Production of native plants for seed, biomass, and natural products

A Dissertation

SUBMITTED TO THE FACULTY OF

UNIVERSITY OF MINNESOTA

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Craig C. Sheaffer, advisor

March 2020

Acknowledgements

The research presented in this document would not have been possible without the love and support of countless mentors, colleague, friends and family. Foremost, I am forever grateful to my advisor, Dr. Craig Sheaffer, whose direction, support, understanding, and unending generosity made it possible for me to complete my doctorate. I am also eternally thankful for my unofficial co-advisor, Dr. Adrian Hegeman, for his kindness, intellectual brilliance, and support throughout my degree program. I am also incredibly grateful to Dr. Susan Galatowitsch and Dr. Clay Carter for their guidance, wisdom, and constructive and insightful commentaries. Though not on my committee, Dr. Jacob Jungers was incredibly generous with his time, advice, and support in all aspects of this research.

While a doctoral student, I was supported by grants from the Minnesota Department of Agriculture through the AGRI Crop Research Grant Program and the Specialty Crop Block Grant Program. Additional support came through the Minnesota Institute for Sustainable Agriculture gift fund, graciously provided by Leanna Forcier. Further support was provided by the University of Minnesota, including the Hueg-Harrison Graduate Fellowship, the Mark and Jean Schroepfer Fellowship, the Nancy Jo Ehlke Fellowship, and Annie's Sustainable Agriculture Scholarship.

The Sustainable Cropping Systems Lab, under the direction of Dr Craig Sheaffer, and the Plant Metabolomics Lab, under the direction of Dr. Adrian Hegeman, were constant sources of support. I thank Joshua Larson, Brett Heim, Lindsay Wilson, and Katherine Bohn for their invaluable assistance with field work and the processing of field samples. I thank Dr. Dana Freund for her extensive guidance in the laboratory and for her moral support throughout my entire program. I thank Dr. Arthur Eschenlauer for his unceasing patience and the seemingly endless hours he spent training me to process large metabolomics datasets.

I could not have completed my dissertation without the love and support of friends and family members, too numerous to list all here. I thank Courtney Jones for her entertaining conversations about "all things science", and also for her encouragement to take time to just sit back and relax. I thank Katherine Sammons for her stimulating conversations and practical perspective. I am so very grateful to my parents, Thomas and Kay Freund, for encouraging my endless questions as a child and for putting an emphasis on education and exploration from an incredibly young age. I thank my sister and brother-in-law, Anna and Rick Peterson, for their nonstop support and reassurance. I am forever grateful for my husband, Mark Saxhaug, for his unwavering support throughout this entire program. I thank Mark for his assistance with field work on the hottest summer Saturdays and mundane laboratory tasks in the evenings. This would not have been possible without his love, understanding, support, and extraordinary sense of humor that could lighten any mood.

Finally, I am thankful for several relatives who, although they have not been with me several years, still inspire me every day. My grandfather, John William Freund, who was

my first instructor of the natural world and taught me the names of wildflowers, trees, and animals. My Great Uncle Raymond Kabot, who loved farming but also took the time to wander the prairies of southwest Minnesota to find flowers. My Great Aunt Barbara Teckla Kabot, who taught me gentleness, kindness, and how to manage a farm and home through love and compassion. Finally, my Great Aunt Frances Edna Kabot for her hard work ethic, adventurous spirit, support of education, and whose love of the outdoors lead to endless hours lost in the garden. I dedicate this dissertation John, Raymond, Barbara, and Frances.

Abstract

Native and naturalized perennial plants are important components of sustainable agricultural systems, providing a wide range of ecosystem services including marketable products. Although demand for these products is increasing, there is limited information on the establishment and production of native and naturalized perennial plants. Through field and greenhouse studies, four native and naturalized perennial plant species were evaluated for their seed yield, biomass yield, and phytochemical (natural product) production. Canada milk vetch (*Astragalus canadensis* L.), purple coneflower (*Echinacea purpurea* Moench [L.]), and showy tick trefoil (*Desmodium canadense* L.) were evaluated in the field for seed and biomass production over three consecutive years. Seed yield decreased in all three species over time, whereas biomass yields over time varied by species. Seed and biomass yields, on a per hectare basis, decreased as the number of rows decreased, and there was little effect of plant community richness or diversity. An untargeted metabolomics approach was taken to evaluate phytochemical production in purple coneflower tissues. Several bioactive compounds were provisionally identified in purple coneflower tissues, and seed tissue possessed a similar profile to that of root. Although the plant community did not affect the overall metabolomic profile of purple coneflower, levels of specific compounds in leaf, stem, and root tissue were affected. Similar biomass yield and metabolomics approaches were applied to fireweed (*Chamaenerion angustifolium* (L.) scop., syn. *Epilobium angustifolium* L.) grown in a hydroponic system. Shoot dry weight increased logistically with increasing mineral concentration of the nutrient solution. Although the abundance of most provisionally identified compounds decreased with increasing mineral nutrient concentration,

commercially-relevant oenothein B and miquelianin were not affected by mineral nutrient treatments. Native and naturalized perennial plants on the agricultural landscape can provide numerous ecosystem services, including marketable products such as seed for restoration plantings, biomass for fuel and forage, and bioactive phytochemicals for medicinal and supplemental purposes. In order to incentivize the establishment of native and naturalized perennials for such marketable products, research on the productivity of specific plants is needed. The research presented in this dissertation provides the groundwork for four native and naturalized Minnesota species.

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Introduction

The strategic inclusion of native and naturalized plants onto the modern agricultural landscape can lead to multiple environmental benefits by creating pollinator and wildlife habitat, minimizing excess nutrient run-off, and decreasing soil erosion (Isaacs et al., 2009; Zauzo and Pleguezuelo, 2009; Sidhu and Joshi, 2016). Monoculture and polyculture systems of native and naturalized plants can be additional streams of income for Minnesota farmers, as they provide marketable products in the form of biomass, seed, and phytochemicals (natural products). This dissertation explores methods to establish and evaluate native and naturalized perennial species for seed and biomass production, and to assess phytochemical production in response to experimental treatments.

The demand for locally-procured native seeds

Over 50 companies and producers in Minnesota market native and naturalized perennial plant seed to governmental agencies, landscape and restoration companies, and private landowners. Recent reports of widespread honeybee colony collapse and the decline in native pollinator populations has shifted the demand from grass-dominant native seed mixes to wildflower-dominant seed mixes (Milstein, 2005; Williams et al., 2015). Governmental programs are also contributing to a rise in interest of growing native plants. State and federal conservation programs such as the Conservation Reserve Program (CRP) encourage farmers to convert environmentally-sensitive land out of crop production and into plant species that will "improve environmental health and quality". As of June 2018, 9.2 million hectares (22.7 million acres) of land in the United States

were enrolled in CRP. As crop profitability per acre falls in Minnesota, United States (Steil, 2016), farmers may enroll more land in the CRP and thus there will be greater demand for native seed.

Uses for native and naturalized plant biomass

The cultivation of perennial plants, especially mixtures (polycultures), is increasingly garnering attention throughout the Midwest for their ability to provide revenue in the form of bioenergy and also provide multiple ecosystem services in agricultural areas (Werling et al., 2014). Perennial polycultures in Minnesota have typically been utilized for biomass and bioenergy studies (Jungers et al., 2015; Gamble et al., 2016). The Energy Independence and Security Act of 2007 set a mandatory renewable fuel standard requiring transportation fuel in the United States, by 2022, to contain a minimum of 36 billion gallons of renewable fuels annually. Native polyculture biomass can be converted into energy, and annually-harvested biomass from monoculture and polyculture plots can potentially be sold for energy to, at least partially, meet these federal requirements (Jungers et al., 2013). Additionally, native plants, especially legumes, can be utilized as forage crops. Forage legumes have the ability to provide high quality livestock feed in addition to marketable seed, soil cover, and pollinator resources such as nectar and pollen (Sheaffer et al., 2003).

Beneficial natural products from native plants

Plants produce a wide variety of biochemical compounds (metabolites, phytochemicals, natural products), some of which are essential to plant growth and development whereas

others are beneficial to adaptation to environmental conditions. Many plant-derived chemicals are key active ingredients in various human products: pharmaceutical drugs, flavors, fragrances, pigments, pesticides, and food additives (Hussain et al., 2012). Consumer demand for natural ingredients in food, medicines, and personal care products has increased over the past two decades, and the organic personal care product market alone is estimated to be worth over 25 billion dollars by 2025 (Grand View Research, Inc., 2019; Kim and Chung, 2011). Field collections and subsequent laboratory analyses have demonstrated that several native and naturalized plant species collected in Minnesota contain compounds with high antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*, which may be useful in personal care product applications (Borchardt et al., 2008; Borchardt et al., 2009). Previous work at the University of Minnesota under the Forever Green Initiative, an agriculture research program to develop new crops and high-efficiency cropping systems, has also developed methods for the analysis of metabolomic profiles from these native and naturalized plant species (Martin et al., 2014).

Why cultivate native plants for seed, biomass, and natural products?

Establishment of native plants on agronomic landscapes is a favorable alternative to wild collection as it alleviates many of the problems associated with wild collection including, but not limited to, destruction of native habitats and loss of biodiversity (Chen et al., 2016). This may also allow for control of agronomic conditions that may influence production. Cultivated plant materials may have additional advantages: certain cultivars may produce greater concentrations of desirable phytochemicals, consistent

phytochemical concentrations may be achieved due to the uniformity of cultivation, and supplies can be grown close to or in the area of demand. Additionally, there is a trend towards cultivation since cultivated material can be organically certified (Laird and Pierce, 2002). However, phytochemical concentration could potentially be lower in cultivated stocks and there is little information on specific agronomic practices that optimize the concentration of natural products in many of these perennial species (Gillitzer et al., 2012).

The use of untargeted metabolomics in agronomic and ecological studies

Metabolomics is the systematic study and characterization of the set of metabolites (phytochemicals or natural products) present in a biological system or sample. Metabolomics studies are typically categorized by their approach, either as targeted or untargeted. Targeted metabolomics approaches involve the measurement of defined groups of chemically-characterized and biochemically-annotated metabolites. Targeted approaches are used when specific, known metabolites are being analyzed. Untargeted metabolomics approaches are comprehensive analyses of all of the measurable metabolites in a sample, including chemical unknowns. Studies on the metabolomic response of plants to certain biotic interactions, such as the diversity of the plant community, are scarce. The four perennial plant species examined in this research have been previously shown, under natural plant community conditions, to produce phytochemical compounds of economic interest, but their capacity to produce compounds has not been evaluated in production settings.

In summary

Strategic placement of native perennial plants on Minnesota's landscape has been identified as a way to improve the environment, especially in agricultural areas. Native perennials grown alone or in polycultures can sequester carbon, capture lost agricultural inputs, prevent soil erosion, increase water filtration, and provide wildlife and pollinator habitat. However, the use of native perennial plants on the agricultural landscape is limited by lack of economic return for producers. Perennial species from Minnesota can provide marketable seed and biomass (Mangan et al., 2011; Jungers et al., 2015; Freund Saxhaug et al., *in review*), and are also sources of biologically-active phytochemicals (Borchardt et al. 2008; Borchardt et al., 2009; Gilitzer et al., 2012).

Early work on the production of native and naturalized plant species for marketable outputs requires establishment under agronomic conditions and examination of the seed, biomass, and phytochemicals produced. Canada milk vetch (*Astragalus canadensis* L.), purple coneflower (*Echinacea purpurea* Moench [L.]), and showy tick trefoil (*Desmodium canadense* L.) were established at two locations and examined for seed and biomass production (**Chapter 2**). Untargeted metabolomics approaches were utilized to examine production of phytochemicals in response to location and agronomic design in purple coneflower (**Chapter 3**). Due to initial difficulties in field establishment, fireweed (*Chamaenerion angustifolium* (L.) scop.) was established in hydroponic culture to examine biomass and phytochemical production in response to nutrient treatments (**Chapter 4**).

Chapter 1

Cultivation of native plants for seed and biomass yield[1]

[1]This chapter has been accepted pending revision at *Agronomy Journal*.

ABSTRACT

Establishing native perennial plants on the agricultural landscape can improve ecosystem services and provide marketable products such as seed for restoration plantings and biomass for renewable energy. Native perennials of economic and ecological interest should be examined in different planting configurations over time to determine their suitability for sustained production. Canada milk vetch (*Astragalus canadensis* L.), purple coneflower (*Echinacea purpurea* L.), and showy tick trefoil (*Desmodium canadense* L.) were established at two locations in Minnesota to evaluate seed, and vegetative biomass yields. These forbs were established in six different agronomic designs: three strip designs (1-row, 3-rows, and 6-rows) and three community designs (monoculture, low-richness polyculture and high-richness polyculture). Seed yields averaged 2995, 950, and 1157 kg ha^{-1} for Canada milk vetch, purple coneflower, and showy tick trefoil in the first year, declining for all over time. Biomass yields averaged 6743, 2725, and 2869 kg ha^{-1} in the first year for Canada milk vetch, purple coneflower, and showy tick trefoil, declining by 98% for Canada milk vetch biomass and increasing by 40% for showy tick trefoil over time. Seed and biomass yields were the lowest in 1-row strip design and greatest in the community designs, with little difference between monocultures and polycultures. Results suggest that production is maximized in community designs, and purple coneflower and showy tick trefoil have the potential for multi-year yields.

INTRODUCTION

Diversification of the agricultural landscape has been posited as a means to reduce the negative environmental consequences associated with agriculture (Frison et al., 2011; Isbell et al., 2017). Increased biodiversity on agricultural lands can create, improve, and maintain ecological services (Asbjornsen et al., 2013; Kremen and Miles, 2012), especially through diversification with native perennial plants. For example, native plants have been used to intercept N fertilizer run-off to prevent contamination of surface waters (Franklin et al., 2015). Furthermore, mixtures of native perennial species have been found to increase pollinator abundance on agricultural landscapes, reduce sediment runoff from annual row-crop systems, and reduce N and P loss from croplands (Abu-Zreig et al., 2004; Grudens-Schuck et al., 2017; Schulte et al., 2017; Hernandez-Santana et al., 2013; Zhou et al., 2014).

In addition to ecosystem services, marketable products can be harvested from native perennial plantings and may provide economic incentives for agricultural producers to add and expand acreage of native perennials. Demand for native seed, especially locally procured seed, is growing as ecological restoration efforts and interest in native plants increase (Meissen et al., 2017; Smith, 2017; White et al., 2018). Aboveground biomass is another potentially marketable product. Perennial plants, especially legumes, can be utilized as forage crops (McGraw et al., 2004; Pierce et al., 2017). Natural products such as essential oils and antimicrobial phytochemicals can be extracted from native perennial biomass (Borchardt et al., 2008; Borchardt et al., 2009) and consumer demand for these natural ingredients in food, medicines, and personal care products has been rapidly

increasing over the past two decades (Muir et al., 2005; Peppin et al., 2010; Kiehl et al., 2014; Kim and Chung, 2011; Transparency Market Research, 2015; Market Envision, 2017).

Desire to improve the sustainability of agricultural systems and diversify agricultural products for farmers and consumers has also led to an interest in cultivating and domesticating wild annual and perennial plants. There have been several promising ongoing efforts to domesticate and improve native plant species for marketable products. For example, *Silphium integrifolium* Michx. is being developed as a perennial oilseed crop (Reinert et al., 2019). The annual plant pennycress (*Thlaspi arvense* L.) is being examined for potential benefits as a cover crop and as an oilseed for biodiesel feedstock (Johnson et al., 2015). Intermediate wheatgrass (*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey) was identified as having several attractive traits for domestication, and is currently being developed as a perennial grain crop (DeHaan et al., 2018). Selection and domestication efforts are useful for developing high-yielding cultivars, but the subsequent loss of genetic diversity in the plant population may limit success in adaptation to changing climatic and environmental conditions (Burton and Burton, 2002). Production practices should be examined for the ability to improve yields while preserving genetic diversity, and thus long-term viability of plant populations (Basey et al., 2015).

