traditionally used in medicine. Therefore, the goal of this study was to investigate aspects of the fruits of four Echinacea taxa native to North America: *E. purpurea, E. pallida var. angustifolia, E. pallida var. pallida,* and *E. atrorubens var. paradoxa.* The alkylamide and phenolic contents of the fruits were analyzed, showing that Echinacea fruits yield valuable metabolites which could, with additional research, be incorporated into a variety of natural health products. Additionally, the effect of the fruit coatings on germination rates under different conditions was studied. Fungal endophyte and epiphyte species present within these fruits were identified to gain a better understanding of how these microbes may contribute to the health of the plant, phytochemical content, and the overall quality of Echinacea herbal products.

Acknowledgements

A huge thank you to everyone who helped me out with this project, it wouldn't have been possible without you! Thanks in particular to Emma for sequencing troubleshooting and for sharing her pathogen strains, to Veronica and Savanah for the isolation and assay work, to Johnathan for help with fungal IDs, and to Rui and Ammar for demystifying HPLC. Shout out to Kaitlyn for joining me in the struggle! Finally, thanks to my family for their encouragement, and to my supervisors who supported me throughout this endeavor.

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1 Chapter: Echinacea and its modern medicinal uses

Echinacea has been growing in popularity over recent decades, currently making up approximately 10% of the total herbal products market in North America (Romero *et al.* 2009). There are 10 different taxa of Echinacea, 4 species and 8 varieties, according to a recent classification by Binns and others in 2002, each having different morphology, range, and phytochemistry (Binns *et al.* 2002 c). Commercial Echinacea preparations most commonly contain one or a mix of three of these taxa: *E. purpurea, E. pallida var. angustifolia*, and *E. pallida var. pallida*. Of these three, *E. purpurea* is used most frequently, making up about 80% of commercial production (Qu *et al.* 2005).

Originating in central North America, Echinacea was used by First Nations groups as a treatment for cuts, burns, cough, throat infections, and fungal infections such as thrush (Shemluck 1982). Despite its many historical uses, most Echinacea products are currently advertised as non-specific immune stimulants for the prevention of respiratory infections due to regulatory restrictions in the United States (Stuart & Wills 2003). However, Echinacea extracts have shown a remarkable variety of applications, including analgesic, anti-inflammatory, and antibacterial activity (Abbasi *et al.* 2007a, Cruz *et al.* 2014). Recent studies have confirmed the antifungal activity of Echinacea, supporting its ethnobotanical use. Echinacea extracts effectively inhibit the growth of *Candida*, even strains resistant to amphotericin B (Merali *et al.* 2003). Other fungal species inhibited by Echinacea extracts include *Botrytis cinerea* and *Fusarium oxysporum* (plant pathogens), and *Trichophytan mentagrophytes*, *Aspergillus fumigatus*, and *Penicillium brevicompactum* (animal pathogens) (Dahui *et al.* 2011, Merali *et al.* 2003, Zabka *et al.* 2011, Shama *et al.* 2008).

This variety of uses can be attributed to the array of phytochemicals produced by Echinacea including, but not limited to, alkylamides, echinacoside, caffeic acid derivatives (cichoric, caftaric, and chlorogenic acid, CADs), cynarin, and polysaccharides. A large body of literature on the effects of these metabolites has accumulated and been thoroughly reviewed (Murthy *et al.* 2014, Manayi *et al.* 2015), though new uses continue to be reported. Briefly, echinacoside, polysaccharides, and cichoric acid, are reported, through various pathways, to stimulate the immune system, while alkylamides are anti-inflammatory. Cichoric acid, caftaric acid, and echinacoside also function as strong antioxidants. Cynarin aids in digestion and can help lower cholesterol, and chlorogenic acid inhibits intestinal glucose uptake. Both CADs and alkylamides are also thought to be antimicrobial. To our knowledge, no studies have looked at the effects of CADs alone, but Cruz and others (2014) confirmed that alkylamides have antifungal activity, functioning by disrupting the cell wall/membrane complex of *Saccharomyces cerevisiae*.

Echinacea supplements on the market today are commonly an extract of the roots, aerial parts, or both, depending on the brand (Qu *et al.* 2005). However, different commercial brands use plants grown under a variety of conditions, with different harvesting times and preparation methods, leading to problems with product standardization (Abbasi *et al.* 2007b, Stuart & Wills 2003, Jones *et al.* 2009). There are also inherent genetic and phytochemical differences between Echinacea species and varieties. For example, *E. purpurea* roots completely lack echinacoside, a common constituent in other species, but have very high levels of certain alkylamides, of which only trace amounts are found in *E. pallida* var. *pallida* and *E. pallida* var. *angustifolia*

(Binns *et al.* 2002b, Murch *et al.* 2006). Cichoric acid tends to be highest in *E. purpurea,* and cynarin is found mainly in *E. pallida* var. *angustifolia* (Murthy *et al.* 2014). Due to obligatory outcrossing and environmental effects, there can also be significant variation between individuals of the same taxa, particularly in *E. pallida* var. *angustifolia* (Kapteyn *et al.* 2002, Binns *et al.* 2002 a, Abbasi *et al.* 2012, Chuang *et al.* 2010, Liu *et al.* 2006).

FLOWERHEADS

Purpurea	Angustifolia	Pallida	Paradoxa
CADs	CADs	CADs	**Alkylamides**
Alkylamides	Alkylamides	Alkylamides	**Echinacoside**
	Echinacoside	**Echinacoside**	

STEMS

Purpurea	Angustifolia	Pallida	Paradoxa
Alkylamides	*Alkylamides*	?	?
Cichoric acid	*Cichoric acid*		

LEAVES

Purpurea	Angustifolia	Pallida	Paradoxa
Cichoric acid	Cichoric acid	**Cichoric acid**	Alkylamides
Caftaric acid	Caftaric acid	*Alkylamides*	Echinacoside
Cynarin	*Cynarin*		

ROOTS

Purpurea	Angustifolia	Pallida	Paradoxa
Alkylamides	**Echinacoside**	Echinacoside	**Echinacoside**
Cynarin	**Cynarin**	*Alkylamides*	CADs
			Alkylamides

Figure 1.1: Localization of bioactive compounds in *E. purpurea, E. pallida var. angustifolia, E. pallida var. pallida,* and *E. atrorubens var. paradoxa.* Compounds in bold are present at high concentrations; italics indicate trace content (Binns *et al.* 2002 b, Erelner *et al.* 2015, Sloley *et al.* 2001, Kabganian *et al.* 2003, Stuart & Wills 2003)

Different parts of individual plants likewise show distinct secondary metabolite profiles (Figure 1.1). In *E. purpurea* and *E. pallida* var. *angustifolia,* the roots generally have the highest levels of alkylamides, echinacoside, and cynarin, whereas flowerheads contain the highest concentration of CADs. The leaves have low levels of cichoric acid, caftaric acid, and cynarin, and are rarely used in natural health products (Chen *et al.* 2009, Qu *et al.* 2005, Stuart & Wills 2003, Kabganian *et al.* 2003). This pattern of localization differs from *E. atrorubens* var. *paradoxa,* where most of the alkylamides are located in the flowerheads, and both flowerheads and roots contain high concentrations of echinacoside (Chen *et al.* 2009). *E. pallida var. pallida* flowerheads also have fairly high echinacoside content, and this is one of the few Echinacea taxa where the leaves have a substantial content of secondary metabolites, specifically cichoric acid (Erelner *et al.* 2015). Information on the localization of polysaccharides is not available, but they have been isolated from extracts of both the roots and aerial parts of *Echinacea purpurea* (Manayi *et al.* 2015).

Echinacea flower heads, when used in medicine, are taken before seed set (Binns *et al.* 2002b, Qu *et al.* 2005, Stuart & Wills 2003), so there is very limited information available on the fruits of Echinacea. A better understanding of fruit chemistry, physiology, and ecology could prove valuable for cultivation and breeding as well as for the development of value added products for the Echinacea industry. Therefore, the goal of this project was to study key aspects of the fruits of Echinacea. I analyzed four different Echinacea taxa: *E. purpurea, E. pallida var. angustifolia, E. pallida var. pallida,* and *E. atrorubens var. paradoxa,* to compare results between different species as well as between species traditionally considered medicinal or ornamental.

2 Chapter: Analysis of the fruits of Echinacea

2.1 The potential of Echinacea fruits

Seed oil from members of the Asteraceae family, such as sunflower oil, is regularly used for both dietary and industrial purposes. Based on the limited research available, the fruits of Echinacea may also be a source of medicinal phytochemicals and other useful products. Previously, Echinacea fruits have been called achenes, however, more recent classifications state that achenes are derived from superior ovaries (Marzinek *et al.* 2008). Since Echinacea fruits are derived from inferior ovaries, the more appropriate term for Echinacea fruits is cypselae.

The extracted oil from all three commercial Echinacea taxa is high in oleic acid, palmitic acid, and linoleic acid as well as vitamin E, making Echinacea oil very nutritious (Oomah *et al.* 2006, Vandyshev *et al.* 2009). The oil yield ranges from 13-23%, depending on taxa and fruit size, with *E. purpurea* seeds generating the greatest volume of oil. Echinacea extracted oil also contains a number of medicinal compounds including germacrene-D, which possesses antimicrobial properties, and alkylamides (Mirjalili *et al.* 2006, Oomah *et al.* 2006). Although *E. pallida* var. *pallida* contains very little, the cypselae of *E. purpurea* and *E pallida* var. *angustifolia* contain 0.75 and 1.06 mg of alkylamide isomer pair dodeca-2E,4E,8E,10E/Z tetraenoic acid isobutylamide per gram, respectively (He *et al.* 1998). Even though I could not find any studies specifically measuring phenolic content of Echinacea fruits, caffeic acid derivatives are routinely derived from flower heads, and are present in flower heads following seed set, albeit at lower concentrations (Liu *et al.* 2007). However, localization of either the oils or metabolites within the cypsela has yet to be adequately explored.