Introducing high-value native species into agronomic settings allows for the preservation of wild stands as well as greater uniformity of the end product due to the use of specific

agronomic practices (Lubbe and Verpoorte, 2011). Specific seed production practices such as fertilization (Grabowski, 2005; Norcini et al., 2006), irrigation (Shock et al., 2018), and direct seeding methods have been described for some perennial wildflower species (Fischbach et al., 2006; Houseal, 2007). However, apart from species with long-established medicinal value such as *Achillea millefolium* and *Echinacea* sp. (Rohloff et al., 2000; Li et al., 1998; Shalaby et al., 1997), there is limited information on the effect of agronomic practices on biomass yield of most native plant species (Ladouceur et al., 2018).

To optimize agronomic outputs of species with economic and environmental potential, native perennial plants of interest should initially be examined in a variety of systems, including monocultures and polycultures (Nevill et al., 2016). Valuable native perennials, such as switchgrass (*Panicum virgatum* L.), can be cultivated in monocultures for use as bioenergy feedstock (Schmer et al., 2008). However, growing grass-forb mixtures can result in greater biomass productivity than growing monocultures of the same species (Mangan et al., 2011; Jungers et al., 2015), and these mixtures may require less agronomic inputs than monocultures while providing additional ecosystem services (Carlsson et al., 2017; Sanderson et al., 2005). While production of native forbs and grasses in monocultures may provide logistical advantages for large-scale seed and biomass production (Cook-Patton and Agrawal, 2014), cultivation of native species in mixtures can lead to greater yields (Picasso et al., 2008) and is recognized as providing the greatest ecosystem benefits (Isbell et al., 2011).

Three perennial forb species – purple coneflower (*Echinacea purpurea* L.), showy tick trefoil (*Desmodium canadense* L.), and Canada milk vetch (*Astragalus canadensis* L.) – were selected for study based on their potential value as a harvestable crop. Showy tick trefoil and Canada milk vetch have potential as native forage species (McGraw et al., 2004; Ristau, 2000), and their seed are highly valuable, priced at over $50 per kilogram from leading seed producers in the Midwest. All three species contain valuable natural products with antimicrobial potential and other medicinal properties (Borchardt et al., 2008). Purple coneflower has a long history of use as a medicinal plant and is a popular herbal supplement (Barrett, 2003; Barnes et al., 2005). Although less widely studied, showy tick trefoil contains phytochemicals that may potentially be useful in the treatment of various disorders (Taylor et al., 2009) and Canada milk vetch has a history of use in Native American cultures for treatment of upper respiratory ailments (Gilmore, 1991). From an ecosystem service perspective, all three species support pollinator and wildlife populations. Purple coneflower appeals to generalist pollinators (Wist and Davis, 2005), while Canada milk vetch appeals to bumblebee species (Platt et al., 1974), and showy tick trefoil seed and biomass are consumed by various bird species and white-tailed deer (Miller and Miller, 2005). Purple coneflower root yields are influenced by planting arrangement (Parmenter and Littlejohn, 1997), however, beyond this, these three species have not been evaluated for seed and biomass yield in this capacity. Yield differences in response to planting arrangement have been reported in other native perennial species (Serajchi et al., 2017). Our objective was to evaluate biomass and seed productivity of these three species in response to strip, monoculture, and polyculture planting configurations.

METHODS AND MATERIALS

The study was established in June 2015 at the University of Minnesota Agricultural Experiment Stations at Becker and Rosemount, MN and seed and biomass annually harvested from 2016 to 2018. The soil at Becker (45°23'14.8"N 93°53'31.4"W) is a Hubbard-Mosford complex (sandy, mixed, frigid Entic Hapludolls) and the soil at Rosemount (44°41'21.0"N 93°04'26.3"W) is a Port Byron silt loam (fine-silty, mixed, superactive, mesic Typic Hapludolls). Soil samples were collected from a 15-cm depth prior to the establishment of the experiment and analyzed for N, P, K and pH. Soils at Becker had an average N (nitrate), P (Bray extractable), K, and soil water pH of 8 kg ha^{-1}, 29 mg kg^{-1}, 106 mg kg^{-1} and 6.6, respectively. Soils at Rosemount had an average N, P, K, and soil water pH of 25 kg ha^{-1}, 31 mg kg^{-1}, 115 mg kg^{-1}, and 6.2, respectively. No fertilizer was applied during the study. The previous crop was rye (*Secale cereal* L.) at Becker, and pennycress (*Thlapsi arvense* L.) at Rosemount. Weather data shown in Table 1.1 was compiled from cli-MATE for Rosemount and Becker (Midwestern Regional Climate Center, 2018).

The experimental design at each location was a randomized block design with treatments in a split plot arrangement. There were three replicates at each location. Six different planting configurations (hereafter referred to as “designs”) were the main whole plot treatments (Figure 1.1). These were: three strip designs (1-row, 3-rows, and 6-rows) and three community designs (monoculture, low-richness polyculture and high-richness polyculture). Strip designs consisted of one, three, or six 1.83-meter long rows of a focal

forb species, each planted 0.15 meters apart. The rows of the focal forb species were bordered by a 1.83-meter by 0.91-meter strip of a native grass mixture on both sides. Native grasses were drilled into a firm seedbed with a plot seeder. During the establishment year of 2015, the grass borders were mowed in July and August to control broadleaf weed species. In 2016 through 2018 weeds were manually removed from the grass border. Community designs consisted of nine 1.83-meter long rows of the focal forbs planted 0.15 meters apart. The monoculture community design was composed solely of the focal forb. The low- and high-richness polyculture designs included additional species seeded among the focal forb plant species.

Split plot treatments were three flowering perennial forb species (hereafter referred to as "focal forbs"): purple coneflower (*Echinacea purpurea* L.), showy tick trefoil (*Desmodium canadense* L.), and Canada milk vetch (*Astragalus canadensis* L.). Focal forb species were seeded by hand at a depth of 2 centimeters with a target plant density of 20 plants per linear meter. Additional species in the polyculture designs were selected based on their native status and plant height – grasses native to the area and of short stature were chosen so the focal forb species would not compete for light. Additional forb and legume species were chosen based on phenology, bloom type, pollinator attraction, and nitrogen-fixing potential of legumes (Table 1.2). All seeds were purchased from Prairie Moon Nursery in Winona, MN. Seeding rates of grass and additional forb species in the polyculture plots were 16.8 kg of pure live seed (PLS) per ha^{-1} based on recommendations for prairie restoration in Minnesota (Fuge, 2000). After accounting for the weight of the focal forb seeds sowed in the polyculture plots (determined from the

target plant density), grass or grass and additional forb seed was added to bring the plot total up to 16.8 kg ha^{-1} PLS. For the low-richness polyculture plots, the weight of the focal forb seed sowed was subtracted from the recommended 16.8 kg ha^{-1} and the remaining amount was split evenly among the 5 native grass species, accounting for differences in species seed weights and germination tests conducted prior to establishment of the experiment. For the high-richness polyculture plots, the remaining kg ha^{-1} PLS was split 60:40 by weight between the additional forbs and grasses. Similar to the low-richness polycultures, the grass component was split evenly among the grass species and the additional forbs were seeded in equal amounts after accounting for seed size and initial germination test results. Additional forbs and grasses were broadcast seeded into their respective polyculture designs after the focal forb species were seeded. Broadleaf and grass weedy species were manually removed from the experimental plots on a weekly basis from May to October throughout all years of the experiment.

Plant counts were conducted in May 2016, approximately one year after establishment. All individual plants within every row were counted at both sites. At Becker, this resulted in an average of 9, 30, and 21 plants per linear meter for Canada milk vetch, purple coneflower, and showy tick trefoil. At Rosemount, this resulted in 9, 26, and 18 plants per linear meter for Canada milk vetch, purple coneflower, and showy tick trefoil. No difference in plant density across treatments was found. Counts were not taken in subsequent years due to extensive branching from purple coneflower and showy tick trefoil plants leading to an inability to distinguish individual plants.

Seed yield

Seed from the three focal forbs was harvested by hand from August to October (Supplementary Table A.1), depending on individual species maturation rates and prior to shattering, from the center 0.91 meters of each row (Figure 1.1B). Canada milk vetch and purple coneflower seeds were harvested when the pods and seed heads were dry and black. Purple coneflower seed heads were collected on multiple dates throughout the fall to avoid seed loss due to shattering. Showy tick trefoil seeds were harvested when seed pods were dry and turned from green to brown. Seed heads and pods were dried in forced-air ovens for 72 hours at 35°C to report yields on a dry matter basis; however, this practice can affect seed viability and should not be used by production-scale seed producers. Seed heads and pods were threshed using a Wintersteiger LD 180 ST4 for Canada milk vetch, a laboratory belt thresher for purple coneflower, and a Wintersteiger LD 350 for showy tick trefoil. After threshing, all seeds were cleaned using a laboratory aspirator and hand sieves to remove chaff and separate seeds. This process removed empty purple coneflower achenes and desiccated and deformed Canada milk vetch and showy tick trefoil seeds. Dried and cleaned seeds were weighed to allow expression of weight on a dry matter basis.

Biomass yield

Biomass was harvested to a 7.5 cm height from the center 0.91 meters of each row at the end of each growing season following seed harvest but prior to complete senescence and leaf loss (Supplementary Table A.1). Biomass from each row was harvested separately,

dried for 72 hours at 60°C, and weighed. Biomass from all focal forb rows of the strip and community designs were summed for a per plot focal biomass measurement.

To further assess productivity of the experimental plots, grass biomass was harvested from the strip design plots and additional species present were harvested from the community design plots. For the grass biomass sampling in strip design plots, a 0.91-meter by 0.15-meter strip was harvested perpendicular to the focal forb rows. For the community design plots, all additional species present were harvested from a 0.91-meter by 0.76-meter area in the center of each plot. The focal biomass yield and the yield of these different components were summed on a per plot basis (hereafter referred to as "total biomass yield").

Statistical analyses

All statistical analyses were conducted using the program R (R Core Team, 2018). Biomass and seed yields were expressed in kilograms per hectare (kg ha^{-1}). Locations were analyzed separately because the initial full model showed the interactions of location with design and species to be significant. Mixed effects models were conducted using the "nlme" package (Pinheiro et al., 2018). Response variables were transformed when necessary to meet model assumptions. The response variables – focal forb biomass, focal seed biomass, and total biomass – were analyzed separately using mixed-effects ANOVA models with design, focal species, and year as fixed effects with block considered a random effect and year evaluated as a repeated measure. Tukey's least

significant difference test was used to compare least square means using the “emmeans” package ($P < 0.05$) (Lenth et al., 2018).

For the polyculture plots, changes in focal seed and focal biomass yield as a function of the surrounding plant community diversity were explored. The diversity metrics used were species richness and the Shannon diversity index $H' = -\Sigma p_i \log(p_i)$. Species richness was the number of species present in each plot. Shannon diversity was based on the dry weight proportion of each species within the group of species present in a polyculture plot. The focal species was not included in the calculation of species richness and Shannon diversity. Weedy species were not included in these analyses as they were not present in the experimental plots due to regular weed removal. To explore the effect of diversity on seed and biomass yield, the response variables of focal seed biomass and focal forb biomass were analyzed using mixed-effects ANOVA models for each location, consistent with earlier analyses. Design and diversity metric were treated as fixed effects while block was considered a random effect.

RESULTS

Through combined analyses of the data, there was a significant location by species by design by year interaction for focal seed yield, focal biomass yield, and total biomass yield ($P<0.0001$). Therefore, separate analyses were conducted on the two different locations. In subsequent location-specific models, there were significant interactions between the main effects for all response variables. This finding, along with knowledge

of biological differences between the species, led to separate analyses for each of the three focal species.

Seed yield

Design and year were significant predictors of focal forb seed yield for all species at both locations (Table 1.3). There was a significant interaction between these predictors for Canada milk vetch at both locations. For all focal species, 1-row strip designs typically had the lowest yields. Community designs consistently had the highest seed yields, and there were few differences noted between the three different community designs (Supplementary Table A.2).

To assess general trends in seed yield over multiple years of harvest, designs within location and species were averaged and Tukey's least significant difference test was used to compare least square means (Figure 1.2). Over the course of the experiment, Canada milk vetch seed yields declined to the point of having no seed pods produced in 2018. Canada milk vetch seed yield decline was significant at both locations. The weevil *Acanthoscelides aureolus* (Horn) was observed in Canada milk vetch seed pods in Rosemount at harvest, but not in Becker. In 2017 and 2018, Japanese beetles (*Popillia japonica* Newman) aggressively consumed showy tick trefoil flowers in Rosemount. Although showy tick trefoil yields decreased over time at both locations, this decrease was not statistically significant.

Biomass yield

Design was a significant predictor of focal biomass yield for all species at both locations, and year was a significant predictor of focal biomass yield for all except purple coneflower in Becker (Table 1.3). There was a significant interaction between these predictors for Canada milk vetch and showy tick trefoil. Similar to focal seed yield, strip designs were typically lower yielding than community designs and the three community designs were statistically similar for all focal species at both locations (Supplementary Table A.3). As with focal seed yield, focal biomass yield over time was examined by averaging the designs within location and focal species, and comparing yields over the three years of harvest (Figure 1.3). Canada milk vetch biomass yields declined at both locations, showy tick trefoil yields increased at both sites, and purple coneflower yields did not change significantly over the three years of the experiment.

Total biomass yield

Total biomass yield, which was the combined biomass of the focal species and grass component of the strip row designs and the combined biomass of the focal species and additional species of the community designs, was affected by design for all focal species at both locations (Table 1.3). Year was a significant predictor in all cases except for Canada milk vetch in Becker, however there was a significant interaction between year and design for Canada milk vetch at this location. For Canada milk vetch at Rosemount – where there was a significant interaction between design and year – the community designs had greater total biomass yield than the strip designs in year one, but this pattern did not persist in years 2 and 3 (Supplementary Table A.4). Although F tests from the

ANOVA indicated significant differences in total biomass yield between designs, means separation within location-focal species combinations showed very few differences between designs (Supplementary Table A.4). For the few instances where designs were significantly different, there were no consistent patterns across focal species or locations (Supplemental Table A.4).

Effects of richness and diversity on focal seed and biomass yield

There were no differences in species richness or diversity across years in Becker; however, both response variables declined in purple coneflower and showy tick trefoil plots in Rosemount (Supplementary Table A.5). Species richness and Shannon diversity did not significantly influence focal species seed or biomass yield for any focal species-location combination (data not shown).

DISCUSSION

Seed yield

A major challenge with seed production from perennial species is related to evolutionary tradeoffs between life span, resource allocation to root tissue, and seed yield. Over time, perennial plants increasingly allocate resources to storage structures, such as roots, and less to seed production (Vico et al., 2016). Purple coneflower root biomass is reported to peak in the third or fourth year of growth (Li, 1998), so the decrease in seed yield noted in Rosemount may be due, in part, to nutrient allocation over time in purple coneflower.

Another potential mechanism of seed yield decline with stand age could be related to changes in plant density. We were unable to measure plant density in years 2 and 3 because of resource limitations related to large plot sizes and challenges associated with assigning stems to unique individual plants. However, if a decrease in plant density was the main driver of seed yield reductions, it could be expected that biomass yields would also decrease to some degree. We observed similar patterns in decreased seed and biomass yields through time for Canada milk vetch, but opposite trends for showy tick trefoil where seed yields declined (though not statistically) and biomass yields increased. The primary driver of seed yield decline may vary by focal species and could include insect herbivory, pathogen pressure, or intra-specific competition with increasing plant density. Some species-specific explanations are offered below.