The most recent study on the localization of alkylamides in Echinacea examined alkylamide content in a total of 36 different Echinacea tissues. Particularly high concentrations of alkylamides were found in petals and disc flowers, and moderate concentrations were also noted in receptacles of mature flower heads. However, this study did not analyze flower head or seed samples after seed set (Rizhsky *et al.* 2016). To our knowledge, the only authors that have touched on the topic of Echinacea cypselae anatomy and phytochemical localization are Schulthess *et al.* (1991). They found that most of the alkylamides are located in the fruit wall around the seed which is shed during germination.

With further examination of the cypselae, it was determined that this outer coating comprises two distinct layers, the perianth and pericarp, which result from the inferior ovary location within the flower (Marzinek *et al.* 2008). The perianth is a porous, woody material which is derived from the corolla and calyx. Interior to the perianth is the pericarp, which is a thin layer derived from the ovary wall. Schulthess described "secretory canals" running in the ribbing of the cypselae between the seed and the pericarp, based on cross-sections of the cypselae. When the whole cypselae are dissected, these structures appear to be more like glands arranged in rows held together by fibrous material along the length of the seed. Therefore I refer to these structures as "secretory glands" rather than canals (figure 2.1).

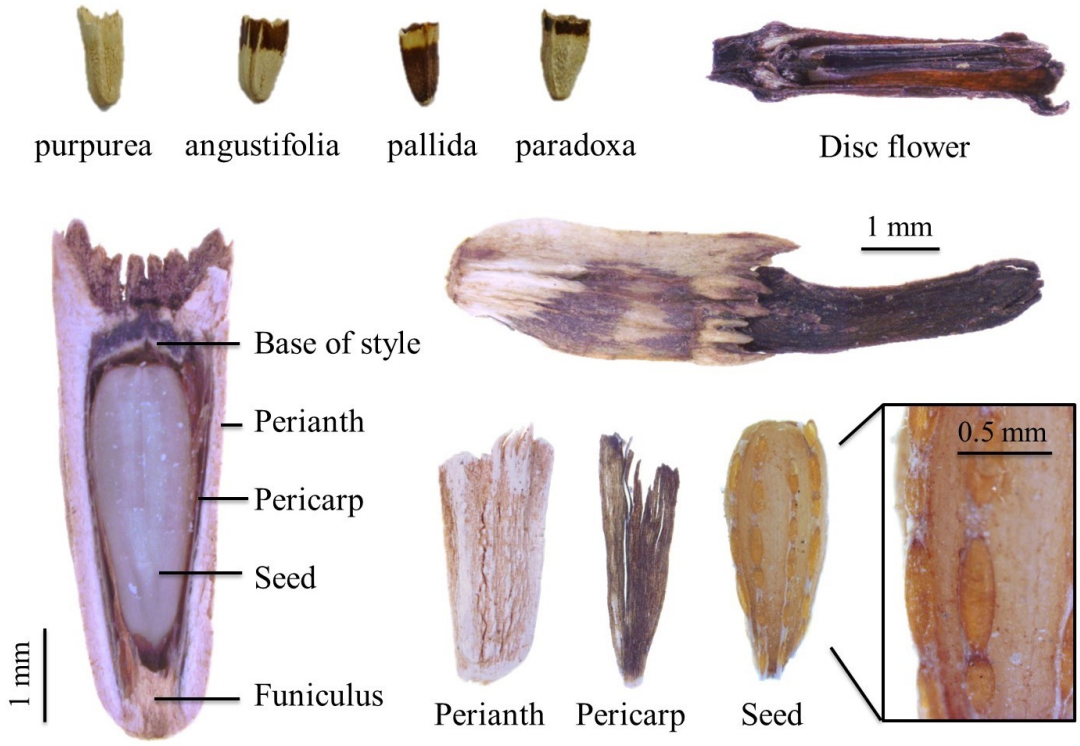

Figure 2.1: External colouration of cypselae from different Echinacea taxa, fruit and flower attachment, and internal cypselae and disc flower anatomy of *E. purpurea*, with detail of the secretory glands along the outside of the seed. Images captured using a Zeiss KL 2500 LCD microscope with an axiocam 1Cc1 camera.

Further study of compounds within the fruits and flower heads would be beneficial to gain a better understanding of phytochemical localization in regards to plant and seed development, and to facilitate diversified uses for Echinacea in medicinal products. This chapter includes analysis of alkylamide and phenolic content in the various portions of the cypselae, to determine where these metabolites are localized. Both of these groups of compounds are active in natural health products, therefore information on their localization in Echinacea is useful for developing high quality medicines and value-added products from often unused plant material. Additionally, alkylamides and CADs are thought to be antimicrobial, and therefore may contribute to

the protection of developing seedlings. Therefore, germination studies were conducted aimed at determining the effect of the removal of cypselae coatings on germination under various conditions, comparing the germination of whole fruits in soil to seeds on soil and seeds on sterile agar.

2.2 Echinacea cypselae analysis methods

2.2.1 Plant material

Four Echinacea taxa were used in these studies. *E. purpurea* and *E. pallida* var. *angustifolia* were provided by Trout Lake Farms (Trout Lake, WA, USA, harvested in 2011). *E. pallida* var. *pallida,* and *E. atrorubens* var. *paradoxa* were purchased from Richters Herbs (Goodwood, ON, Can., purchased March 2015). Cypselae are defined as the intact seed, perianth and pericarp, as it was harvested from the plant (Figure 2.1).

2.2.2 Cypsela layer metabolite extraction

Approximately 40 cypselae from each of the four Echinacea varieties were dissected with forceps and a razor blade into perianth, pericarp, and seed fractions (Figure 2.1). The weight of each fraction was between 0.115 g and 0.220 g for seeds, 0.018 g to 0.056 g for pericarp, and 0.045 g and 0.057 g for perianth. Metabolites were extracted in 2 ml of 80% ethanol (HPLC grade) in glass vials. Each vial was vortexed for 15 seconds, and then placed in a Branson 5210 sonicator bath for thirty minutes, briefly vortexed again, and then placed on a shaker for approximately three hours at 250 RPM. The samples were allowed to settle for 30 minutes, and the supernatant was then transferred to a fresh glass tube using a glass Pasteur pipette. This protocol was repeated, and the two extracts were pooled for a total of approximately 4 ml for each fraction. Following the extraction of the seed fraction, which is termed the “outer seed” extract,

the seeds were crushed with a mortar and pestle and a forth extract of the crushed seeds was prepared as described above to produce an "inner seed" extract.

All extracts were dried under nitrogen, then re-suspended in 1 ml of methanol (HPLC grade) and passed through a 0.2 μm syringe filter (Chromatographic Specialties Inc., Ontario, Canada) prior to HPLC analysis.

2.2.3 Cypsela layer phytochemical analysis

An Agilent 1100 series high pressure liquid chromatograph was used, with a LUNA C18(2), 150 × 2.1 × 5 μm column and a linear solvent gradient of water and acetonitrile (with 0.1% trifluoroacetic acid in each) was used. Gradient protocol was 10% acetonitrile for 1 minute, linear transition from 10-100% acetonitrile over 19 minutes, then holding at 100% acetonitrile for 5 minutes. Column conditions were 0.4 ml/min, 220 bar and 50 °C. Agilent 1100 series diode array detector (DAD, λ = 268 nm for alkylamides and λ = 330 nm for phenolic compounds) was used to qualitatively analyze the extracts with Chemstation software (Agilent Technoligies, Ontario, Canada). Concentration of phytochemicals was determined as peak area divided by dry mass of tissue extracted. The limit of detection was determined to be 15 units, and so smaller peak areas were not considered (given a value of 0).

2.2.4 Germination tests

For each of the four Echinacea taxa, 25 whole cypselae were sown directly in BX Pro-mix (Premier Horticulture Inc., Quakertown, PA) potting soil and placed in the greenhouse. Another 25 cypselae of each taxon were de-coated (pericarp and perianth removed) to obtain seeds. Seeds were sterilized with 3% sodium hypochlorite for 15 minutes, immersed in 70% ethanol for 30 seconds, and then rinsed thoroughly with sterile

distilled water three times. Sterile seeds were sown on a water agar medium and kept out of direct light. Germination of whole cypselae in soil and seeds on water agar was recorded daily, as was total seedling survival after 30 days. Tests of whole cypsela germination on media were impeded by challenges with contamination, and the data was not included. Differences in germination were assessed using a chi-square test with the statistics program R. In a second experiment, germination rates were determined for 15 whole cypselae and 15 sterilized seeds sown in BX Pro-mix soil in the greenhouse.

2.3 Cypselae analysis results

2.3.1 Cypsela layers and phytochemical analysis

Due to the number of different alkylamides, and the fact that several of them are isomers, definitive isolation and identification of all peaks using HPLC-DAD is complicated by co-eluting and isobaric compounds. Alkylamide peaks from *E. purpurea* root extract were given numbers between 1 and 7, which will be used as a reference to identify peaks in the extract analysis (figure 2.2). The identities of these alkylamides are 1 and 2: Undeca-2E/Z,4Z/E-diene-8,10-diyonic acid isobutylamide, 3: Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide, 4: Undeca-2E,4Z-diene-8,10-diyonic acid 2-methylbutylamide, 5: Dedeca-2E,4Z-diene-8,10-diyonic acid 2-methylbutylamide, 6 and 7: 2E,4E,8Z,10E/Z-dodecatetraenoic acid isobutylamide. The same was done for the identification of phenolic compounds (figure 2.3). Although some of the peaks in the 330 nm chromatogram could not definitively be identified, based on their UV absorbance profile they are likely caffeic acid derivatives, or other phenolics.