Peak seed yields from Canada milk vetch occurred in the first harvest year, and yields significantly decreased in the following years. This temporal trend of decreasing seed yields is similar to that reported by Houseal (2007), one of the few references for seed yield of native species over several years of harvest. Canada milk vetch seed yields in 2016 and 2017 were nearly ten-fold greater than the average of 112 to 224 kg ha^{-1} reported by Houseal (2007), indicating that there may be a wider range of potential seed yields from native species than previously reported. Although Canada milk vetch had high initial seed yields, it is unlikely to be profitable beyond the second year of harvest. Similar to Canada milk vetch, showy tick trefoil seed yields were five to six times greater than reported by Houseal (2007). In Rosemount 2017 and 2018, low showy tick trefoil seed yields were potentially due to beetle herbivory of flowers. The flowering of showy

tick trefoil in Rosemount coincided with the emergence of Japanese beetle adults in Rosemount these years, and the majority of damage to the plant was inflicted on the flowers. The substantial presence of Japanese beetles in Rosemount, especially on showy tick trefoil, may also have deterred pollinators, thus contributing to the lower seed set. Marketable seed harvests from showy tick trefoil are likely not possible without Japanese beetle control.

In this experiment, community designs, which had larger continuous areas of the focal species, consistently had greater seed yields than strip designs (Supplementary Table A.2). Additionally, plots with a greater number of rows in the strip designs typically exhibited greater seed yields. This is likely due to the inclusion of the grass border in strip designs, which reduced the total area of focal forbs within a given design. Greater seed yields in community design could also be due, perhaps in part, to pollinator dynamics and pollination success in response to patch size. Previous work on seed production of wildflower species has shown that greater wildflower population sizes and density produce increased seed yields. Seed yield from Canada milk vetch is the greatest at high planting densities and bumblebee (*Bombus*) species, one of the main pollinators of Canada milk vetch, tend to favor larger patches of flowers and visit multiple flowers within a patch, leading to greater pollination success and seed yields (Platt et al., 1974). Similarly, seed yields from several native flowering species were found to increase with increasing size of patch and density (Dauber et al., 2010). It is important to note that the spatial scale of this experiment was smaller than the range of most pollinator species, which can have foraging distances of over one kilometer (Carvell et al., 2016). Future

work on native flowering species of interest for seed production should consider the effects of planting density and pollinator interactions on seed yields.

Biomass yield

Canada milk vetch aboveground biomass yield has previously been shown to decrease over time in annually-harvested polyculture systems and showy tick trefoil aboveground biomass has been shown to increase (Jungers et al., 2015), which is similar to trends observed in this experiment. Increases in showy tick trefoil biomass may be a result of increased branching as individual plants age. In the first year, showy tick trefoil plants generally consisted of a single stem. In subsequent years, individual plants grew four to eight additional shoots from the original rootstock, making showy tick trefoil an attractive species for biomass production over several years. Purple coneflower is typically cultivated for root biomass, and this is the first study to specifically address aboveground biomass yields in addition to seed yields. Biomass yields did not significantly change over the course of this experiment, indicating that this could be a potentially sustainable species for biomass production.

Production in mono- and polyculture plots typically resulted in greater yields than cultivation in strip row designs, although there was no significant difference detected between the community designs. Ecological theory suggests that greater species diversity leads to greater overall productivity of polycultures (Tilman et al., 2014), but focal biomass yields were not found to be greater than those from monocultures (as described in Cardinale et al., 2011). However, this lack of difference between the three community

designs may be misleading. Differences or changes in the density of the additional species seeded in polyculture designs may affect yields of the focal species, or vice versa, disguising a more accurate interpretation. For example, purple coneflower polycultures in Rosemount were observed in the field to exclude both planted and weedy species, and this is reflected in the decline of species richness and diversity. This decline in the added grasses and forbs is perhaps due to the potential allelopathic effects of coneflower root exudates (Viles and Reese, 1996). Purple coneflower polyculture plots in Rosemount lacked additional species and therefore were very similar to monoculture plots, which could account for the lack of difference between community designs.

Alternatively, the similarity in yields from mono- and polycultures may be due to dynamics of the established plant community in the polyculture designs. For a focal species to produce greater biomass in a polyculture than a monoculture (to "overyield"), the polyculture plant community often includes species that complement the focal species (Picasso et al., 2011). For example, planting legumes into a polyculture of warm-season grasses can result in overyielding of warm-season grasses by means of increased nitrogen fertility provided by the legumes (DeHaan et al., 2010). In this study, species richness and Shannon diversity were found to have no effect on focal biomass yields. Additional species included in the polyculture designs were selected based on traits other than complementarity with the selected focal species which may explain the lack of any noticeable effect of diversity on yields. To promote overyielding of the focal species selected for this study, further investigation of traits conducive to complementarity, such as mycorrhizal association for increased phosphorus cycling (Li et al., 2014), is required.

Total biomass yield

Although most of the mean comparisons for total biomass yield between designs for the focal species were not statistically significant, community designs tended to have higher total biomass yields than strip designs. However, this was not the case for Canada milk vetch in 2018. Greater yields from the strip designs are attributed to biomass from the grass component, which at times accounted for over 95% of the total biomass. Among showy tick trefoil plots, no one community design was consistently the highest yielding, and elimination of the highest-yielding design typically eliminated any difference in means among the remaining designs. Although design influenced focal biomass yields, total biomass yields varied little in response to design across the focal species. This suggests that a given area dedicated to a focal forb had a similar level of productivity whether seeded with the focal species only, the focal and polyculture species, or focal species rows bordered by native grasses. Although this research mainly focused on yields from the focal forbs, the grass and polyculture species may also provide additional economic and ecological benefits to producers. For example, native grasses can be harvested for bioenergy feedstock (Jarchow et al., 2012) and native flowering species may support pollinator populations vital to pollinator-dependent crops (Williams et al., 2015).

CONCLUSION

To successfully cultivate native perennial forbs for continual seed and biomass yield, it is crucial to examine their performance over time and in response to various production practices. In this experiment, different planting configurations were utilized to assess

seed and biomass yields of the three forb species over three consecutive years. Community designs generally produced greater seed and biomass yields than strip row designs on a per hectare basis, suggesting that larger continuous plantings of these native forbs rather than fewer rows are favorable for greater yields. However, diverse communities may make mechanical harvesting challenging compared to rows or monocultures. Canada milk vetch biomass and seed yields declined over the three years of study, indicating that this species may not be well-suited for long-term cultivation and harvest without replanting. Showy tick trefoil, however, is a promising candidate for long-term biomass harvest, as yields increased over the course of the experiment. Plant community richness and Shannon diversity did not influence focal seed or biomass yield. Total biomass yield varied in response to design across species, although elimination of the highest-yielding design within species and environment typically eliminated any difference in means, indicating evenness in the productivity of the land.

The establishment of native perennials is a promising way to increase biodiversity on the agricultural landscape with the potential to provide additional income to the grower as well as increased ecosystem services. Demand is increasing for native seed and other bio-based products from perennial plant biomass, but the dynamics of native perennial species grown in agronomic settings need to be examined before there is widespread establishment specifically for marketable products. Although perennial species can persist and even re-seed for decades, longer-term studies need to be conducted to justify their establishment for seed and biomass as a sustainable income for growers.

ACKNOWLEDGMENTS

Thank you to Joshua Larson, Brett Heim, Lindsay Wilson, Katherine Bohn, and past and current member of the Sustainable Cropping Systems Laboratory at the University of Minnesota for assistance in the field and laboratory. Funding for this research was provided by the AGRI Crop Research Grant Program from the Minnesota Department of Agriculture and the Hueg-Harrison Fellowship.

Table 1.1. Average mean daily temperature (°C) and mean monthly total precipitation (mm) from 2016 to 2018.

	Becker, MN[a]								Rosemount, MN							
	Monthly Rainfall				Air Temperature				Monthly Rainfall				Air Temperature			
Month	**2016**	**2017**	**2018**	**30-year Average**	**2016**	**2017**	**2018**	**30-year Average**	**2016**	**2017**	**2018**	**30-year Average**	**2016**	**2017**	**2018**	**30-year Average**
	----------mm----------				----------°C----------				----------mm----------				----------°C----------			
Apr.	49.5	83.6	32.3	72.8	7.3	8.4	1.1	7.1	56.4	115.6	50.3	78.9	8.4	9	1	7.7
May	66.8	143.5	30	98.1	14.3	13.2	17.5	14.2	69.3	182.4	108.7	113	15.6	13.5	18.6	14.6
June	63.8	69.9	91.9	113.9	19.3	19	19.8	19	81.3	91.4	154.4	129.1	21	20.4	21.4	20
July	164.8	112.3	133.1	102.7	21	21.2	21.1	21.3	120.7	138.7	111	114.8	24.8	22.3	22	22.2
Aug.	117.1	130	87.1	105.3	20.6	17.9	20.4	20.1	178.3	128.8	99.3	113.8	21.3	18.9	21.1	20.8
Sept.	128.8	71.4	82	69.7	16.4	17.2	16.6	15.9	132.8	42.4	153.7	83.7	17.9	17.9	17.4	16.7
Oct.	47	160.5	103.6	67.4	10.2	9.4	6.1	8.6	62.2	98.6	68.8	66	11.1	9.6	6.2	9.5
Total/Average[b]	637.8	771.1	560.1	627.6	15.6	15.2	14.7	15.2	701	797.8	746.3	710.5	17.2	15.9	15.4	15.9

[a] Plots located in Becker were irrigated at a rate of 25.4 mm per week from June to September in 2015, and May to September in 2016, 2017 and 2018.
[b] Totals are presented for monthly rainfall columns, while averages are presented for 30-year average and temperature columns.

Table 1.2. Additional species included in the low-richness and high-richness polyculture plots. All listed species were seeded in the high-richness plots. Only the grass species were seeded in the low-richness polyculture plots.

Scientific name	Common name	Characteristics
Elymus trachycaulus	Slender wheatgrass	Cool season grass, dense root system, moderately drought resistant
Bromus kalmii	Prairie brome	Cool season grass, short rhizomes with fibrous roots
Schizachyrium scoparium	Little bluestem	Warm season grass, drought tolerant, deep and fibrous root system
Bouteloua curtipendula	Side-oats grama	Warm season grass, dense and shallow root system
Sporobolus heterolepis	Prairie dropseed	Warm season grass, fibrous and short-rhizomatous root system
Dalea purpurea	Purple prairie clover	Nitrogen-fixing legume, purple flowers, blooms June-September
Liatris aspera	Blazing star	Highly attractive to many diverse pollinators, purple flowers, blooms July-October
Amorpha canescens	Lead plant	Nitrogen-fixing legume, shrub like, purple flowers, blooms June-August
Asclepias tuberosa	Butterfly weed	Highly attractive to pollinators, orange flowers, blooms June-August

Table 1.3. Mean seed, focal biomass, and total biomass yield F-value and significance for main effects and interactions for the three focal species in Becker and Rosemount.

	Becker						Rosemount					
	Canada milk vetch		Purple coneflower		Showy tick trefoil		Canada milk vetch		Purple coneflower		Showy tick trefoil	
	F-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*
Seed Yield												
Design	42.77	<0.0001	16.47	<0.0001	42.05	<0.0001	77.36	<0.0001	104.68	<0.0001	12.75	<0.0001
Year	17869.4	<0.0001	40.48	<0.0001	22.98	<0.0001	27402.6	<0.0001	37.60	<0.0001	194.22	<0.0001
Design x Year	11.03	<0.0001	1.59	NS†	1.02	NS	19.72	<0.0001	2.03	NS	2.18	NS
Focal Biomass												
Design	7.43	0.0001	14.38	<0.0001	48.45	<0.0001	20.28	<0.0001	18.41	<0.0001	14.99	<0.0001
Year	141.44	<0.0001	2.13	NS	13.15	<0.0001	464.62	<0.0001	6.59	0.0038	21.06	<0.0001
Design x Year	2.38	0.03	0.24	NS	0.75	NS	5.69	<0.0001	1.29	NS	2.63	0.02
Total Biomass												
Design	1.95	<0.0001	3.83	0.0074	12.54	<0.0001	2.91	0.027	2.87	0.029	4.62	0.0025
Year	59.86	NS	4.76	0.015	21.26	<0.0001	289.11	<0.0001	13.92	<0.0001	15.44	<0.0001
Design x Year	5.46	0.0001	1.89	NS	0.46	NS	9.86	<0.0001	1.13	NS	2.11	NS

† NS, not significant at the 0.05 probability level.

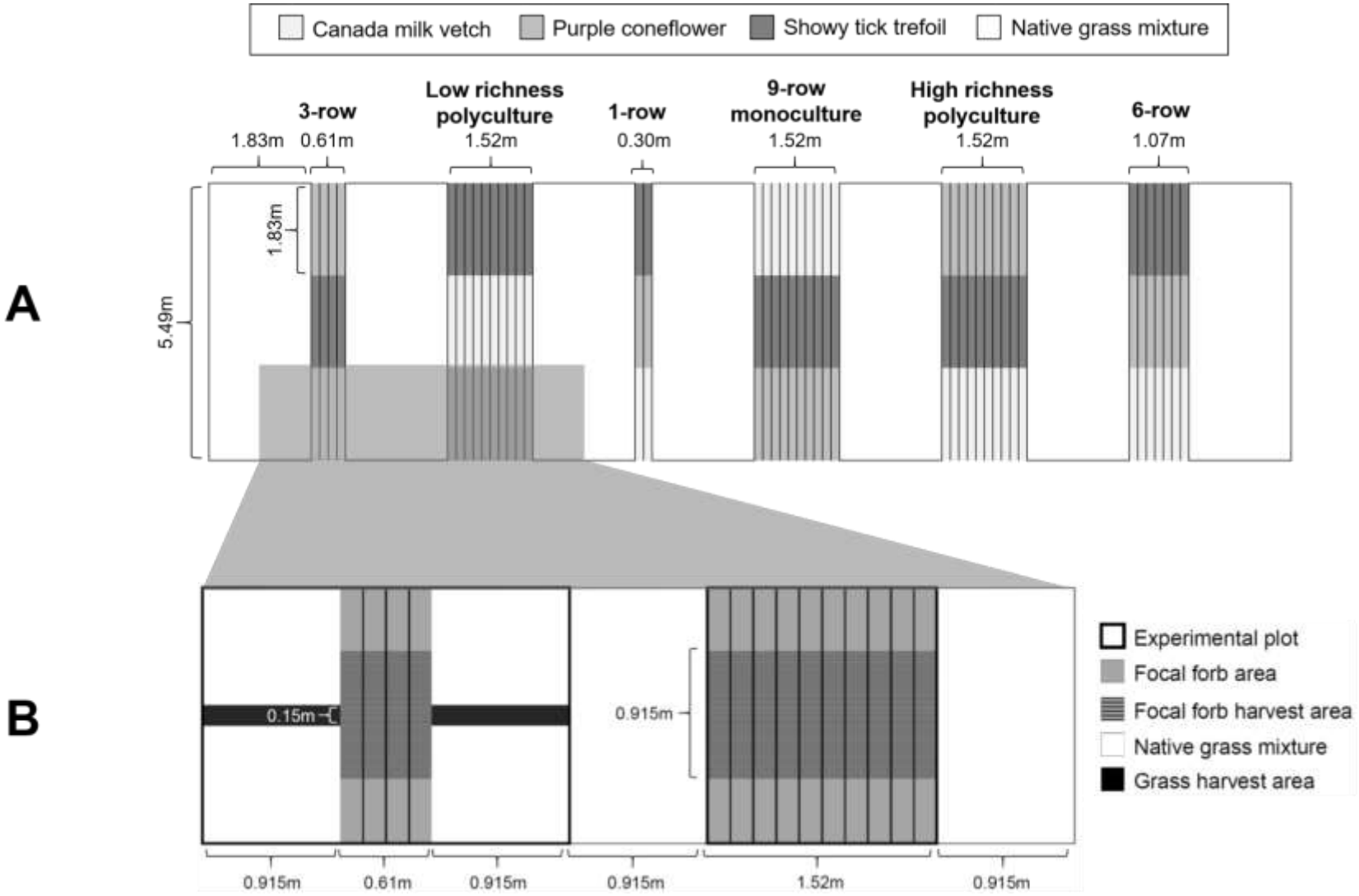
Canada milk vetch
Purple coneflower
Showy tick trefoil
Native grass mixture
A
3-row
1.83m
0.61m
Low richness polyculture
1.52m
1-row
0.30m
9-row monoculture
1.52m
High richness polyculture
1.52m
6-row
1.07m
1.83m
5.49m
B
0.15m
0.915m
0.915m
0.61m
0.915m
0.915m
1.52m
0.915m
Experimental plot
Focal forb area
Focal forb harvest area
Native grass mixture
Grass harvest area

Figure 1.1. Schematic of a single block from the field experiment (A) with plot dimensions. Enlarged panel (B) shows the harvest areas for the focal forbs and grass. Focal forbs were harvested from every design, while grass was only harvested from strip row designs.