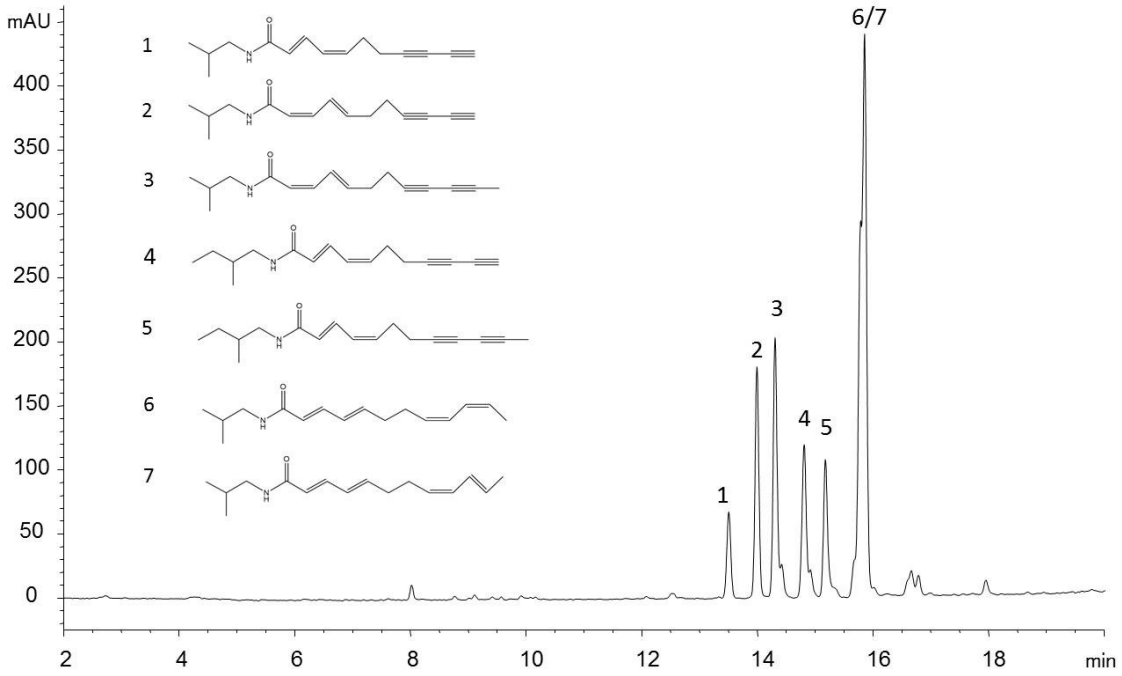

Figure 2.2: HPLC-DAD chromatogram (λ = 268 nm) of *E. purpurea* root extract used as a reference for the identification of alkylamides in the cypsela layers, with structures of the principle alkylamides found in *E. purpurea* root extract and Echinacea cypselae

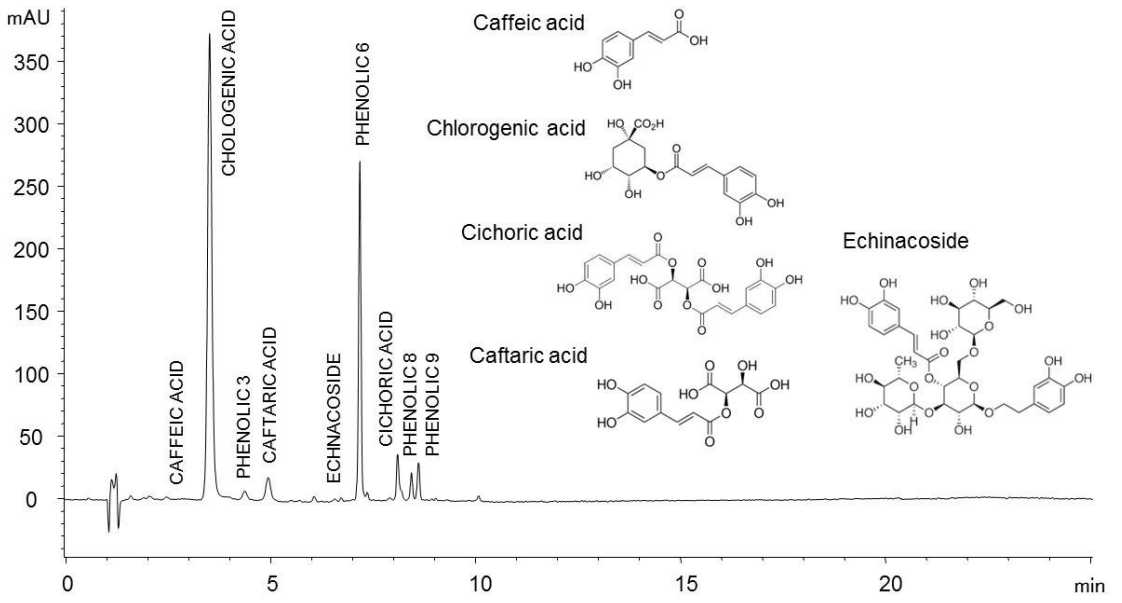

Figure 2.3: HPLC-DAD chromatogram (λ = 330 nm) of *E. pallida var. angustifolia* crushed seed ethanolic extract, as a reference for the phenolics in the cypsela layers of Echinacea with structures of the principle phenolics found in Echinacea cypselae

In every Echinacea taxon and in all cypsela parts, alkylamides 1, 4, and 6/7 were present in various concentrations. Only a tiny amount of alkylamide 3 was present in the cypselae of *E. purpurea* and *E. pallida var. angustifolia*. Alkylamides 2 and 5 were only found at detectable concentrations in *E. atrorubens var paradoxa*, which had the greatest overall diversity of alkylamides (figure 2.4). When comparing alkylamide content between the various cypsela parts, the pericarp extract consistently contained the highest concentrations, always making up at least half of the total alkylamides in the cypsela. The outer seed and perianth extracts were similar in terms of their alkylamide concentration, but the division of alkylamides between these two differs among taxa. For example, the outer seed extract has a higher concentration of alkylamides than the perianth extract in *E. purpurea*, but not in *E. atrorubens var. paradoxa*. The inner seed extract invariably contained very low concentrations of alkylamides.

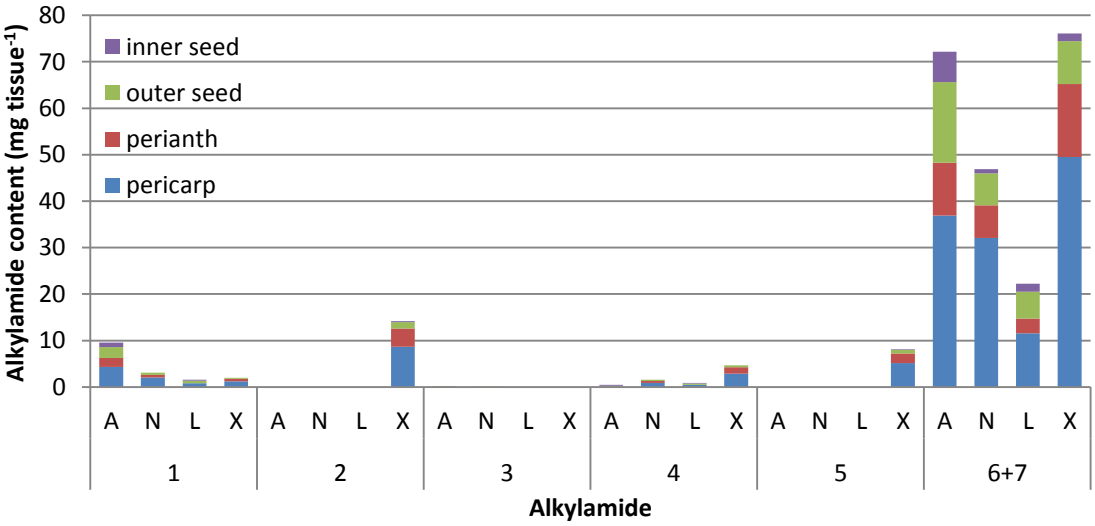

Figure 2.4: Localization of alkylamides within the cypselae of *E. purpurea* (A), *E. pallida var. angustifolia* (N), *E. pallida var. pallida* (L), and *E. atrorubens var. paradoxa* (X) Based on the area under the peaks from the HPLC-DAD chromatogams (λ = 268 nm). Bars represent contribution of individual portions of the cypsela to the total amount of the specific alkylamide.

Several phenolic compounds were detected in the cypselae of Echinacea, including the three CADs, and echinacoside. Chlorogenic acid was particularly concentrated in the inner seed extracts, as well as in the pericarp extracts of *E. pallida var. angustifolia*. Indeed, in comparison to alkylamides, the inner seed extract is quite rich in phenolics, making a substantially larger contribution to total phenolic content in every taxa. In the case of several phenolics, one third more of the total content is found in the inner seed extract, particularly phenolic compound 6 and chlorogenic acid. *E. pallida var. angustifolia* and *E. pallida var. pallida* contain a substantial portion of their phenolic compounds in the perianth extract, especially caffeic acid, phenolic 3, echinacoside, cichoric acid and phenolic 8. The outer seed extract was generally a small contributor to total phenolic content. In contrast to alkylamides, the pericarp extract contained only moderate amount of phenolics, though this changed considerably between taxa. For example, the pericarp extract contains almost 1/3 of the chlorogenic acid in *E. pallida var. angustifolia*, but had no echinacoside in the case of *E. purpurea* or *E. pallida var. pallida*. In general, *E. pallida var. pallida* holds little of any phenolic compound in the pericarp (figure 2.5).

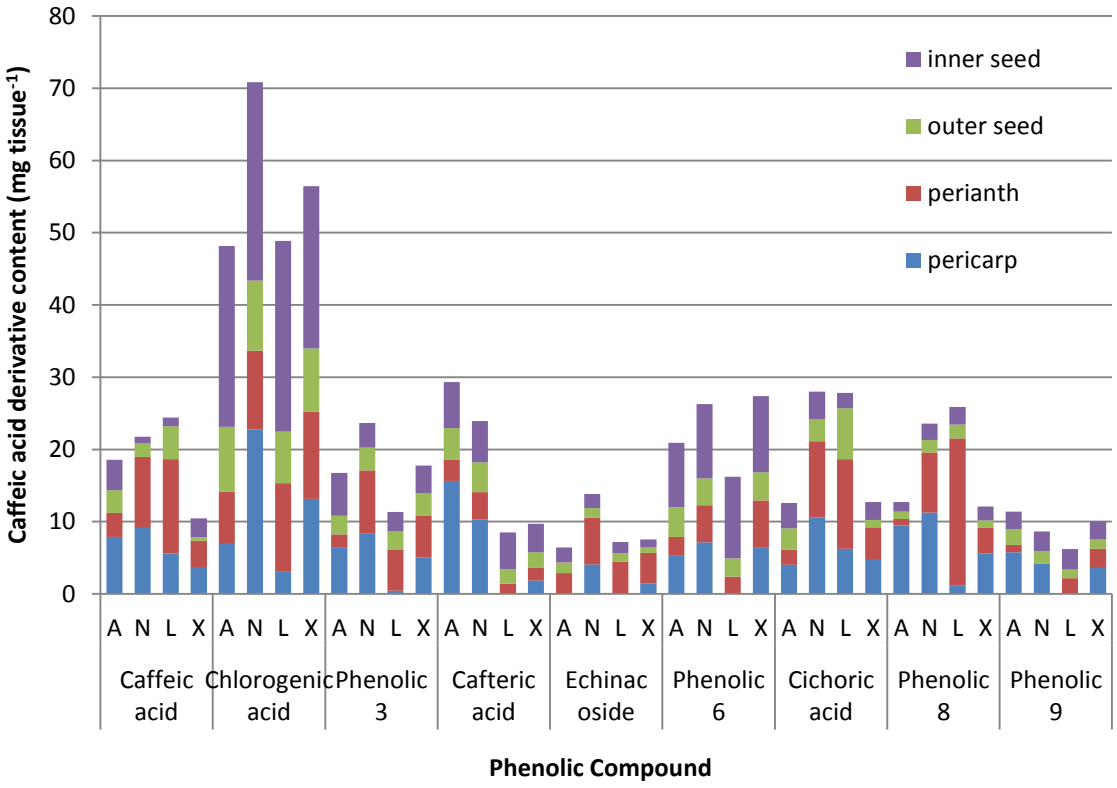

Figure 2.5: Localization of phenolics within the fruits of *E. purpurea* (A), *E. pallida var. angustifolia* (N), *E. pallida var. pallida* (L), and *E. atrorubens var. paradoxa* (X). Based on the area under the peaks from the HPLC-DAD chromatogams (λ = 330 nm). Bars represent contribution of individual portions of the fruit to the total amount of the specific phenolic.

When comparing the sum content of alkylamides (based on peaks identified in figure 2.2) in cypselae between Echinacea taxa, *E. atrorubens var. paradoxa* had the highest content and greatest diversity of alkylamides. *E. purpurea* had the next greatest content, having a similar content of alkylamide 6/7 and a higher content of alkylamide 1, but containing no detectable amount of other alkylamides. *E. pallida var. pallida* cypselae contained the lowest concentration of alkylamides, with only around one quarter the content of *E. atrorubens var. paradoxa*. This contrasts with the results for phenolic content (based on peaks identified in figure 2.3), where *E. pallida* var. *angustifolia* cypselae had the highest aggregate content. *E. purpurea* and *E. pallida var. pallida*

cypselae had virtually identical phenolic concentrations (despite differing ratios of individual phenolics), adding to approximately 75% of the concentration in *E. pallida* var. *angustifolia*. Finally, *E. atrorubens var. paradoxa* cypselae had the lowest phenolic content, though it was only slightly less than *E. purpurea* and *E. pallida var. pallida*. (figure 2.6).

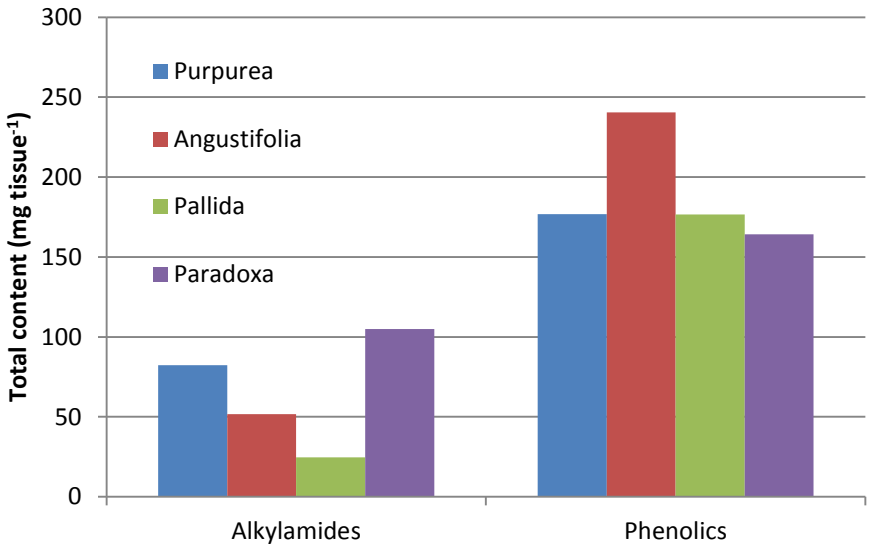

Figure 2.6: Total measured alkylamides and phenolics, sum of areas under the peaks of HPLC-DAD chromatograms, in all cypsela tissues for *E. purpurea, E. pallida var. angustifolia, E. pallida var. pallida,* and *E. atrorubens var. paradoxa.*

2.3.2 Germination:

When placed in soil, intact cypselae of *E. purpurea* and *E. pallida var. pallida* had higher frequencies of germination than did those of *E. pallida var. angustifolia* and *E. atrorubens var. paradoxa* (table 2.1). Overall, seeds with perianth and pericarp removed had low germination compared to intact cypselae when germinated in the soil. Intact cypselae of *E. purpurea* germinated at a significantly higher rate than seeds. Of

the seeds that germinated in soil, several did not survive until the end of the 30 day trial period. Intact *E. pallida var. pallida* cypselae had a borderline significant (p=0.05011) increase in germination compared to the seeds, but this increase became significant when taking into account overall survival.

For all Echinacea taxa there was a significantly greater germination frequency in seeds sown in sterile agar medium in comparison to those sown in soil (chi-squared test, $p < 0.05$). *E. purpurea* seeds germinated at a slightly lower rate on medium compared to whole cypselae on the soil, though this difference was not significant (table 2.1). When whole cypselae were sown on agar media, they were overtaken by the growth of endogenous microbes and none were able to germinate.

Table 2.1: Germination rate of Echinacea seedlings during a 30 day trial period, under three different conditions. 'Seed' refers to cypsela with perianth and pericarp removed. n=35, 15, 25 for cypsela-soil, seed-soil, and seed-agar treatments, respectively. Superscript letters denote significance at $p = 0.05$

	Germination (%)		
	Cypsela - Soil	Seed - Soil	Seed - Agar
E. purpurea	75.3^a	6.7^b	63.0^a
E. pallida var. angustifolia	11.3^a	6.7^a	93^b
E. pallida var. pallida	62^a	26.7^a	93^b
E. atrorubens var. paradoxa	0^a	6.7^a	87^b

At the end of the 30 day germination period, *E. purpurea* and *E. pallida var. pallida* appeared more robust after emerging from the soil. *E. purpurea* seedlings were generally the healthiest, the fastest growing, and were eventually the first to produce flowers. In comparison, the few *E. pallida var. angustifolia* and *E. atrorubens var. paradoxa* plants that survived had slower growth, and developed fewer leaves. All seedlings that were germinated in sterile medium tended to be a bit delicate, with leaves

and roots that were smaller than those grown in the soil. However, they were able to be transplanted into the soil after a few weeks of growth in nutrient medium. Most of the seedlings that were transferred to soil survived, and resembled soil grown seedlings after a couple months of growth.

2.4 Discussion: cypsela morphology, phytochemistry and germination

Since Echinacea cypselae do not currently have commercial value as a source of phytochemicals, there has been very little study on their phytochemical content. To remedy this knowledge gap, I determined whether Echinacea cypselae are a potential source of medicinal products by assessing alkylamides and phenolics in the various parts of the cylselae.

Echinacea cypselae contain several bioactive metabolites, including alkylamides, caffeic acid derivatives, and low levels of echinacoside. The localization of individual compounds within the cypsela is different for phenolics and alkylamides. Alkylamides are more concentrated in the pericarp, whereas phenolics are localized more in the inner seed and perianth. While this may theoretically allow for the isolation of several different extracts from the cypsela, each enriched with a unique class of compounds, the process of dissecting the seeds was extremely time consuming and would not be a feasible process for producing natural health products. Previous studies have noted that in oil extracted from Echinacea, fruits contain enough of these metabolites to have a medicinal effect in rodents (Yu *et al.* 2013). Therefore, extraction of the whole fruit would be the most efficient way to derive medicinal products from Echinacea cypselae.

While our methods may have overestimated the weight of outer seed tissue (since the weight of the whole seed was considered), the outer seed extract consistently had

considerable alkylamide content. This leads me to believe that metabolites, particularly alkylamides, may be concentrated in the secretory glands around the seeds (figure 2.1). In the future, the glands should be isolated and re-tested to confirm. Alkylamides are strong defensive metabolites, so concentration of alkylamides in the cypsela coatings likely helps to protect the seed. In contrast, phenolics were distributed throughout the cypsela, with the inner seed extract having relatively higher phenolic content compared to the outer seed extract. It is unclear whether this is due to the fact that phenolics are localized in the interior of the seed, or whether crushing the seed liberated these metabolites from the tissues.