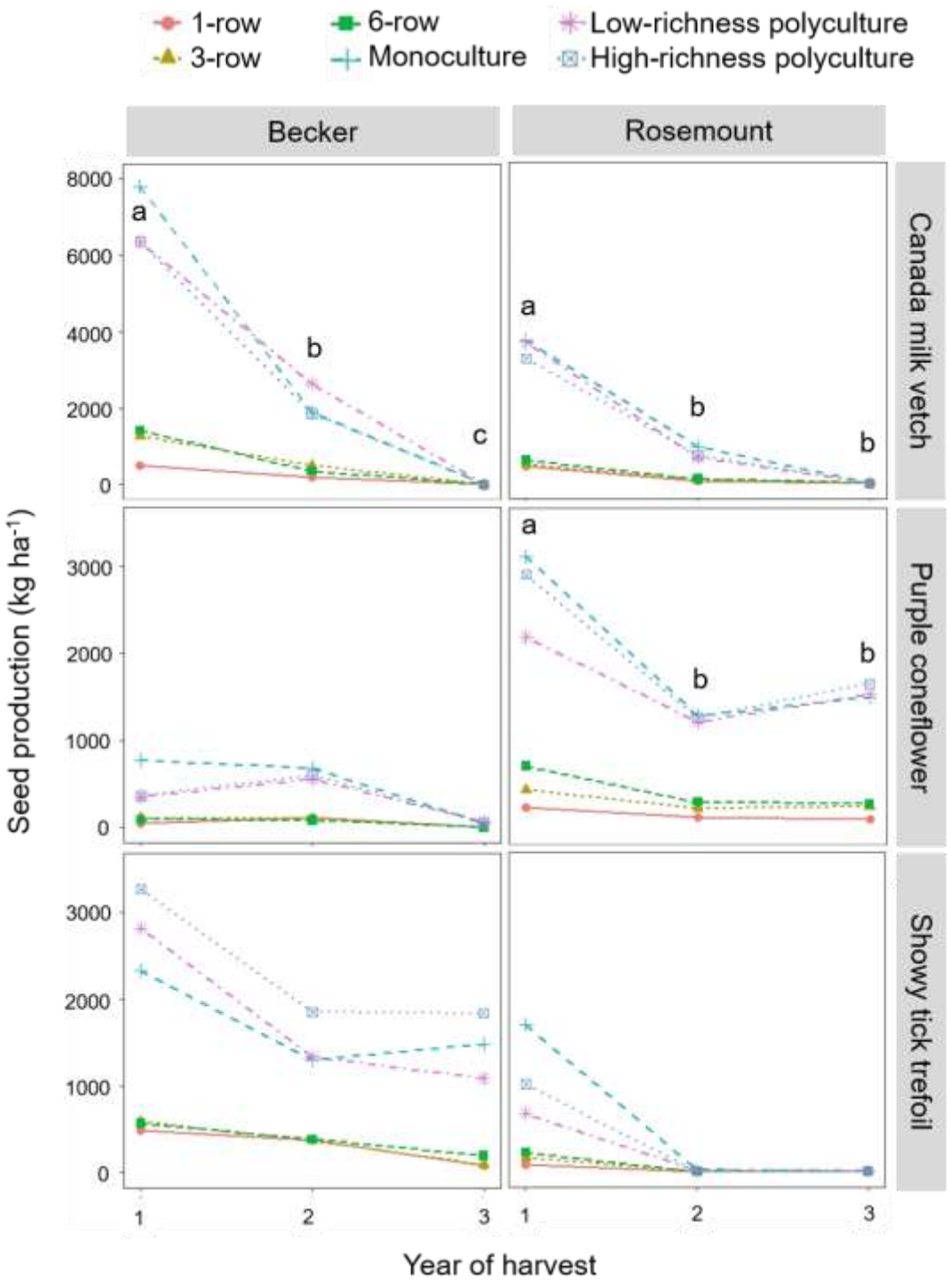

Figure 1.2. Mean focal forb seed yield by species and agronomic design at two locations for three years. Error bars were omitted from this figure for clarity (data in supplementary materials). Letters denote a significant difference of the average of all designs within a year across 3 years of harvest. Absence of letters indicates no significant change in mean focal seed yield.

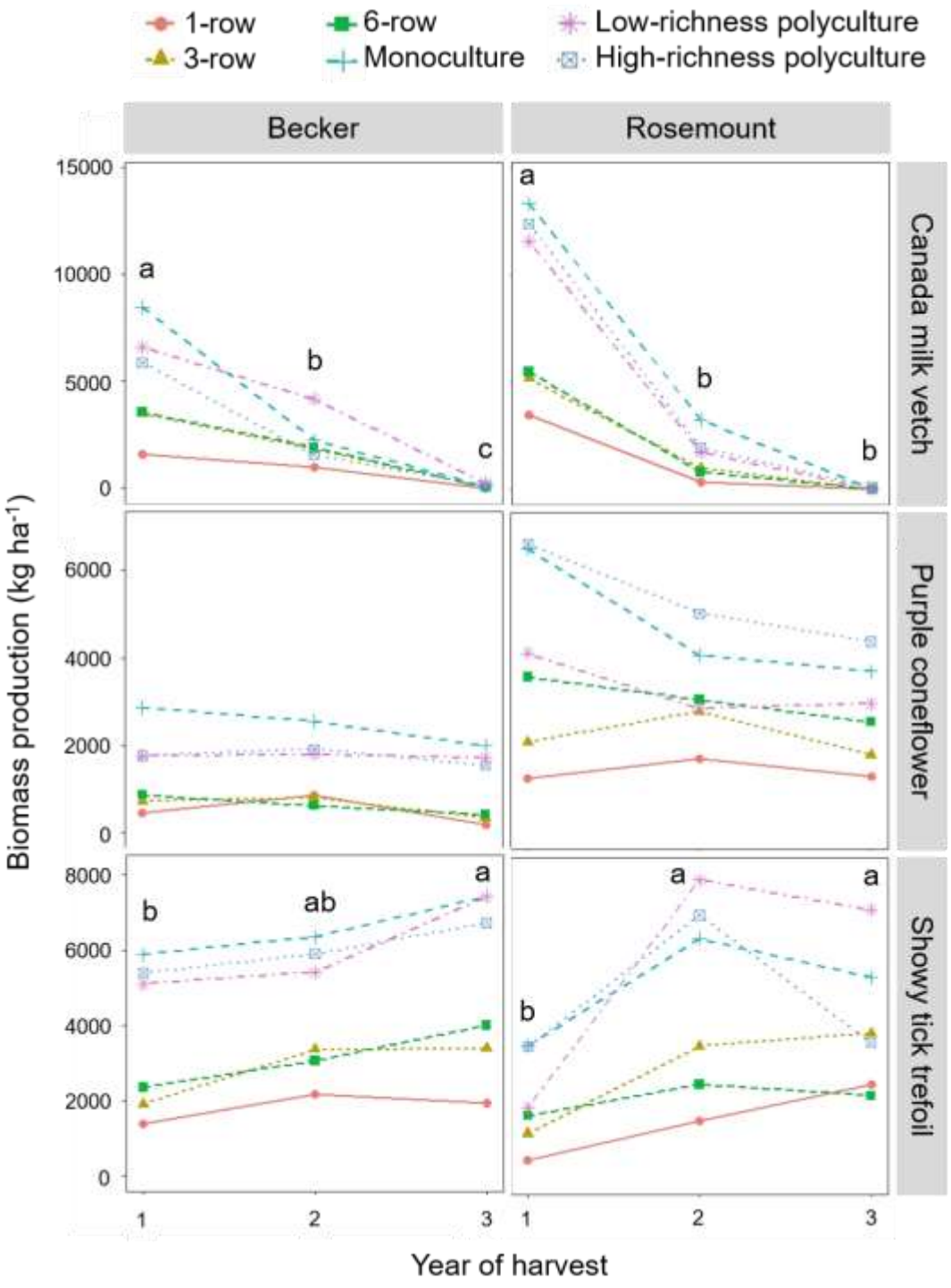

Figure 1.3. Mean focal forb biomass yield by species and agronomic design at two locations for three years. Error bars were omitted from this figure for clarity (data in supplementary materials). Letters denote a significant difference of the average of all designs within a year across 3 years of harvest. Absence of letters indicates no significant change in focal biomass yield.

Chapter 2

Field production of purple coneflower (*Echinacea purpurea* Moench [L.]) for beneficial phytochemicals

ABSTRACT

Plants species from the genus *Echinacea* are widely used as dietary supplements for immunomodulatory purposes and for treatment of upper respiratory ailments. Purple coneflower (*Echinacea purpurea* Moench [L.]) is the most widely utilized and cultivated species of *Echinacea*. Cultivation of purple coneflower has mainly focused on methods to increase biomass yields and there are few reports of field-level changes to alter phytochemical content. Experimental plots were established at two locations in Minnesota to evaluate the effect of site and agronomic design on the phytochemical profile of purple coneflower, as well as the relative content of several provisionally identified compounds. Five different tissues types were harvested, and extracts were prepared and analyzed using liquid chromatography-mass spectrometry. Site of production affected the levels of several caffeic acid derivatives in leaf, stem and root tissue, but agronomic design had little to no effect on phytochemical content. Tissue types were chemically distinct, likely due to alkamide content, and all tissue types contained phytochemicals of medicinal interest. The chemical content of purple coneflower seed, which is uncommon in *Echinacea* supplements, was found to be similar to that of root. Our findings suggest that seed tissue may be used either in place of, or in addition to, root tissue in purple coneflower preparations, thus preserving the rootstock and allowing for continued harvests in successive years.

INTRODUCTION

Consumer demand for herbal dietary supplements has been increasing for the past 15 years in the United States, with sales surpassing $8 billion in 2017 (Smith et al., 2018). *Echinacea* is one of the most popular dietary supplements, with total sales around $85 billion annually (Smith et al., 2018). The genus *Echinacea* (Asteraceae) is comprised of ten herbaceous perennial flowering species native to North America, and three of these species are used medicinally: narrow-leaf coneflower (*Echinacea angustifolia* [DC.] Hell.), pale purple coneflower (*Echinacea pallida* [Nutt.] Nutt.), and purple coneflower (*Echinacea purpurea* [L.] Moench) (Barrett, 2003). *Echinacea* supplements are mainly utilized as chemo-preventative agents or as a treatment for upper and lower respiratory illnesses (Barnes et al., 2005; Grimm and Müller, 1999). *Echinacea* products have also been implicated in the enhancement of natural killer cell activity (Currier and Miller, 2000), production of cytokines in macrophages (Burger et al., 1997; Sharma et al., 2009), antioxidant and antiviral activity (Hu and Kitts, 2000; Vimalanathan et al., 2005), and the stimulation of *in vivo* and *in vitro* phagocytosis (Bauer et al., 1988). Although results of clinical trials pertaining to the effectiveness of *Echinacea* have been inconsistent (Turner et al., 2000; Barrett et al., 2002; Schoop et al., 2006; Karsch-Völk et al., 2015), use as a supplement is nonetheless highly popular and production is growing (Riggs and Kindscher, 2016; Miller and Yu, 2004).

No single class of metabolites has been shown to be responsible for the bioactivity of *Echinacea*, but rather several different classes appear to be important (Barnes et al., 2005). Polysaccharides and glycoproteins have been isolated from *E. purpurea* and have

been shown to possess immunostimulatory and anti-inflammatory properties (Melchart et al., 2002; Bodinet et al., 2002). Phenylpropanoids, including caffeic acid glycosides and caffeic esters of quinic and tartaric acid, have shown antioxidant, antibacterial, and antiviral activity and are commonly reported in *Echinacea* samples though in differing amounts across the three medicinal species (Hu and Kitts, 2000; Binns et al., 2002). Echinacoside is reportedly the major phenolic component of *E. angustifolia* and *E. pallida* roots while cichoric acid is the major phenolic component of *E. purpurea* roots and aerial tissues (Pietta et al., 1998; Wills and Stuart, 1999). Over 20 different alkamides have been identified from root and inflorescence tissue, and exhibit immunostimulatory activity (Goel et al., 2002; Matthias et al., 2004). Similar to the phenylpropanoid compounds, alkamide content varies by species. Ketoalkenes and ketoalkynes with a carbonyl in the 2-position are the predominant alkamides in *E. pallida*, while alkamides derived from undeca- and dodecanoic acid are common in *E. angustifolia* and *E. purpurea* (Bauer and Woelkart, 2005).

Purple coneflower (*Echinacea purpurea* [L.] Moench) is the most widely cultivated of the *Echinacea* species and is reported to have high levels of caffeic acid derivatives and alkamides compared to other *Echinacea* species (McKeown, 1999; Miller and Yu, 2004). While root and rhizome tissues are the major tissues of interest in other *Echinacea* species, flower and aerial tissues from purple coneflower, which can be harvested in the first year of growth, also contain phytochemicals of interest (Perry et al., 1997; Vimalanathan et al., 2005; Manayi et al., 2015). In addition to therapeutic benefits, purple coneflower may provide several ecological benefits to an agricultural landscape. It is a

drought-tolerant, long-lived perennial that is easily established from seed as well as from transplants and can thrive in a variety of soil types, making it sustainable option for marginal lands (Kindscher, 2016). Single plants can produce numerous flowering heads each season and support a diverse mixture of pollinator groups including native bees, honey bees, flies, beetles, wasps, and butterflies (Wagenius and Lyon, 2010; Wist and Davis, 2005). The potential of enhanced ecosystem services along the marketability of bioactive phytochemicals make purple coneflower an attractive alternative crop for producers.

Although wild-harvested *Echinacea* may be considered more desirable than cultivated plants due to a perceived higher chemical content and wild-harvested purple coneflower root and floral tissues have been found to contain higher levels of cichoric acid, chlorogenic acid, and certain alkamides (Binns et al., 2002b; Kindscher and Riggs, 2016), cultivation may provide an opportunity to optimize phytochemicals of interest while preserving native *Echinacea* stands. Agricultural practices, such fertilization and irrigation, have been shown to affect the phytochemical content of other medicinal species (Zhu et al., 2009; Baghalian et al., 2011; Osuagwu and Edeoga, 2012; Tavarini et al., 2015; Shiwakoti et al., 2016). Fertilization and time of harvest are currently the practices of greatest interest for the enhancement of beneficial compounds in purple coneflower. Application of fertilizer, in the forms of biodynamic compost and conventional nitrogen, has been found to positively influence alkamide content of *Echinacea* species (Kindscher and Riggs, 2016; El-Gengaihi et al., 1998) while nitrogen fertilization has also been shown to decrease production of phenolic acids in purple

coneflower in the first year of production (Berbec et al., 1998). With concerns about nutrient runoff from agricultural lands, more sustainable cultivation strategies should be pursued to develop systems that optimize phytochemical content of purple coneflower while limiting chemical inputs.

Biotic and abiotic environmental factors strongly influence the plant metabolome (Brunetti et al., 2013; Bundy et al., 2009; van Dam and van der Meijden, 2018). Higher phytochemical content in wild populations may be due to complex community and soil conditions such as allelopathy or organic matter, so cultivating purple coneflower in a mixed plant community similar to a native environment may increase yields of desired phytochemicals, but it may also risk a reduction in desired phytochemicals (Binns et al., 2002b). To examine the effect of the plant community on the phytochemical content of purple coneflower, five tissue types were harvested from plants grown at two sites in Minnesota in six different agronomic designs, ranging from single rows to polyculture communities. Liquid chromatography-mass spectrometry analyses were performed to examine the effects of location, plant community, and tissue type on metabolites with known bioactivity as well as the overall metabolomic profile of purple coneflower.