In general, I found that *E. atrorubens var. paradoxa* contains a greater concentration and diversity of alkylamides than any of the other taxa (figure 2.4). *E. atrorubens var. paradoxa* differs from the commercial species in that its phytochemicals, particularly alkylamides, are concentrated in the flower head, which could explain the discrepancy (Chen *et al.* 2009). Conversely, *E. atrorubens var. paraxoda* cypselae contained the lowest concentration of phenolics, although not greatly different from *E. purpurea* and *E. pallida var. pallida*. This is somewhat unusual as the flower heads of *E. atrorubens var. paraxoda* are generally lacking in caffeic acid derivative, whereas *E. purpurea* and *E. pallida var. pallida* are known to be relatively rich (figure 1.1) (Binns *et al.* 2002 b, Erelner *et al.* 2015). The phytochemical content in Ecinacea cypselae varies somewhat from the phytochemical content in the flower heads, where *E. purpurea* has a much higher content of caffeic acid derivatives, and both *E. pallida var. pallida* and *E. atrorubens var. paradoxa* have substantial amount of echinacoside (figure 1.1) (Binns *et al.* 2002 b, Erelner *et al.* 2015, Sloley *et al.* 2001, Kabganian *et al.* 2003, Stuart & Wills

2003). However, it is not unexpected for the content and ratios of phytochemicals to change during the maturation of the seed heads. Additionally, since it has been demonstrated that Echinacea phytochemical content can vary, these results may vary with a different seed source.

This study did not determine whether there was a role of these metabolites in germination, or what that role may be, however this would be an interesting topic to pursue. To my knowledge there have been no studies analyzing the effects of alkylamides on germination. However, evidence has been found that alkylamides may act as a plant growth regulator, promoting the development of root systems in *Arabidopsis* (Lopez-Bucio *et al.* 2006). Several phenolics are considered allelochemicals, and are known to be inhibitory to the germination of seedlings. This includes caffeic and clorogenic acid, which can moderately inhibit the germination of several crop and weed species (Williams & Hoagland 1983).

There have been several studies looking at the germination of Echinacea species and how it can be improved with various treatments (Chuanren *et al.* 2004, Parmenter *et al.* 1996, Romero *et al.* 2005). In agreement with this study, untreated *E. pallida var. pallida* and *E. purpurea* cypselae germinate at rates of around 70%-85%, and appear to have no stratification requirement (table 2.1). On the other hand, *E. pallida* var. *angustifolia* germination rates can be as low as 0-5% but stratification treatments can improve germination to rates comparable to *E. purpurea* and *E. pallida var. pallida* (Chuanren *et al.* 2004, Parmenter *et al.* 1996, Romero *et al.* 2005). To my knowledge, no papers have been published assessing the germination of *E. atrorubens var. paradoxa.*

In this germination experiment, cypselae were not pre-treated with a stratification period (the seed I received from Trout Lake Farms and Richters had not been stratified), and so the germination rates of the whole cypselae on the soil are comparable to rates found in other studies. No cypselae of *E. atrorubens var. paradoxa* germinated indicating that, similar to *E. pallida* var. *angustifolia*, cypselae of this variety may require long stratification periods (table 2.1). In the future, it may be beneficial to repeat these germination trials with stratification pre-treatment in order to more clearly see the results for *E. pallida* var. *angustifolia* and *E. atrorubens var. paradoxa* in the soil treatments. Nevertheless, comparatively high germination and seedling survival rates were achieved when cypsela coats were removed and seeds were sown on agar medium.

When bare seeds were sown in soil, germination rates generally decreased in comparison to cypselae in soil. Notably, some seeds in the soil treatment rotted away before the end of the 30 day trial period, suggesting that the cypsela coats may enhance germination by protecting germinating seeds from environmental microbes. Parmenter and others (1996) found that while partially removing seed coats from *E. pallida* var. *angustifolia* cypselae did not affect germination of non-stratified seeds, germination was halved in treated seeds which had experienced two or more weeks of stratification. It is possible this additional time was sufficient to allow microbes to colonize the (initially sterile) soil and infect the seedlings. Given these results, it is likely that the perianth and pericarp coatings act as a physical barrier to infection by environmental microbes, since removing part of the coat results in decreased germination. These coatings may also be providing chemical defense, as they contain antimicrobial alkylamides and CADs. Germinating seedlings need to produce these defensive metabolites *de novo*, and do not

produce levels of alkylamides comparable to the whole cypsela until they are 14 days old (Schulthess *et al.* 1991). These cypsela coatings also contain microbial species which, as discussed in subsequent chapters, may play a role in the defense of the seed and in overall plant health.

Conversely, germination rates of seeds on agar medium were significantly increased, with rates over 85% germination (table 2.1). Increases in germination upon removal of the cypsela coatings has previously been noted when *E. pallida var. angustifolia* is sown on media or filter paper (Chuanren *et al.* 2004, Sorensen & Holden 1974). Since there were no nutrients in the germination medium in this study, this increase must have been due to some other factor. It has been noted that high humidity, as would have been found in the sealed plates, can increase germination rates (Romero *et al.* 2005). However, there are also several seed-coat associated factors which impose dormancy and could have limited germination in whole cypselae, such as mechanical constraint, limited access to water, or production and retention of inhibitors (Kelly *et al.* 1992). Since the entire coat was removed in this study, it is impossible to determine which of these factors could have resulted in increased germination with removal of the coats. More research will be necessary to determine whether or not the cypsela coats impose dormancy, and if so, by what mechanism.

3 Chapter: Fungal species inhabiting Echinacea cypselae coatings

3.1 Introduction to Echinacea's fungal inhabitants

Nearly all plant life on Earth has developed close relationships with endophytes, which are diverse assemblages of microbes that asymptomatically inhabit plant tissues (Wilson 1995), and epiphytes, which colonize the surface of the plant (Lindow & Brandl

2003). Endophytes can be found inter- or intracellularly within most plant tissues, including the roots, leaves, stem, and flowers (Moszczynska *et al.* 2013, Lata *et al.* 2006, Saikkonen *et al.* 1998, Aly *et al.* 2011). Although these colonists inhabit different spaces within the plant, both endophytes and epiphytes can contribute to the functional characteristics of a plant, with plant phenotypes often a product of both plant and microbial gene expression (Partida-Martinez & Heil 2011, Friesen *et al.* 2011).

Plant-associated microbes can exist in a range of interactions with the plant, from incipient parasites and decomposers to mutualists or neutral colonists. These interactions may shift over the lifetime of the plant, leaf or tissue, and with ecological conditions (Yuan *et al.* 2011, Saikkonen *et al.* 1998, Carroll 1988, Aly *et al.* 2011, Lindow & Brandl 2003). Even where these microbes have been identified in plants, there is still a limited understanding of the relationship between endophytes, epiphytes and the host plant, and how they may factor into plant development, phytochemistry, or disease transmission.

There is good evidence to suggest that microbial plant colonists can confer a variety of benefits to the host plant. One of the first benefits to be identified was protection against herbivory by fungal endophytes (Saikkonen 2006, Hyde & Soytong 2008, Vega 2008, Sumarah *et al.* 2010). Both endophytes and epiphytes may provide protection to the host plant against pathogens, either by directly competing (by physical or chemical competition), or by inducing/enhancing the plant defense response (Arnold & Lutzoni 2007, Saikkonen *et al.* 1998, Yamaji *et al.* 2011, Farre-Armengol *et al.* 2016). Improved tolerance to abiotic stresses has also been linked to microbial colonists in several species (Aly *et al.* 2011, Friesen *et al.* 2011). In particular, increased resistance to drought and heavy metal contamination has been noted in plants colonized by endophytes

(Yuan *et al.* 2011). Upregulated production of antioxidant compounds by the plant in response to fungal invasion or production of antioxidants by the endophytic fungi may be responsible for this phenomenon (Yuan *et al.* 2011, Arnold & Lutzoni 2007, Aly *et al.* 2011). Some leaf epiphytic cyanobacteria even have the ability to fix atmospheric nitrogen, making it available to the plant (Friesen *et al.* 2011). It has also been proposed that microbial colonists help to improve germination by producing plant growth hormones or protecting the seedling from environmental pathogens (Aly *et al.* 2011, Friesen *et al.* 2011, Yamaji *et al.* 2001). Therefore, microbes in the cypselae may have an influence on the germination of Echinacea, its growth, or its phytochemical content. It is important to identify these species to understand the impact they may have on Echinacea's cultivation and chemical profiles.

Although no studies have assessed epiphyte populations in Echinacea, an array of fungal endophytes have been identified in Echinacea leaves, roots and stems. These include species from the genera *Alternaria, Aspergillus, Botrytis, Ceratobasidium, Cladosporium, Colletrotrichum, Epicoccum, Fusarium, Glomerela, Gibberella, Mucor, Mycoleoptodiscus, Penicillium, Pleosporales, Phoma, Rhizopus, Sclerotinia, Trichoderma*, and *Ulocladium* (Rosa *et al.* 2012, Moszcynska *et al.* 2013). Several of these genera, including *Alternaria*, *Botrytis*, and *Fusarium* contain species that are harmful plant pathogens, so it is still unclear which of these species may benefit the plant and which may be latent pathogens.

Plant-associated microbial assemblages are often extremely diverse; each plant can contain hundreds of individual species, which may be systemic or localized to a certain tissue (Aly *et al.* 2011, Carroll 1995, Saikkonen *et al.* 1998, Arnold & Lutzoni

2007, Lindow & Brandi 2003). There may also be considerable variation in microbe assemblages between plants. For example, assemblages in mature plants can differ from seedlings, plants in dense groups may have more diverse assemblages than isolated plants, and the same species growing in different regions often have different colonists (Aly *et al.* 2011, Carroll 1995, Frisen *et al.* 2011).

It is possible that differences in microbial assemblages may be partly responsible for variability in the content of active phytochemicals between and within Echinacea species. Indeed, Rosa and others (2012) studied the antifungal activity of extracts from the endophytes they had found colonizing Echinacea roots. Certain extracts, particularly from species of *Fusarium*, had significant antifungal activity when tested against the fungal phytopathogen *Colletotrichum spp*. In several cases, this activity was even stronger than the inhibition caused by industrial antifungals. To determine whether or not plant-associated microbes are also contributing to variability in Echinacea phytochemitry, and to understand the role they play in the defense of the plant, these species and the metabolites they produce must be assessed.

Contamination of Echinacea cypselae with microbes is a well-known phenomenon (Abbasi *et al.* 2007 b, Choffe 2000). It is unknown what effects the microbes colonizing Echinacea cypselae may have on the germination of the plant in field conditions, the transfer of disease, protecting the seed, or the overall health of the plant. Contamination with fungal plant pathogens, for example, is known to be a problem for Echinacea cultivation, especially in humid areas (Kindscher *et al.* 2008). Microbes within the plant may contribute to these difficulties, yet no studies have identified the microbial species in the cypselae. Considering how ubiquitous these

microbes appear to be in Echinacea seed stocks (Abbasi *et al.* 2007 b), it is possible that, rather than being pathogenic, some of them may actually be beneficial.

In this study, a survey of fungal colonists found in and on the cypselae of *E. purpurea*, *E. pallida var. angustifolia, E. pallida var. pallida,* and *E. atrorubens var. paradoxa* was conducted. In addition, endophytes from the roots of *E. pallida var. angustifolia* were identified and compared to the species found in the cypselae and recovered in previous studies. Extracts of the cypselae microbes were further assayed for diagnostic Echinacea phytochemicals and antifungal activity against common plant pathogens. Results provide a clearer understanding of Echinacea's relationship with environmental, epiphytic, and endophytic fungi and could help improve cultivation and disease prevention.

3.2 Methods for microbial colonist survey and antifungal assessments

3.2.1 Survey of fungal species in cypsela coats and roots

Fifty seeds each of *E. purpurea, E. pallida var. angustifolia, E. pallida var. pallida,* and *E. atrorubens var. paradoxa* were dissected using forceps and a razor blade. Small fragments of pericarp and perianth (figure 2.1) were placed on potato dextrose agar (PDA) plates, which were then incubated at room temperature for approximately one week in sterile conditions. During the incubation period, emerging colonies were transferred using a sterile needle to individual plates and grown for several days.

E. pallida var. *angustifolia* seeds were sown in BX Pro-mix soil and allowed to grow for approximately 9 months in the greenhouse. A section of *E. pallida* var. *angustifolia* tap root, 5 – 7 cm in length, was harvested from each of three different plants. Root sections were washed with tap water and air dried for 12 hours. Roots were

then sterilized with 3% sodium hypochlorite for 15 minutes, immersed in 70% ethanol for 30 seconds, and then rinsed thoroughly with sterile distilled water three times. Roots were then thinly sliced with a sterile scalpel blade and transferred to PDA petri plates. Plates were sealed with parafilm and left at room temperature for one week with emerging colonies subsequently transferred to separate PDA plates, as described above.

3.2.2 DNA extraction and species identification

Fungal isolates were separately grown on PDA plates for ~60 days at room temperature. Mycelia were harvested and 1-2 g (wet weight) of mycelium was ground in a mortar with 200 µl of extraction buffer (2% triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl, pH adjusted to 8 using 6 M HCl, 1 mM EDTA). Homogenized samples were then transferred to a sterile microfuge tube and an additional 200 µl of extraction buffer was added. Samples were vortexed briefly and 0.5 g of 0.5 mm glass beads were added to each sample. Tubes were shaken in a Retsch MM301 mixer mill for 2 minutes at maximum speed, and then placed on ice for five minutes and the mixer mill treatment was repeated. Samples were then centrifuged for 10 minutes at 13,000 RPM (r_{ave} = 8 cm, 15, 000 ×g). The supernatant was transferred to a fresh microfuge tube and 400µl of 24:1 chloroform:isoamyl alcohol was added. Tubes were vortexed briefly, centrifuged for 3 minutes at 15, 000 ×g, and the aqueous layer was transferred to a fresh tube. Two volumes of ice cold 95% ethanol were added and tubes were placed on ice for 5-10 minutes. Tubes were centrifuged for 10 minutes at 15, 000 ×g and the supernatant was discarded. Pellets containing nucleic acids were washed gently with 70% ethanol and then dried for 2 minutes under vacuum. Pellets were re-suspended in 200 µl sterile

distilled water and a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, Delaware, USA) was used to estimate DNA content.

Fungi were identified to genus or species level based on DNA sequencing of the nuclear rDNA internal transcribed spacer (ITS) (Toju *et al.* 2012) and subsequent morphological examination by light microscopy. For *Penicillium* species, β-tubulin sequencing was used to confirm species identity. PCR reactions comprised 5 µl of template DNA (approximately 10 ng/µl) in a 50 µl reaction mix of 1x Phusion HF buffer, 200 µM of each dNTP, 0.5 µM each primer, 3% dimethyl sulfoxide, and 1 unit Phusion DNA polymerase. PCR conditions were 95 °C for 6 min, then 35 cycles of 94 °C for 40 sec, 47 °C for 50 sec, 72 °C for 40 sec, and a final extension at 72 °C for 6 min for the ITS sequences and 5 cycles of 94 °C for 60 sec, 68 °C for 90 sec, 72 °C for 120 sec, decreasing annealing temperature 1 °C per cycle, then 25 cycles of 94 °C for 60 sec, 64 °C for 90 sec, 72 °C for 120 sec, with a final 10 min elongation period at 72 °C for the β-tubulin sequence (Toju *et al.* 2012, Samson *et al.* 2004). PCR primer sequences used were ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), ITS5 (5′-GGAAGTAAAAGTCGTA ACAAGG-3′), Bt2a (5′- GGTAACCAAATCGGTGCTGCTTTC -3′), and Bt2b (5′- AC CCTCAGTGTAGTGACCCTTGGC -3′) (Toju *et al.* 2012, Glass & Donaldson 1995).

PCR amplicons were purified (Geneaid Biotech Ltd, New Taipai City, Taiwan) and both strands were sequenced at Genome Quebec (Montreal, QC, Canada). For species identifications, the Basic Local Alignment Search Tool (BLAST) at NCBI was used to identify Genbank entries matching our ITS sequences. Species identities based on ITS sequences were confirmed by Jonathan Mack (Carleton University) by morphological examination of fungal cultures using light microscopy.

3.2.3 Preparation of fungal extracts

Fungal species from Echinacea cypselae were each separately grown in test tubes containing 5 ml of half strength potato dextrose broth for 4 weeks at room temperature. The culture tubes containing mycelium and broth were placed at -20 °C overnight, transferred to -80 °C for one hour and then lyophilized in an E-C Super Modelyo freeze-dryer for approximately 40 hours until dry. Dried samples were transferred to fresh tubes and powdered with a small metal spatula. Five ml of half strength potato dextrose broth was lyophilized and processed as above to act as a PDB carrier control. Final weights of the dried samples ranged between 1-9 mg.

Dried samples were extracted in 10 ml of 70% ethanol. Each sample was vortexed for 15 seconds, and then placed in a Branson 5210 sonicator bath for thirty minutes. Tubes were briefly vortexed again, and placed on a shaker for approximately three hours at 250 RPM before being centrifuged for 5 minutes at 1000 RPM (R_{ave} = 13cm, 150 ×g) in a swinging-bucket rotor. The supernatant was transferred to a fresh tube, concentrated in a speed-vac and then lyophilized.

3.2.4 Detection of Echinacea-like metabolites in fungal extracts

Extracts of each fungal culture were re-suspended in HPLC grade methanol and analyzed using a Shimadzu Ultra performance liquid chromatography – electrospray ionization – mass spectrometry (UPLC-MS) system (Mandel Scientific Company Inc, Guelph, ON, Canada). The system consisted of a binary pump (LC30AD), a column thermostat (CTO20a), a high performance autosampler (SIL- 30AC), a photodiode array detector (SPD-M20A) and a mass selective detector LC2020. Electrospray source was operated in positive and negative ionization modes (Table 1). The nebulizing gas flow

was set at 1.5 L/min and drying gas flow was at 10 L/min. The desolvation line temperature and heat block temperature were set at 300°C and 450°C, respectively. Detection of Echinacea-like substances was attempted by co-chromatographic comparison of the retention time and mass data for reference standards of Echinacea phenolics and alkylamides. Single ion monitoring (SIM) (Shimadzu Lab Solutions software; 5.41.240), based on the mass-charge ratios of positive and/or negative ions observed for reference standards, was used to extract related molecules and detect Echinacea metabolites with more sensitivity. The PBD broth extract was analyzed by the same method and used to eliminate components of the media in the results.

3.2.5 Antifungal assays

Extracts of the fungal cultures were re-suspended at a concentration of ~0.5 mg/ml in 25% ethanol and were tested using broth dilution assays for inhibition of the growth the plant pathogens *Alternaria solani* (196949), *Fusarium oxysporum* (DAOM 215464), and *Botrytis cinerea* (1B907b) (AAFC, Ottawa, ON, Canada).

Twofold dilution series of each extract and the carrier solvent control (25% ethanol) were carried out in a microtitre plate (96-well, flat bottomed). A similar dilution series was done with hygromycin B (initial concentration = 2500 µg/ml; Bioshop, Burlington, ON, Canada) as a positive antifungal control. Dilution series were made up such that the final volume in each well was 100 µl. Stock solutions of macerated mycelium from fungal pathogens in 25% glycerol were diluted in PDB such that there were ~800 colony forming units (CFU)/ml. 100 µl of this diluted pathogen stock (~80 CFU) was added to each well. Microtitre plates were incubated at room temperature for approximately four days. Growth of fungal pathogens was analyzed using a Gen5 plate

reader by optical density readings at 595 nm and visually confirmed by eye. Minimum inhibitory concentrations (MICs) were determined as the concentration of extract which resulted in a 50% reduction in fungal growth compared to control wells of pathogen and media with no inhibitors.