METHODS

Field experimental design

The field experiment was established in June 2015 at University of Minnesota Agricultural Experiment Stations in Becker (45°23'14.8"N 93°53'31.4"W) and

Rosemount (44°41'21.0"N 93°04'26.3"W), MN. Mean annual precipitation April to October was 90.0 mm in Becker and 99.9 mm in Rosemount. Mean annual temperature April to October was 15.2°C in Becker and 15.9°C in Rosemount.

Purple coneflower was established in six different planting designs at each site with three replications. Purple coneflower seeds were sown at a depth of 2 centimeters with a target plant density of 20 plants per linear meter in one row, three rows, six rows, nine rows, nine rows seeded with five native grass species (low-richness polyculture), and nine rows seed with five native grass species and four native forb species (high-richness polyculture). All rows were 1.83 meters long and 0.15 meters apart. Experimental plots were separated by a native grass mixture. Further details on the experimental design are given in (Freund Saxhaug et al., *in review*).

Plant collection and processing

Flower, leaf, stem and root tissue were harvested July 14, 2016 in Rosemount and July 20, 2016 in Becker. Seed was harvested September 14, 2016 in Rosemount and September 20, 2016 in Becker. Six individual coneflower plants were randomly selected for sampling from each experimental plot. Selected plants were harvested from the only row in the one-row design, the center row in the three-row design, the center two rows in the six-row design, and the center three rows of the nine-row and polyculture designs. All selected plants were from the center 0.9 meters of the rows and the center rows of the plots. Selected plants were dug up using a small trowel taking care to preserve the entire

root system. Tissue-specific processing details are described in Table 2.1. Plant samples were flash frozen on dry ice, secured in coolers, and transported to the Plant Metabolomics laboratory at the University of Minnesota in St. Paul. Leaf, stem and seed tissue samples were stored at -80°C in the dark until extraction. Root and flower tissue samples were lyophilized, ground into a fine powder, homogenized, and stored in the dark at room temperature until extraction.

Phytochemical extraction and metabolite profiling

Extractions of plant samples were completed at the Plant Metabolomics laboratory and were carried out at room temperature (25°C). Leaf, stem, and seed tissue samples in microcentrifuge tubes were removed from the freezer, thawed, and weighed. For flower and root samples, 20 milligram aliquots of ground tissue were dispensed into microcentrifuge tubes prior to extraction. A 2.5-millimeter tungsten carbide bead was added to each sample with 70% isopropanol at a rate of one milliliter per 200 milligrams of fresh frozen sample or one milliliter per 20 milligrams of lyophilized and ground sample. A SPEX SamplePrep model 2010 Geno/Grinder® was used to pulverize samples for five minutes at 1500 rpm. Samples were subjected to centrifugation for five minutes at 14000 G, followed by transfer of the supernatants (extracts) into a sterile microcentrifuge tubes. All extracts were stored at -80°C in the dark prior to LC/MS analysis.

Metabolomic profiles were obtained using the Ultimate® 3000 HPLC Q Exactive™ (Thermo Scientific), a C_{18}-reversed-phase ultra-performance liquid chromatography-electrospray ionization-hybrid quadrupole-orbitrap mass spectrometer with an autosampler and sample vial block maintained at 4°C. Chromatographic separations were performed using a sample injection volume of 0.5 µL, and an Acquity reversed-phase C_{18} HSS T_3 1.8 µm particle size, 2.1 × 100 mm column (Waters), with column temperature 40°C, and flow rate 0.40 mL/min. The solvent system consisted of solvent A (water including 0.1% formic acid) and solvent B (acetonitrile including 0.1% formic acid). The 25-minute gradient program was as follows: initial 2% B, 2 min 2% B, 1 min 15% B, 5 min 30% B, 1 min 50% B, 4 min 70% B, 3 min 98% B, 1 min 98% B, 1 min 2% B. The MS conditions were as follows: full scan mass range 115-1000 *m*/*z*, resolution 70,000, desolvation temperature 350°C, spray voltage 3800 V, auxiliary gas flow rate 20, sheath gas flow rate 50, sweep gas flow rate 1, S-Lens RF level 50, and auxiliary gas heater temperature 300°C. Data were acquired using Xcalibur™ software version 2.1 (Thermo Scientific). Samples were analyzed by tissue in five consecutive batches, cleaning the sweep cone between different tissues. Several controls were included in each batch of samples: HPLC-grade water was used as an internal laboratory control, tissue-specific pools were used as batch controls, and a universal pool of all tissue types was used to align individual samples in the data processing steps. Sample analysis order was randomized within each tissue sample set.

Data processing and statistical analyses

Raw format files were converted to the open mzML format using the ProteoWizard tool msconvert (Chambers et al., 2012). Data acquired in the first two minutes and the final six minutes of each sample run were excluded from the analysis because of noise. Converted mzML files were compressed into ZIP format using the 7-Zip software then uploaded to the Galaxy platform Workflow4metabolomics (W4m) to preprocess, annotate, and perform statistical analyses of the metabolomic data collected from the Q Exactive™.

The following processes were performed for both positive and negative ionization mode prior to use of the Join +/- ions tool (Eschenlauer et al., 2018). Thermo Scientific Xcalibur software was used for *m/z*-domain centroid fitting, and features were picked from the centroided data using the W4m XCMS Set tool (Guitton et al., 2017) with the following parameters: "centWave" method, maximum ppm *m/z* deviation 3 ppm, minimum peak width 2 seconds, maximum peak width 5 seconds, signal/noise threshold 3, minimum difference in *m/z* for peaks with overlapping retention times -0.001, peak limits based on second derivative, prefilter "3,1e5", and noise filter 0.

Differences in chromatographic retention time were reconciled using the W4m XCMS Group tool (Guitton et al., 2017). The Group tool was run before the retention correction with the following parameters: "density" grouping method, bandwidth six seconds, minimum fraction of samples 0 and width of overlapping *m/z* slices 0.006, with a

maximum of 50 groups to identify in a single *m/z* slice. Retention correction was performed with the W4m XCMS Retcor tool (Guitton et al., 2017) using the following parameters: “peakgroups” method, 50 missing samples allowed in retention-time correction groups, 1 extra peak allowed in retention time correction groups, LOESS smoothing method with a degree of smoothing for local polynomial regression fitting of 2 and gaussian fitting.

Low-intensity features in samples were estimated using the “chrom” method in the W4m XCMS FillPeaks tool (Guitton et al., 2017). The W4m CAMERA tool (Guitton et al., 2017) was used to annotate feature lists using default parameters. Features of the following classes were eliminated using the W4m Quality Metrics tool (Guitton et al., 2017): features with intensities threefold greater in blanks than in samples, features with no variance across samples, samples with no variance across features, data for blanks, and data for pools, features not annotated by CAMERA as the most abundant mass in a given isotopic envelope. The Join +/- ions tool (Eschenlauer et al., 2018) was used to combine resultant peak lists for data collected in positive and negative ionization modes. Values in the dataMatrix were log base 2 transformed (Kohl et al., 2012).

Several features were provisionally identified by comparing *m/z* values and UV absorption maxima of ions and fragments with previously published datasets and by performing mass searches of the Human Metabolome Database (HMDB). The program R (R Core Team, 2018) was used to conduct statistical analyses. Mixed effects models were

conducted using the “nlme” package (Pinheiro et al., 2018) to examine the effect of site and planting design on the mean relative intensity of select features, with block considered a random effect. The “emmeans” package (Lenth et al., 2018) was used to conduct Tukey’s least significant difference test comparing least square means. Multivariate statistical analyses by principal components analysis (PCA) were conducted to visualize differences in metabolomic fingerprints of the five tissues, the two sites, and the six planting designs treatments.

RESULTS

To investigate changes in metabolomic profiles and changes in the relative intensity of specific metabolites from purple coneflower grown under six different cultivation conditions at two sites, five tissue types were harvested and processed for LC-MS analysis. 2743 peaks were detected and 27 were provisionally identified as compounds previously reported in purple coneflower (Table 2.2).

Untargeted analyses of the overall metabolomic profile

Principal components analysis (PCA) was utilized to explore the effect of tissue, site and cultivation design on the overall metabolomic profile (all features) of purple coneflower. Based on differences in environmental conditions between the two sites including, but not limited to, soil type and precipitation, data from the two sites were analyzed separately. The metabolic profiles of the five different tissues show separation from one another at each field site, mostly along the second principal component (Figure 2.1A-B).

The second principal component (PC) explained 10% of the total variability at Becker and 11% at Rosemount. Metabolomic profiles of seed tissue samples from Becker were separated from all other tissues, where seed profiles obtained from Rosemount were not as distinct from other tissues. Leaf tissue at both field sites appeared to separate from other tissues along the first PC, which explained 49% and 45% of the total variability at Becker and Rosemount, respectively. Differences in the overall metabolomic profiles between the two field sites were assessed for each tissue type using PCA (Figure 2.2A-E). The two field sites were not distinguishable by PCA for either flower or seed tissues. Stem and leaf tissues were showed slight separation along the second PC, accounting for 13% and 16% of the total variability. Root tissue from the two sites was also distinguishable, although the points form an arch shape. There is no apparent effect of agronomic design on the overall metabolomic profile for any of the five tissues using PCA (Supplementary Figure B.1A-E).

Effect of tissue, site, and agronomic design on provisionally identified features

Select features were provisionally identified as 16 phenolic compounds and 11 alkamides based on comparison with previously published mass and UV absorption data sets as well as library mass searches (Table 2.2). A heatmap was created to visualize differences between the five tissues (Figure 2.3). Shading of the blue color is associated with the mean of the relative abundances of the ions detected in each sample ($\log_2$ of the intensity). Hierarchical clustering was used to group tissues and features. Cichoric acid, caftaric acid, chlorogenic acid, caffeic acid, and echinacoside are the major phenolic components of *Echinacea* extracts (Miller and Yu, 2004). These five compounds were

detected in all of the tissues analyzed, with the exception of echinacoside that was absent in seed tissue. Provisionally identified phenolic compounds tended to be less abundant in seed and root tissue, whereas alkamides tended to be more abundant. A correlation matrix of the 27 features shows that many of the alkamides are positively correlated, but are negatively correlated with several phenolic compounds (Figure 2.4).

The metabolomic tissue profiles based on the select 27 features show distinctions between tissues along the first PC (Figure 2.1C-D). The first PC explains 44% of the variation at Becker, and leaf, flower and stem tissue are completely separated from seed and root tissue. There iss greater overlap of stem and seed tissue at Rosemount, and the first PC explains 39% of the variation. Alkamides are the greatest contributors to the difference between tissues at both sites (Supplementary Figure B.2). As with the overall metabolomic profile, slight differences between the two sites are notable in leaf, stem and root tissue (Figure 2.2F-J). Differences between sites were examined further by plotting the mean intensity of the select features from Becker versus Rosemount, in which a slope of 1 would indicate similarity between sites (Figure 2.5).

All major phenolic compounds of *Echinacea* were found to be significantly different between the two sites with the exception of echinacoside in root tissue (Figure 2.5; Supplementary Table B.1 and B.2). No differences in alkamide content between sites were found. Again, there is no discernible effect of agronomic design on the profile of the common features (Supplementary Figure B.1F-J).

Influence of agronomic design in leaf tissue

Differences due to agronomic design were not apparent through principal components analyses, so mixed effects analyses were utilized to examine the effect of design on individual select features. Potential differences in the levels of caffeic acid, caftaric acid, and cichoric acid were detected in leaf tissue from Rosemount (Figure 2.6; Supplementary Table B.3). These three phenolic compounds exhibited the same trend: as the number of rows in a design increased, the mean intensity increased. No differences between the nine-row monoculture or two polyculture designs were detected, although the low-richness polyculture always had the highest relative intensity. No other differences due to agronomic design were found for the major phenolic compounds of *Echinacea* or the 11 provisionally identified alkamides in any other tissue.

DISCUSSION

Purple coneflower is a popular medicinal herb widely utilized for its immunostimulatory and anti-inflammatory properties, as well as for treatment of various other ailments (Manayi et al., 2015). The chemistry of purple coneflower is well documented, and caffeic acid derivatives and alkamides are considered the most important contributors to the bioactivity of coneflower extracts (Barnes et al., 2005). Although many medicinal plants, including *Echinacea* species, are wild-collected, there is a trend towards cultivation (Chen et al., 2016). Cultivation of medicinal species such as purple coneflower may lead to higher yields of active compounds through greater control and

manipulation of growth conditions, and thus a greater economic return for the producer. In this research, the effects of tissue type, site of production, and agronomic design on the phytochemical profile of purple coneflower were examined through use of a field experiment and liquid chromatography-mass spectrometry analyses.

Site and tissue type influence metabolomic profile

In contrast with previously reported high variability of phenolic and alkamide content from geographically distinct populations of purple coneflower (Berbec et al., 1998; Wills and Stuart, 1999), analysis of all detected features and of the 27 select features showed little difference between the two experimental sites with the exception of some differentiation in metabolomic profile of stem, leaf and root tissue (Figure 2). Other experiments involving purple coneflower harvested from distinct geographical populations have determined no differences in the concentration of the phenolic caffeic acid derivatives caftaric acid, chlorogenic acid, chicoric acid, and echinacoside (Liu et al., 2007). In this experiment no significant differences were found between sites for any tissue in regard to alkamide content, however, further analysis of the top phenolic compounds revealed higher relative intensities from Becker in stem, leaf and root tissue for caffeic acid, caftaric acid, chlorogenic acid, cichoric acid, and echinacoside in leaf and stem tissue. Seed and flower tissue were not significantly different between sites for any specific feature.

Levels of caffeic, caftaric, chlorogenic, and cichoric acid were all significantly higher at Becker than at Rosemount. Soil and environmental characteristics may account for the differences in relative intensities of phenolic compounds between the two sites in this experiment. Soil at the Becker site is described as a sandy Hubbard-Mosford complex whereas the soil at Rosemount is Port Byron silt loam. Average precipitation was greater Rosemount and N was nearly threefold greater at Rosemount than at Becker (Freund Saxhaug et al., *in review*). Water and nutrient availability have previously been reported to influence the phenolic content of purple coneflower: irrigation reduces the yield of phenolic compounds while drought stress stimulates the production of cichoric acid (Yousef et al., 2013; Gray et al., 2003), and higher nitrogen availability reduces phenolic content in purple coneflower (Yousef et al., 2013). In contrast to several phenolic acids, alkamide content did not significantly differ between experimental sites. Developmental stage of harvest, rather than abiotic and biotic conditions, is currently considered the major driver of alkamide content of purple coneflower (Gray et al., 2003; Letchamo et al., 1999; Qu et al., 2005; Thomsen et al., 2012). Experimentation in highly controlled environments like greenhouses or in hydroponic systems (similar to Zheng et al., 2006) may help elucidate the effects of environmental influences on caffeic acid derivatives, and potentially alkamides, produced by purple coneflower to provide direction for field production recommendations.

Unlike leaf and stem tissue, the metabolomic profiles of flower and seed tissue were not strongly influenced by site of production. This inconsistency may be due to differences in the functions of these tissues. Mature leaves and stems are considered source tissues,

actively producing photosynthate that is transported to sites of active growth in the plant. Flowers and seeds are points of photosynthate delivery, consumption, and storage and are considered sink tissues. There is, perhaps, some general tendency toward maintaining a stable biochemical profile in sink tissues in response to fluctuating environmental conditions. Through work on the medicinal plant *Tithonia diversifolia* ([Helms.] A. Gray), leaf and stem tissue were found to be strongly influenced by geographic origin, whereas root and inflorescence tissue were not (Sampaio et al., 2016). Maintenance of consistent metabolomic profiles of flower and seed tissue may be due to other ecological and evolutionary pressures, such as interactions with pollinators (Verdonk et al., 2003), chemical requirements for dormancy and germination (Finch-Savage and Leubner-Metzger, 2006), or protection of seeds from predators (Cervantes-Hernández et al., 2019). Although there is a limited body of research on the environmental effects on tissue-specific metabolomes of medicinal plants, flower and seed tissue may maintain relatively similar metabolomic profiles across geographically-distinct sites of cultivation, thus resulting in a more consistent end product.