3.3 Results of fungal colonist survey

3.3.1 Isolation of fungi from Echinacea cypselae

Seven fungal species were isolated from the perianth and pericarp of *E. purpurea*. Six were isolated from *E. pallida var. angustifolia* cypselae, with an additional ten species isolated from the roots. Eight species were isolated from *E. pallida var. pallida,* and seven from *E. atrorubens var. paradoxa.* Overall, Fusarium species were the most common, and were isolated from all samples except the cypselae of *E. pallida var. pallida*. In particular, *E. atrorubens var. paradoxa* had numerous Fusarium species. *Rhizopus stolonifera* was obtained from three of the four Echinacea species, and Aspergillus species were isolated from both *E. pallida var. pallida* and *E. atrorubens var. paradoxa*, as well as from the roots of *E. pallida* var. *angustifolia*. Despite these similarities, in several cases a fungus was only recovered from a single Echinacea taxon (Table 3.1). For example, *Rhodotorula mucilaginosa* was recovered only from *E. purpurea*. Notably, there were five different strains of *Trichoderma sp.* isolated from roots of *E. angustifolia*, whereas representatives of this genus were not recovered from any cypselae. *F. oxysporum* was the only species in common between *E. pallida var. angustifolia* roots and cypselae. However, *A. alternata* was found in the roots of *E. pallida var. angustifolia* and the cypselae of *E. purpurea* (Table 3.1). Finally, we were unable to identify two fungi recovered to species (Table 3.1).

Table 3.1: Fungal species identified in the cypselae coatings of four different Echinacea taxa, and in the roots of *E. Pallida var. Angustifolia*

E. purpurea	***E. pallida var. pallida***	***E. atrorubens var. paradoxa***	***E. pallida var. angustifolia***	
Cypsela				**Roots**
Alternaria alternata				*Alternaria alternata*
	Aspergillus flavus, A. niger, Aspergillus sp.	*Aspergillus niger*		*Aspergillus fumigatus*
Aureobasidium pullulans				
	Cladosporium cladosporoides			
Cryptococcus laurentii				
Fusarium sp.		*Fusarium equisetti, F. proliferatum, F. sporotrichoides, F. verticillioides, Fusarium sp.*	*Fusarium equisetti, F. oxysporum, Fusarium sp.*	*Fusarium oxysporum, F. solani*
			Mucor circenelloides	
	Penicillium aurantiogriseum , Penicillium sp.		*Penicillium canescens*	
Phoma sp.				
	Rhizopus stolonifer	*Rhizopus stolonifer*	*Rhizopus stolonifer*	
Rhodotorula mucilaginosa				
	Talaromyces sp.			
				Trichoderma citrinoviride, T. deliquescens, T. hamatum, T. harzianum, Trichoderma sp.
Unknown genus/species				Unknown genus/species

3.3.2 Antifungal activity by fungi isolated from Echinacea cypselae

To determine the role of fungal colonists in protecting Echinacea from environmental microbes, the antifungal effect of extracts from each species was assessed against three fungal plant pathogens. The majority of the fungal extracts demonstrated 50% inhibition of pathogen growth at high concentrations, but this was not significantly different than the carrier alone (table 3.2). However, certain extracts were able to inhibit the growth of at least one of the three plant pathogens tested at much lower concentrations (figure 3.1). These seemed to generally be from the genus Fusarium, though *Penicillium canescens* and *Aspergillus* sp. were effective inhibitors as well. *F. sporotrichoides* was the only extract that effectively inhibited all three plant pathogens; *F. verticillioides* inhibited two out of the three pathogenes (*B. cinerea* and *A. solani*)(table 3.2). In general, *F. oxysporum* seemed to be the least susceptible to inhibition. Five extracts had low MIC50 values for *B. cinerea* and *A. solani*, as opposed to *F. oxysporum*, which was only inhibited by one extract (table 3.2). It is worth noting that several of the species which inhibited *B. cinerea* or *A. solani,* but not *F. oxysporum* were Fusarium species (including the extract of *F. oxysporum* which was isolated as an endophyte from *E. pallida var.* angustifolia). This may indicate that Fusarium spp. have antagonism specific to microbes outside of their own genus. Three of the fungi isolated from the cypselae failed to grow in liquid culture: *Aspergillus flavus, Aureobasidium pullulans*, and the unknown fungi from *E. purpurea*, so there is no data on their inhibition of the fungal pathogens.

Table 3.2: Inhibitory activity of fungal extracts against plant pathogens over a four day growth period (n=1). MIC50 values are the lowest % concentration of the extract which resulted in > 50% growth inhibition when compared to the pathogen grown without inhibitors. ** denotes MIC50 inhibition at a concentration 8× lower than the carrier (25% ethanol).

Extract identity	MIC 50 (%)		
	B. cinerea	*A. solani*	*F. oxysporum*
25% ethanol	**12.5**	**50**	**25**
Hygromycin B (2500 µg/ml)	1.56	0.098	0.024
E. pallida var. angustifolia			
Fusarium equisetti	3.13	25	12.5
Fusarium oxysporum	3.13	6.25**	12.5
Penicillium canescens	1.56**	12.5	6.25
Mucor circenelloides	3.13	25	25
Fusarium sp.	3.13	25	6.25
E. atrorubens var. paradoxa			
Fusarium proliferatum	0.195**	12.5	12.5
Fusarium equisetti	3.13	25	6.25
Fusarium verticillioides	0.195**	0.781**	12.5
Fusarium sporotrichoides	0.781**	6.25**	3.13**
Aspergillus niger	6.25	12.5	6.25
Fusarium sp.	3.13	6.25**	12.5
E. pallida var. pallida			
Cladosporium cladosporoides	6.25	25	12.5
Penicillium sp.	12.5	25	12.5
Aspergillus niger	12.5	25	6.25
Rhizopus stolonifer	3.13	25	12.5
Aspergillus sp.	6.25	6.25**	12.5
Penicillium aurantiogriseum	3.13	25	6.25
Talaromyces sp.	3.13	12.5	12.5
E. purpurea			
Fusarium sp.	3.13	25	6.25
Rhodotorula mucilaginosa	6.25	25	25
Cryptococcus laurentii	6.25	12.5	25
Alternaria alternata	6.25	12.5	12.5
Phoma sp.	6.25	25	25

3.4 Discussion: Fungal inhabitants

Although they have received relatively little research attention, endophytes and epiphytes are now thought to have a much greater impact on plants than previously imagined. Endophytes, in particular, have also become a major source of drug discovery (Elsabai *et al.* 2014). Past studies have surveyed the endophytes present in the roots and leaves of Echinacea, however there has yet to be an analysis of endophytes in Echinacea cypselae. As one hallmark of a symbiotic endophyte is vertical transmission through the propagules (Carroll 1988), identifying endophytes in the cypselae of Echinacea may provide evidence as to which microbes are truly benefiting the plant.

Figure 3.1: Fungal isolates which effectively inhibited pathogenic fungi. A) *F. oxysporum*, B) *F. sporotrichoides*, C) *Fusarium sp.*, D) *F. proliferatum*, E) *P. canescens*., F) *F. verticillioides*, G) *Aspergillus sp.*

I did not surface sterilize the Echinacea cypselae before I dissected them, so species living epiphytically on the surface of the seeds would have also been captured. Surveying epiphytes is equally important as endophytes since epiphytes and spores on the surface or

in the pores of the perianth would be carried on to the next generation of plants through the cypselae, where they may play a role in plant development, phytochemistry, or disease. However, this method makes it impossible to determine which species were endophytes or epiphytes, as it would capture both. Some species can even switch between the two lifestyles. Indeed, several of the fungal species identified are ones which can exist epiphytically on the surface of a plant, entering the tissues to become endophytes when conditions favour the transition. These include *Alternaria alternata*, *Cladosporium cladosporoides*, and species of *Phoma, Aspergillus, Penicillium* and *Aureobasidium* (Arnold 2000, Mukhtar *et al.* 2010).

In this study, seven fungal species were isolated from Echinacea cypselae which had previously been isolated as endophytes from *E. purpurea* leaves, stems, and roots (Rosa et al. 2012, Moszczynska et al. 2013): *A. alternata, C. cladosporoides, F. oxysporum, F. solani, F. sporotrichoides, R. stolonifera*, and *T. hamadum*. Although in this study, not all of these species were recovered from *E. purpurea* (table 3.2). Only four genera isolated from Echinacea cypselae in this study had not previously been reported in Echinacea tissues: *Aureobasidium, Cryptococcus, Talaromyces* and *Rhodotorula*. These genera may be restricted to cypselae or relatively rare as Echinacea colonists in general.

Distinct differences exist between the fungal assemblages of the cypselae and roots of *E. pallida var. angustifolia*. The roots yielded a diverse set of *Trichoderma* isolates, which did not appear in any of the cypselae assemblages. Other than *F. oxysporum*, no species were in common between the *E. pallida var. angustifolia* cypselae and roots. *F. oxysporum* is a generalist plant pathogen and saprotroph and could have

been acquired from the environment, therefore there is no concrete evidence that it was passed directly to the roots. However, disease symptoms were not evident in either the cypselae or the full grown plants, so *F. oxysporum* was not evidently behaving as a pathogen. In any case, the initial assessment does not strongly support the idea that the microbes in the daughter plant were passed down from the cypselae.

It should be noted that this study used a limited sampling of Echinacea materials, and thus provides a preliminary view of microbial distribution patterns in cypsela and roots among Echinacea taxa. Additional sampling of various populations of Echinacea may serve to clarify which of these fungi were beneficial, as assemblages can vary between different populations, but symbionts would likely be ubiquitous in plants throughout Echinacea's range (Carroll 1988).

Overall, microbial colonists in the cypselae of Echinacea are generalist saprotrophic species such as *Aspergillus, Penicillium, Rhizopus, Mucor* and *Talaromyces*; all very common fungal genera in the environment. *Aspergillus* and *Penicillium* are particularly common as airborne spores, and likely colonized the Echinacea cypselae through aerial contact (Hedayati *et al.* 2007, Kung'u 2016, Kwon *et al.* 2001, Naraghi *et al.* 2012, Shukla & Mishra 1992). *Fusarium* can be found in soil, water, and organic materials, and has been found asymptomatically in Echinacea in previous studies (Nucci & Anaissie 2007, Rosa *et al.* 2012). *Rhodotorula mucilaginosa* and *Cryptococcus spp*. are yeasts that are commonly found in aquatic environments (Libkind *et al.* 2003). To my knowledge there is no information on the relationship of these yeasts to plants. However, some endophytes of forest trees have been found in aquatic environments,

potentially needing two different environments to complete their life cycle (Sokolski *et al.* 2006).