Tissues possess distinct metabolomes

Untargeted mass spectrometry-based analysis of all detected features showed differences between the five tissue types at both sites, although tissue types did overlap indicating some shared similarity in metabolomic profile (Figure 2.1). Analysis of the select 27 features showed similar separations and slight overlap among tissues. The findings in this experiment are consistent with previous reports of alkamide content of various purple coneflower tissues. Root tissue is considered to be the main source of alkamides in purple

coneflower, especially C_{12} diene-diene alkamides (Binns et al., 2002b; Cech 2006; Bauer et al., 1989; Perry et al., 1997). In this experiment, root tissue was determined to be a main source of alkamides (Figure 2.3). Although aerial tissues are not considered to be significant sources of alkamides, they do contain higher levels of C_{12} tetraene alkamides and C_{11} diene-diynes (Wills and Stuart, 1999; Perry et al., 1997). Of the aerial tissues, flowers are considered to have the highest levels of alkamides, followed by stems and then leaves (Wills and Stuart, 1999). Flower tissue was found to contain higher levels of alkamides than leaf and stem tissue, but generally less than seed tissue. Alkamide 1 and Alkamide 4 have been previously isolated from seed tissue (He et al., 1998), but this is the first report of additional alkamides and phenolic compounds found in purple coneflower seed. Seed tissue may have the potential to replace root tissue for medicinal use as it was the most chemically similar to root tissue based on the select 27 features (Figure 2.3). Further work must be done on quantifying the phytochemical content of purple coneflower tissues, especially that of seed if it is to be utilized as an alternative to root tissue.

Cichoric acid is considered to be one of the most important phytochemicals with bioactive properties in purple coneflower (Bauer, 1999). Similar to previous reports, flowers harvested in this experiment had the highest cichoric acid content, indicating the importance of including flower tissue in standardized supplements (Supplementary Table B.4) (Wills and Stuart, 1999; Stuart and Wills, 2000: Binns et al., 2002b). Stems contain reportedly lower concentrations of cichoric acid, also consistent with findings from this work, but are still sources of other caffeic acid derivatives such as caffeoylmalic acid.

Seed tissue was not a significant source of cichoric acid compared to the other tissues and overall contained lower levels of phenolic compounds. No single tissue contained the highest amount of all compounds with known bioactive properties, indicating that the most beneficial purple coneflower supplements may be the combination of all tissues together. This may also be the most agronomically effective approach. Seed and flower tissue could potentially replace root tissue in terms of alkamide content, thus preserving the root system for sustained production. Flower and leaf tissue contain high amounts of cichoric acid along with several other caffeic acid derivatives, while stem tissue can provide additional alkamide content. The combination of alkamide-rich tissues along with phenolic acid-rich tissues may also provide a supplement with higher potency, as alkamides have been found to increase the antioxidative activity of cichoric acid (Thygesen et al., 2007). Pooling all tissues together could potentially decrease the workload of producers, as plants could be mechanically harvested in bulk (Thomsen et al., 2018).

Agronomic design influences metabolites in leaf tissue

Purple coneflower plants were established in different agronomic designs of varying row numbers and diversity to test the influence of the plant community on phytochemical production. An effect of agronomic design was found only for caffeic acid, caftaric acid, and cichoric acid in leaf tissue from Rosemount (Figure 2.7). Plants from the one-row treatment had the lowest levels of these phenolic acids while the nine-row had the highest. It was originally predicted that the highest levels of caffeic acid derivatives, which are sometimes produced as defensive compounds (Korkina, 2007), would be

detected in purple coneflower samples from the one-row design due to increased solar ultraviolet radiation and heterospecific competition with the grass edge. (Broz et al., 2010). UV radiation has previously been shown to increase phenolic acid content various plant species (Liu et al., 1995; Lavola, 1998; Luthria et al., 2006), and enhances production of caffeic acid derivatives in purple coneflower hairy root cultures (Abbasi et al., 2007). The composition of the plant community can also affect an individual plant's biochemistry (Scherling et al., 2010), with higher presence of defensive compounds when surrounded by different species (Broz et al., 2010).

There was no significant difference between the nine-row and both polyculture designs, which were designed to vary by the presence or absence of other plant species and not by density or number of rows of purple coneflower. The additional polyculture species had poor germination and growth in Rosemount, such that the polyculture plots more closely resembled monoculture plots (Freund Saxhaug et al. *in review*), possibly explaining the similarity in metabolomic profile among the different agronomic designs.-Although there were no apparent differences in metabolomic profile between agronomic designs, larger plots of purple coneflower (nine-row monoculture, low- and high-richness polyculture plots) had higher levels of several caffeic acid derivatives in leaf tissue. The pattern of higher phenolic acid content in response to increasing number of rows may be attributable to nutrient depletion based on plant community composition (Sherrard et al., 2019). Plant communities with lower diversity, such as monocultures, tend to have higher rates of nutrient depletion (Fornara and Tilman, 2009), and this depletion can lead to

greater production of phenolic acids, such as those found in purple coneflower (Chishaki and Horiguchi, 1997).

A predominant finding of this experiment was that there was little to no effect on phytochemical content and metabolomic profile of purple coneflower due to agronomic design. This may be a result of several unknown interacting ecological factors offsetting one another. Recent research has directed attention to soil and plant microbial communities and their effect on the content and bioactivity of purple coneflower extracts (Haron et al., 2019; Maggini et al., 2017; Maggini et al., 2019). Samples were harvested the year after establishment, so there may not have been sufficient time for plant and soil community dynamics to significantly affect the plant metabolome. Time of harvest is another important factor affecting the content of bioactive compounds in purple coneflower (Stuart and Wills, 2000; Thomsen et al., 2018), and earlier or later season harvests may have yielded vastly different results. These results, however, may be simply due to the stronger influence of the inherent phytochemical profile of purple coneflower over effects of the greater plant community. Clearly several field-level and environmental factors will need to be considered when establishing and maintaining purple coneflower for production of therapeutic phytochemicals.

CONCLUSION

Purple coneflower was established in six different agronomic designs at two different sites in Minnesota. The year after establishment, flower, leaf, root, stem and seed tissue

were collected and extracts were prepared for liquid chromatography-mass spectrometry analyses. Field-cultivated purple coneflower was found to contain several compounds of therapeutic interest previously reported in purple coneflower. Further work should include the quantification of these compounds of interest, especially caffeic acid derivatives and alkamides and the relation of these two chemical classes to determine the best harvest season to optimize the content of both. Phytochemical profile and the mean intensity of specific features were influenced by tissue type. Results suggest that seed tissue contains similar alkamides to that of root tissue and could potentially be used in place of root tissue to preserve purple coneflower stands for multiple years of harvest. Aboveground tissues could be pooled to maximize the content of all select features and eliminate the labor and time cost of separating harvested tissues. Site of production, but not agronomic design, affected phytochemical profile and the level of certain features, indicating a need for further exploration of environmental conditions that may influence bioactive compound production in purple coneflower. Since agronomic design overall had little effect on phytochemical content, recommendations for growing purple coneflower for bioactive phytochemicals depend on the goals of the producer. Purple coneflower could be established with additional native species to improve ecosystem services, or it could be established in single rows for greater ease of harvest. Future work on the field production of purple coneflower should address additional agronomic practices that may influence phytochemical content such as planting density and harvest time as well as adherence with industry standards of *Echinacea* supplements.

ACKNOWLEDGEMENTS

Thank you to Dr. Dana Freund, Lindsay Wilson, Abigail Diering, and the Sustainable Cropping Systems Laboratory at the University of Minnesota for assistance in the field and laboratory. Thank you to Dr. Arthur Eschenlauer and Mark Esler for technical support with Galaxy M. Funding for this research was provided by the AGRI Crop Research Grant Program from the Minnesota Department of Agriculture and the Hueg-Harrison Fellowship.

Table 2.1. Tissue-specific details on the metabolomics harvest of purple coneflower flower, leaf, stem, root, and seed.

Tissue type	Harvest and processing procedure
Flower	Topmost flowering head from the plant was removed and immediately placed in a freezer bag and put on dry ice.
Leaf	Three leavers were removed from the stem at the second, fourth, and sixth nodes (counting from the bottom). A sterile 8-mm diameter leaf punch was used to punch each leaf at the base of the leaf directly over the mid-vein and at the center of the leaf to the left of the mid-vein (approximately 200mg of tissue). All leaf disks from an individual plant were placed in a single 1.5-mL microcentifuge tube and placed dry ice.
Stem	Three 2-mm cross-sections of the stem were cut with a sterile razor directly below the second, fourth, and sixth nodes where the leaves were removed and sampled (approximately 200mg of tissue). Stem cross sections were placed in a 1.5-mL microcentrifuge tube and placed on dry ice.
Root	The underground rhizome, tap root and fibrous roots were separated from the aboveground portion of the plant using sterile clippers, rinsed in a bowl of sterile water, and placed in a freezer bag and put on dry ice.
Seed	Six mature seeds were harvested from the topmost flowering head from the plant (approximately 200mg of tissue), placed in a 1.5-mL microcentrifuge tube, and put on dry ice.

Table 2.2. Features provisionally identified in purple coneflower tissues by UHPLC–Orbitrap-MS.

Feature	Feature assignment	Observed ion	Molecular formula	Monoisotopic mass	Retention time (sec)	Observed *m/z*	Adduct *m/z*	Accuracy (ppm)	Reference(s) for identification
Phenolics									
P01	Protocatechuic acid (3,4-dihydroxybenzoic acid)	M-H	$C_7H_6O_4$	154.0266	405	153.0188	153.0193	3.4	Manayi et al., 2015; Barnes et al., 2005
P02	Coumaric acid	M-H	$C_9H_8O_3$	164.0473	292	163.0396	163.0401	3.0	Pomponio et al., 2002; Bauer 1999
P03	Caffeic acid	M-H	C_9H_8O4	180.0423	265	179.0195	179.0121	3.7	Luo et al., 2003; Abbasi et al., 2007; Lee 2012
P04	Quinic acid	M-H	$C_7H_{12}O_6$	192.0634	291	191.0556	191.0561	3.0	Pires et al., 2016; Maggini et al., 2019
P05	Caffeoylmalic acid	M-H	$C_{13}H_{12}O_8$	296.0532	292	295.0451	295.0459	3.0	Human Metabolome Datebase; Grevsen et al., 2008; Hahn and Nahrstedt, 1993
P06	Caftaric acid	M-H	$C_{13}H_{12}O_9$	312.0481	266	311.0399	311.0409	3.0	Cech et al., 2006; Pellati et al., 2011; Luo et al., 2003; Binns et al., 2002c; Thomsen et al., 2018
P07	Vanillic acid hexoside	M-H	$C_{14}H_{18}O_9$	330.0951	253	329.0872	329.0878	4.8	Du et al., 2017; Pomponio et al., 2002; Di Stefano et al., 2019
P08	Chlorogenic acid (3-O-caffeoylquinic acid)	M-H	$C_{16}H_{18}O_9$	354.0951	292	353.0871	353.0878	2.0	Luo et al., 2003; Binns et al., 2002c; Thomsen et al., 2018; Pellati et al., 2005
P09	Ferulic acid hexoside	M-H	$C_{16}H_{20}O_9$	356.1107	309	355.1027	355.1035	1.0	Du et al., 2017; Di Stefano et al., 2018; Pomponio et al., 2002
P10	Feruloylquinic acid	M-H	$C_{17}H_{20}O_9$	368.1107	348	367.1029	367.1035	1.0	Liu et al., 2018; Kuhnert et al., 2010
P11	Cichoric acid	M-H	$C_{22}H_{18}O_{12}$	474.0798	379	473.0709	473.0726	4.0	Cech et al., 2006; Pellati et al., 2011; Luo et al., 2003; Thomsen et al., 2018
P12	Undefined cinnamic acid	M-H	$C_{23}H_{20}O_{12}$	488.0955	460	487.0865	487.0877	2.5	Lu et al., 2012
P13	Cynarin (dicaffeoylquinic acid)	M-H	$C_{25}H_{24}O_{12}$	516.1268	437	515.1173	515.1195	4.3	Zheng et al., 2006; Binns et al., 2002c; Kabganian et al., 2003
P14	Rosmarinic acid glycoside	M-H	$C_{24}H_{26}O_{13}$	522.1373	495	521.1671	521.1646	4.8	Mari et al., 2015; Thygesen et al., 2007; Dalby-Brown et al., 2005
P15	Rutin (rutoside)	M-H	$C_{27}H_{30}O_{16}$	610.1534	383	609.1444	609.1461	2.7	Kurkin et al., 2011; Vimalanathan et al., 2013
P16	Echinacoside	M-H	$C_{35}H_{46}O_{20}$	786.2582	569	785.2622	785.2504	4.7	Binns et al., 2002c; Thomsen et al., 2018; Wang et al., 2009

Table 2.2 continued

Feature	Feature assignment	Observed ion	Molecular formula	Monoisotopic mass	Retention time (sec)	Observed *m/z*	Adduct *m/z*	Accuracy (ppm)	Reference(s) for identification
Alkylamides									
A01	Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide	M+H	$C_{15}H_{19}NO$	229.1467	690	230.1525	230.1539	5.9	Cech et al., 2006; He et al., 1998; Pellati et al., 2011; Luo et al., 2003; Mudge et al., 2011
A02	Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	M+H	$C_{16}H_{21}NO$	243.1623	725	244.1685	244.1701	6.6	Cech et al., 2006; Pellati et al., 2011; Luo et al., 2003; Mudge et al., 2011
A03	Dodeca-2E/Z,4E,10E-triene-8-ynoic acid isobutylamide	M+H	$C_{16}H_{23}NO$	245.1780	744	246.1844	246.1858	5.7	Cech et al., 2006; Luo et al., 2003; Mudge et al., 2011
A04	Dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide	M+H	$C_{16}H_{25}NO$	247.1936	794	248.2007	248.2014	3.0	Cech et al., 2006; He et al., 1998; Pellati et al., 2011; Luo et al., 2003; Mudge et al., 2011
A05	Dodeca-2E,4E,8Z-trienoic acid isobutlyamide	M+H	$C_{16}H_{27}NO$	249.2093	812	250.2185	250.2171	5.8	Cech et al., 2006; He et al., 1998; Pellati et al., 2011; Luo et al., 2003; Mudge et al., 2011
A06	Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide	M+H	$C_{17}H_{23}NO$	257.1780	761	258.1847	258.1858	4.4	Cech et al., 2006; He et al., 1998; Pellati et al., 2011; Luo et al., 2003; Mudge et al., 2011
A07	Dodeca-2E-ene-8,10-diynoic acid 2-methylbutylamide	M+H	$C_{17}H_{25}NO$	259.1936	786	260.1999	260.2014	5.9	Spelman et al., 2009; Mudge et al., 2011
A08	Dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide	M+H	$C_{17}H_{27}NO$	261.2093	836	262.2161	262.2171	3.7	Cech et al., 2006; Pellati et al., 2011; Mudge et al., 2011; Spelman et al., 2009
A09	Dodeca-2E,4E-dienoic acid 2-methylbutylamide	M+H	$C_{17}H_{31}NO$	265.2406	925	266.2480	266.2484	1.6	Bauer et al., 1989; Lopes-Lutz et al., 2010; Mudge et al., 2011
A10	Pentadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide	M+H	$C_{19}H_{27}NO$	285.2093	827	286.2164	286.2171	2.6	Binns et al., 2002c; Mudge et al., 2011
A11	Pentadeca-2E,9Z-diene-12,14-diynoic acid 2-methylbutylamide	M+H	$C_{20}H_{29}NO$	299.2249	660	300.2324	300.2327	1.0	Miller and Yu, 2004; Binns et al., 2002; Mudge et al., 2011

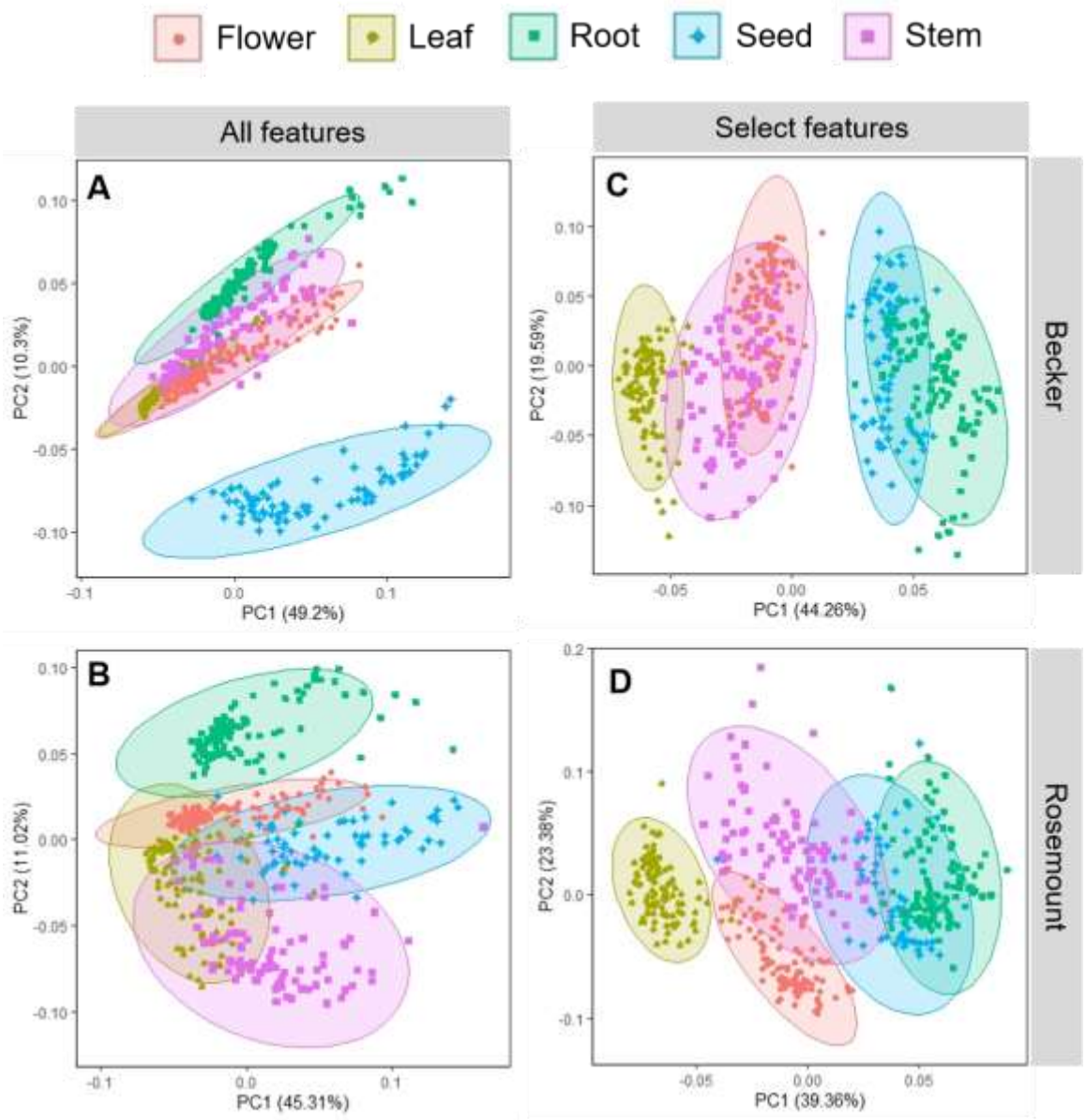

Figure 2.1. Principal components analysis of purple coneflower tissues from Becker and Rosemount for all features (A and B) and the 27 select features (C and D). The percent of variation explained by each principal component is shown along the axes.

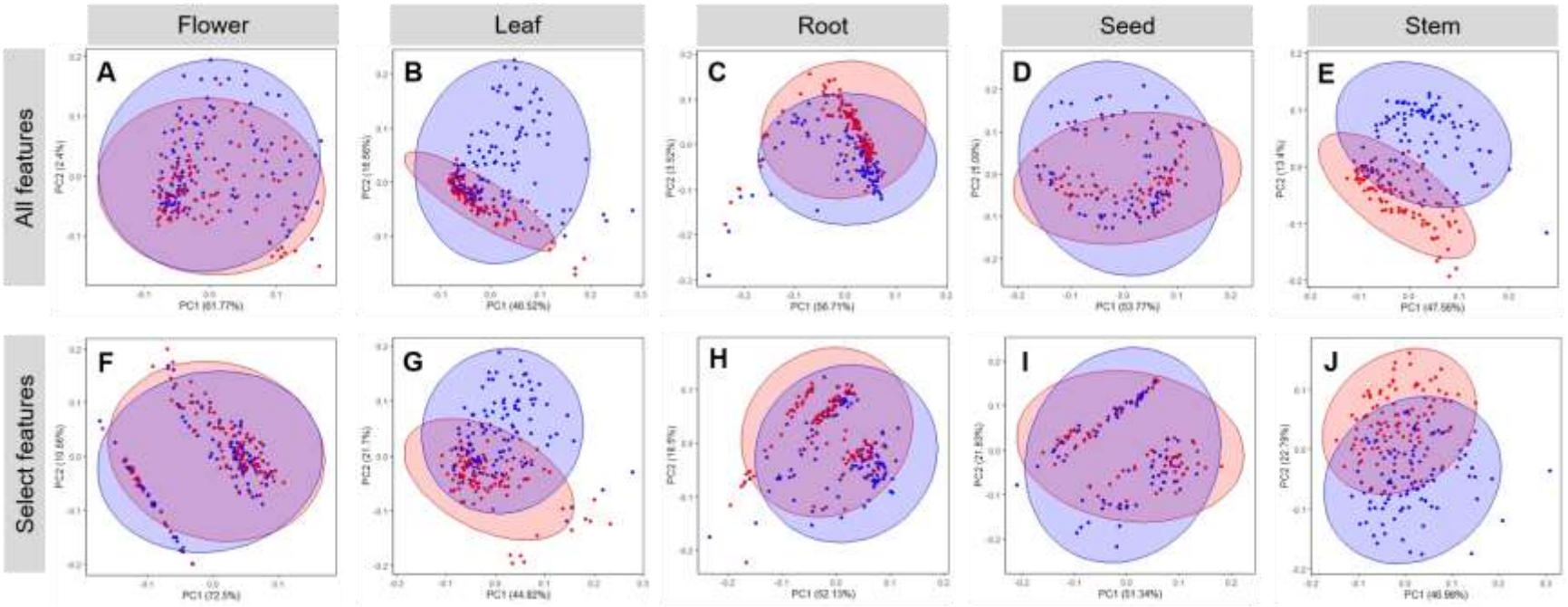

Figure 2.2. PCA scatter of all features (A-E) and select features (F-J) from five tissue types harvested at Becker (red) and Rosemount (blue) sites. The percent of variation explained by each principal component is shown along the axes.

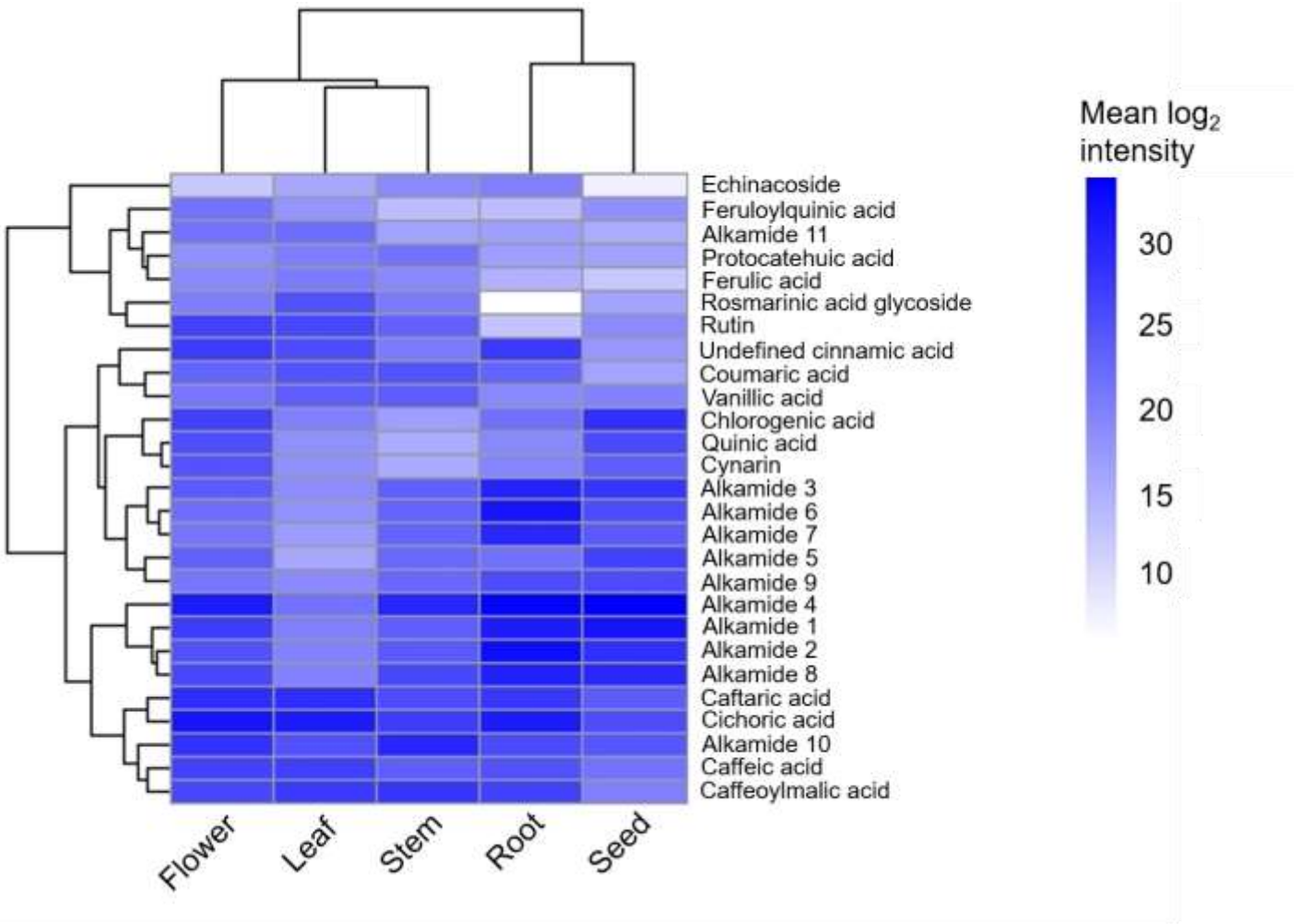

Figure 2.3. Heat map of the mean log_2-transformed intensity values for flower, leaf, root, seed and stem tissue. Hierarchial clustering of metabolomic profiles reveals similarities in profiles between tissues types and similarity in mean intensity between features.

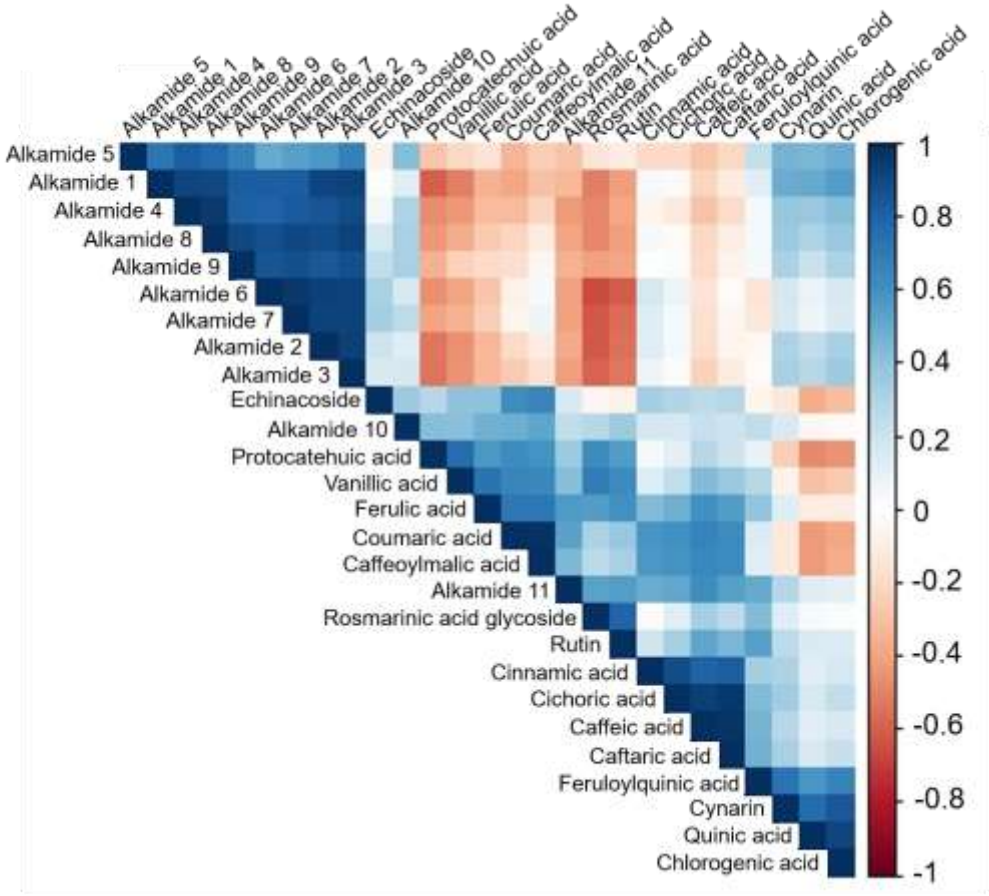

Figure 2.4. Correlation matrix of the 27 select features provisionally identified in purple coneflower tissue samples. Dark red indicates a strong negative correlation whereas dark blue indicates a strong positive correlation. Features were ordered using the "hclust" method which is the hierarchical clustering of correlation coefficients.