A variety of *Trichoderma* species were isolated from the roots of *E. pallia var. angustifolia*. *Trichoderma* are common soil microbes which form symbiotic relationships with the roots of plants, and can parasitize other fungal pathogens (Harman *et al.* 2004). Colonization of plants with *Trichoderma* species has been shown to enhance root growth and productivity, and induces plant defense response which helps protect against pathogens (Harman *et al.* 2004). Since these microbes activate the plant defense response and can't penetrate more than a few cell layers into roots, perhaps explaining why they were not identified in the cypselae (Naraghi *et al.* 2012).

This study, like others using similar methodology, is limited in its capacity to capture the true diversity of the species present in the cypsela tissue. Culture-based studies are inherently flawed in that they favour fast growing microbes, and that symbionts, the species of most interest, may not be able to grow outside of the plant tissue. Some tests have shown that a more diverse array of endophytes will emerge when growth media is supplemented with extract from the host plant (Prior *et al.* 2014, Arnold & Lutzoni 2007). In future experiments, either supplementation of the media with Echinacea extract, or a direct DNA extraction and PCR amplification from the cypsela would be beneficial to further identify symbiotic microbial species.

It is well established that colonization with arbuscular mychorrizal fungi (AMF) benefits plants in terms of growth and increased phytochemical content. This includes Echinacea, where colonization with AMF can significantly increase CAD content (Araim *et al.* 2009, Zubeck &Blaszkowski 2009, Gualandi *et al.* 2014). Although endophytes

and epiphytes are distinct from AMF, they may also have effects on the growth and phytochemistry of the host plant (Gualandi *et al.* 2014). However, the mechanism by which plants benefit is still unclear. The prevailing theory is that fungal colonization can stimulate a defense response in the plant, essentially "priming" the plant to defend itself against pathogenic microbes (Arnold & Lutzoni 2007, Saikkonen *et al.* 1998). However, there has been some evidence to suggest endophytes produce their own defensive compounds and have antifungal activity (Rosa *et al.* 2012).

Although no fungal species isolated from Echinacea cypselae produced compounds which were similar to those found in Echinacea, it is possible that they provide other metabolites which could be beneficial to the plant. To determine whether cypselae microbes play a defensive role, fungi isolated from the various Echinacea taxa were tested for antifungal capabilities against three fungal plant pathogens. In order to capture metabolites which may have been excreted into the media, both the media and the fungi were lyophilized and extracted. Of the seven extracts determined to be effective antifungals, four were derived from cypsela endophytes of *E. atrorubens var. paradoxa*, including the two extracts which inhibited more than one pathogen, all of which were *Fusarium* species.

Several of the fungal species identified in this study have previously been identified to have antifungal activity. *Aspergillus fumigatus* possesses potent antifungal metabolites, some of which are of comparable strength to common antifungal drugs when tested against pathogens such as *F. oxysporum* and *A. solani* (Li *et al.* 2012). Likewise, extracts of *Aspergillus nidulans* were shown to inhibit *Candida* species by interfering with synthesis of the cell wall glucan layer (Demain 1999).

Antifungal activity has also been well documented in *Penicillium canescens*. The main metabolites responsible for this activity include griseofulvin, canescin, culvulinic acid, and tryptophan-containing alkaloids. These compounds are active against a variety of microbes such as *Fusarium* species*, Bacillus subtilis*, *Colletrotrichum truncatum*, and *Rhizoctonia solani* (Bertinetti *et al.* 2009, Nicoletti *et al.* 2007).

Within the *Fusarium* genus, antifungal activity is somewhat contested, with different studies finding either the presence or absence of activity in various species. Rosa and others (2012) reported that *F. oxysporum, F. solani*, and several unidentified *Fusarium* species isolated from Echinacea were effective inhibitors of Colletrotrichum. *Fusarium proliferatum* was found to be inhibitory towards the bacteria *B. subtilis, E. coli*, and *Salmonella typhimurium*, as well as the fungi *B. cinerea, S. sclerotiorum, F. oxysporum*, and *R. solani* (Chowdhary & Kaushik 2015, Li *et al.* 2014). However, Kumar and Kaushik (2013) found no antifungal activity in *F. proliferatum*. In the present study, extracts of *F. proliferatum* inhibited *B. cinerea*, but not *F. oxysporum*. To my knowledge, no studies have been carried out on antifungal metabolites from *F. sporotrichoides* or *F. verticillioides*, which in this study were found to inhibit multiple plant pathogenic fungi tested.

Although *Aureobasidium pullulans*, which was isolated from *E. purpurea*, failed to grow for the antifungal assay portion of this experiment, it has previously been researched as an antifungal biocontrol agent. *A. pullulans* is a common yeast-like fungi that has been isolated both epiphytically and endophytically, and which is effective in preventing various fungal infections in apples, grapes, grapefruit, and sweet cherries (Schena *et al.* 2003, Schena et al. 1999, Wittig *et al.* 1997, Falconi & Mendgen 1993),

and in preventing the post-harvest decay of pears in storage conditions (Sanchez *et al.* 2010, Schena *et al.* 2003).

The present study presents a limited view of the potential interactions between the plant and fungal species present within Echinacea cypselae. These microbes may produce a more diverse (and potentially more active) array of metabolites when in competition with a pathogen, when in the presence of the rest of the endophyte/epiphyte community, or with access to the conditions and phytochemicals present in Echinacea plant tissue. Growing these microbes on media which contains Echinacea extract or performing a cross streak assay with Echinacea microbes and pathogens would provide more information on the metabolism of these colonist species.

Going forward, the active metabolites responsible for the pathogen inhibition observed in the fungal extracts should be identified. This experiment used crude extracts, so it is impossible to get a clear understanding of the effects of specific inhibitory components. Metabolites from *F. sporotrichoides* would be of particular interest for further study as this species appears to have a fairly broad range of antifungal activity. Testing populations of Echinacea from different locations in order to evaluate correlations between fungal assemblages and phytochemical content would also be particularly valuable, as it could provide insight into how endophytes and epiphytes should factor into standardization efforts in industry.

4 Chapter: Conclusions and future directions

Due to the popularity of Echinacea as a natural health product, a huge body of research has accumulated around its medicinal uses and cultivation methods. However, apart from studying methods to break dormancy, which can pose a significant challenge

to Echinacea cultivation, there is still relatively little information about the cypselae of Echinacea. This study showed that the absence of the coatings around Echinacea seeds, the perianth and pericarp, leads to poorer germination when seeds are sown in soil. However germination is significantly increased when de-coated seeds are sown on sterile media compared to seeds sown in the soil. Besides suggesting that the seed coat plays an important role in protecting germinating seedlings, this may represent a way to circumvent Echinacea seed dormancy without the need for long stratification periods or hormone treatments. This culturing technique is similar to "embryo rescue", which has been used successfully in other species (Sharma *et al.* 1996), but to my knowledge is not common practice in Echinacea cultivation.

The cypsela coatings in Echinacea contain a variety of bioactive alkylamides and phenolics. The alkylamides were most concentrated in the pericarp which is in contrast to phenolic compounds, which were localized to a greater extent in the inner seed and perianth. The absence of these metabolites may have contributed to the decrease in germination observed when seeds were sown in soil. Although there does not appear to be a direct correlation between metabolic content and germination, it is interesting to note that the two taxa with the lowest overall germination rates, *E. pallida* var. *angustifolia* and *E. atrorubens var. paradoxa*, were the ones with the highest concentration of phenolics and alkylamides, respectively, in their cypsela coatings. Although these taxa germinated poorly in both soil treatments, future tests with stratified seeds may show a more significant decrease in germination for these taxa without cypsela coats if they are more dependent on the high concentration of phytochemicals. *E. pallida var. pallida*, the

variety with the lowest overall metabolic content in the cypsela coats, had only a modest decrease in germination when bare seeds were sown in soil.

Going forward, it would be wise to further study the phytochemistry of rare Echinacea taxa. *E. atrorubens var. paradoxa*, which is not used commercially, was shown to have particularly high concentrations of alkylamides in its cypselae. Harvesting flower heads, as opposed to harvesting roots, might be a more sustainable system of Echinacea cultivation. A diversity of alkylamide and phenolic compounds have been identified in non-commercial Echinacea taxa (Binns *et al.* 2002 c), but have yet to be adequately studied. The historic reasoning for avoiding these taxa was their scarcity and more challenging cultivation requirements. However, recent advances in tissue culture technology may help to overcome these problems. Propagating Echinacea through tissue culture would allow for the use of potentially more efficacious taxa and also free up the cypselae as potential sources of medicinal products.

Microbes isolated from the cypselae of the four different Echinacea taxa were, overall, consistent with assemblages previously reported from Echinacea roots and leaves. A total of 23 different species were isolated from the cypsela coats of Echinacea, representing 12 genera. Although certainly not conclusive, it is possible that the endophytes and epiphytes present in the cypsela coatings could have an effect – positive or negative - on the germination of Echinacea seeds. Several of these microbes are able inhibit one or more plant pathogenic fungi, which might suggest a beneficial role in the host plant. It is possible that these species protect the plant from external pathogenic microbes, rather than just stimulating a defense response or improving stress tolerance in the host. Further research will be needed to clarify the effect of endophytes and epiphytes

on the phytochemistry and overall health of Echinacea. Plant-associated microbes may represent a good avenue of exploration for the improvement and standardization of Echinacea cultivation.

Echinacea cypselae contain a variety of bioactive metabolites including alkylamides and caffeic acid derivatives, as well as nutritious oils and beneficial microbes. Although there is a lot of research yet to be done, the cypselae of Echinacea have the potential to yield a variety of efficacious and sustainable natural health products.

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