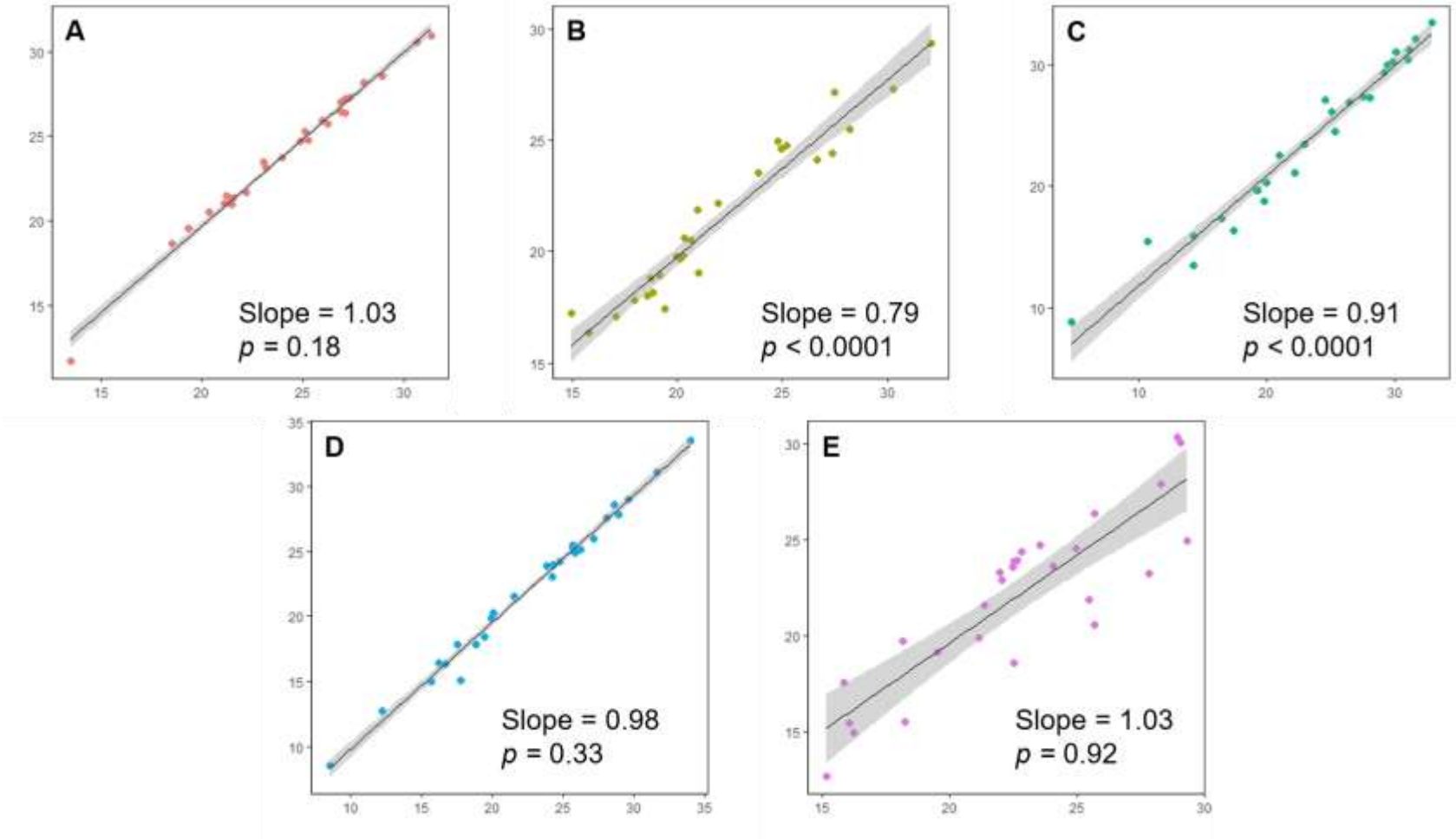

Figure 2.5. Comparison of mean intensity values for select features from Becker and Rosemount for flower (A), leaf (B), root (C), seed (D), and stem (E) tissue. Each point corresponds with a single feature, and Becker values are along the x-axis while Rosemount values are along the y-axis. Reported slope values are the slope of the line generated from the linear model. Reported p-values correspond to the test of the slope for significance against a slope of 1.

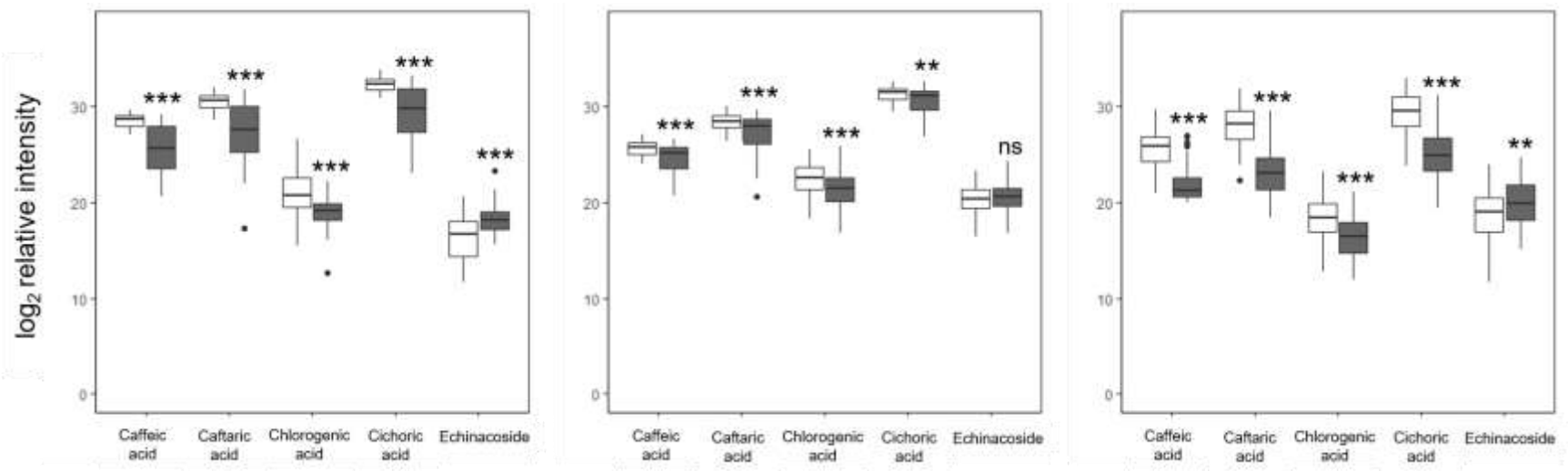

Figure 2.6. Comparison of the mean relative intensity for leaf (A), root (B), and stem (C) tissue between Becker (white) and Rosemount (gray). ns, *, **, and *** indicate not significant or significantly different according to Tukey's least significant difference at the $p<0.05$, $p<0.01$, and $p<0.001$ levels, respectively.

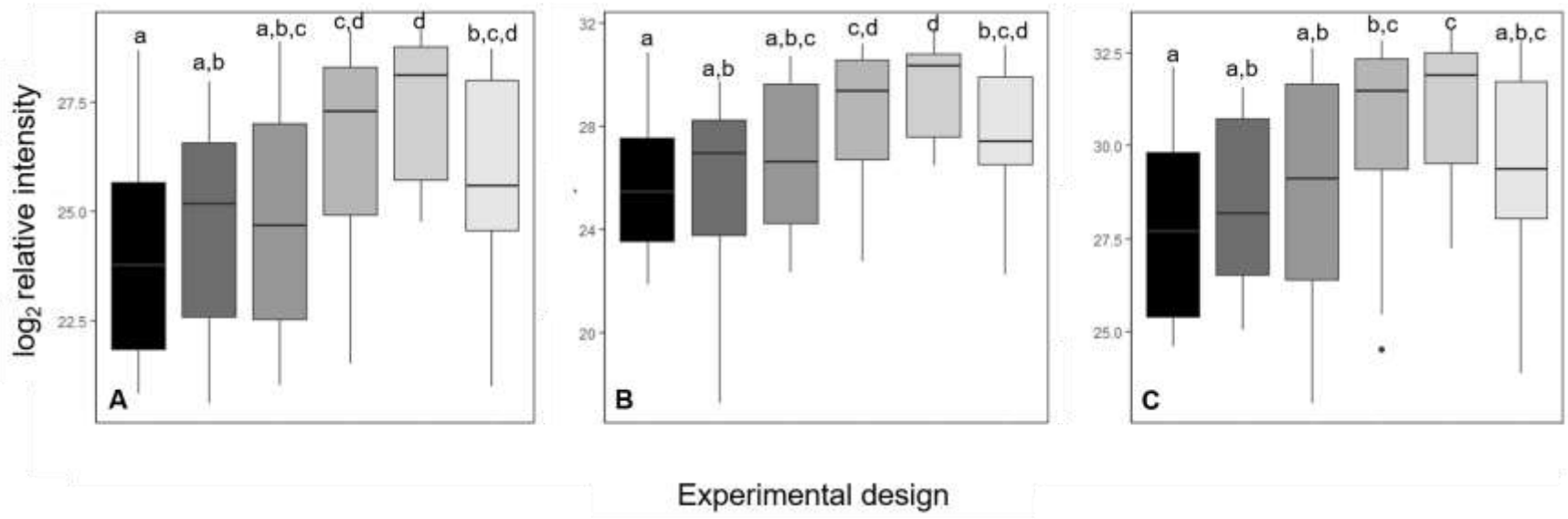

Figure 2.7. Effect of experimental design on the mean relative intensity of caffeic acid (A), caftaric acid (B), and cichoric acid (C) in leaf tissue. Experimental designs from left to right within panel are 1-row, 3-row, 6-row, 9-row, low-richness polyculture, and high-richness polyculture.

Chapter 3

Hydroponic production of fireweed (*Chamaenerion angustifolium* (L.) scop.) for biomass and phytochemical production

ABSTRACT

Fireweed (*Chamaenerion angustifolium* (L.) scop., syn. *Epilobium angustifolium* L.) is a rich source of phenolic compounds and has been utilized worldwide in traditional medicine. Many of these phenolic compounds possess bioactive properties with a wide range of therapeutic effects on human health, but there is little work on the direct production of fireweed for these beneficial phytochemicals. To examine the effects of mineral nutrient concentration on fireweed biomass production and metabolomic profile, fireweed individuals were grown in a greenhouse hydroponic system with varying concentrations of Hoagland's nutrient solution. Plants were harvested at maturity for biomass yield and metabolomic profiling of five tissues. As mineral concentration of the nutrient solution increased, shoot dry weight increased logistically and the root:shoot ratio decreased exponentially. Untargeted metabolomic analyses detected differences in response to nutrient level in leaf, stem and root tissue. Several features were provisionally identified in hydroponic fireweed, similar to those identified in field grown samples, most notably the therapeutic compounds oenothein B and miquelianin (quercetin-3-*O*-glucuronide). Relative abundances of oenothein B and miquelianin were not affected by mineral nutrient treatments, although several other features increased in abundance as mineral nutrient content decreased. Leaf tissue contained higher abundances of most provisionally identified features, while root tissue exhibited the lowest abundances. Hydroponic cultivation of fireweed has the potential to supply raw material for phytochemical demand, but the effects of mineral nutrient amendment need to be reconciled with biomass production.

INTRODUCTION

Plants are invaluable sources of bioactive compounds, many of which are greatly beneficial to human health worldwide. Up to 90% of people in developing countries rely on traditional, plant-based medicines, and over one quarter of prescription medications in developed countries are derived from plant sources (Barata et al., 2016; Hamilton, 2004). With increasing interest in naturally-derived remedies, there is a rapidly growing demand for plant-based medicines worldwide (Chen et al., 2016). Globally, over 60,000 plant species are used utilized for medicinal purposes, but harvest of these species poses a serious threat to conservation of native habitats and biological diversity (Barata et al., 2016; Hamilton 2004; Canter et al., 2005). Cultivation of medicinal plants alleviates ecological disturbances associated with wild collection by centralizing production, and can provide further benefits through the development of high-yielding cultivars and cultivation close to or in the area of demand. Although lower concentrations of desirable phytochemicals and levels of bioactivity have been reported in cultivated stocks (Binns et al., 2002; Conforti et al., 2006; Ryu et al., 2016), evaluation of environmental drivers of phytochemical production in wild plants have helped to determine specific production practices that enhance the content of beneficial phytochemicals (White et al., 2008; Similien et al., 2016). Although agronomic practices have been shown to increase the phytochemical content of some species over wild-collected plants, cultivation in the field can still lead to large variation due to environmental factors difficult to control such as temperature and complex ecological interactions (Macias et al., 2007).

Hydroponic cultivation, systems in which nutrients are delivered to the plant via liquid solutions in lieu of soil, of medicinal plants is an increasingly attractive solution to quantity and quality issues in the production of marketable phytochemicals. Many medicinal plants have been successfully grown in hydroponic culture for harvest of aboveground tissue (Kiferle et al., 2011; Prasad et al., 2012; Kaul et al., 2017; Surendran et al., 2017), root and rhizome tissue (Akiyama et al., 2017; Lu et al., 2018), and multiple tissues (Kim et al., 2010; Pedneault et al., 2014). Hydroponic cultivation allows for year-round production and may aid in habitat conservation, as plants are established in greenhouses and growth chambers rather than competing for valuable land (Maggini et al., 2011). Hydroponic systems also provide opportunities for increasing biomass production and improving the bioactivity and consistency of phytochemical end products on a large scale (Hayden, 2006), and several medicinal species reportedly produce higher levels of beneficial compounds under hydroponic conditions than when grown under field conditions (Pedneault et al., 2000). In situ production of phytochemicals is strongly influenced by temperature, light and nutrient availability, pH, and interaction with soil microorganisms and herbivores (Canter et al., 2005), and these factors can be difficult to manage in agricultural fields. Hydroponic systems allow for greater control of environmental conditions that drive phytochemical production and therefore can provide greater consistency in the end product.

Fireweed (*Chamaenerion angustifolium* (L.) Scop.) is a perennial herbaceous plant used in traditional medicine around the world. Native to the Northern Hemisphere, fireweed has historically been used in traditional North American, European, and Asian societies

as a medicine and food source (Rogers, 2014). Fireweed is a rich source of phytochemical beneficial for human health and ruminant health (Schepetkin et al., 2016; Baert et al., 2015). Extracts of fireweed possess a wide range of bioactivity including anti-inflammatory, antioxidant, antimicrobial, anti-proliferative, and immunomodulatory properties (Juan et al., 1988; Kiss et al., 2011; Onar et al., 2012; Borchardt et al., 2008; Kosalec et al., 2011; Vitalone et al., 2001; Schepetkin et al., 2009). In addition to human health benefits, phytochemical compounds present in fireweed may make the plant suitable as a ruminant supplement to improve nutrition by way of improving utilization of consumed protein, promoting animal growth, and enhancing resistance to intestinal parasites (Baert et al., 2017).

The bioactivity of fireweed extracts has been attributed to the presence and prevalence numerous polyphenolic compounds, most notably flavonoids, phenolic acids, and ellagitannins (Schepetkin et al., 2016; Agnieszka et al., 2018). Flavonoids present in fireweed include kaempferol, myricetin, quercetin, and their monoglycosidic derivatives (Schepetkin et al., 2016). Phenolic acids identified in fireweed extracts include caffeic acid, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, and p-coumaric acid (Granica et al., 2014). Unique to fireweed is the abundance of high molecular weight oligomeric ellagitannins, most notably oenothein B, on which extensive research has been conducted on its bioactivity (Granica et al., 2014; Ramstead et al., 2012; Schepetkin et al. 2009). Oenothein B and miquelianin (quercetin-3-*O*-glucuronide) have been suggested as a basis for commercialization of fireweed extracts due to their abundance in fireweed tissue and evidence of their bioactive properties (Schepetkin et al., 2016; Baert al., 2017).

Abundance of polyphenolic compounds within fireweed and tissue-specificity of compounds has been described (Shikov et al., 2010; Granica et al., 2012; Baert et al., 2015; Baert et al., 2017). Ellagitannins account for up to 15% of fireweed dry mass and 90% of the total polyphenols in flowers, leaves and stems (Baert et al., 2015; Baert et al., 2017). Oenothein B is the most abundant compound overall, and is found in higher concentrations in flowers than in leaves (Shikov et al., 2010; Granica et al., 2012; Baert et al., 2017). Kaempferol-3-*O*-rhamnoside, myricetin-3-*O*-rhamnoside, and quercetin-3-*O*-rhamnoside are the main flavonoids present in flower tissue and are absent in other tissues (Baert et al., 2017). No compounds have been reported in tissues other than flower, leaf, and stem.

There are few reports of biological and environmental factors influencing phytochemical content of fireweed other than the effect of elevation on flavonoids. Greater altitude leads to increased concentrations of flavonoids in aerial tissues, although specific factors responsible for this increase, such as temperature or soil moisture, have not been directly examined (Monschein et al., 2015). There are no reports of specific cultivation techniques or growth conditions, such as nutrient availability, that may influence phytochemical content of fireweed. Nutrient amendment may be one of the most convenient ways to alter the phytochemical profile, especially in a hydroponic system, and potentially enhance beneficial polyphenol content of fireweed. Additionally, higher nutrient availability, especially N and P, increases biomass production of fireweed which would be beneficial in a production system (Bennett et al., 2004; Pinno et al., 2013; Bales and Hirsch-Green, 2019). Effects of added nutrients, especially N, on polyphenol content

have not been examined in fireweed, but they have been addressed in other medicinal plant species. Higher levels of rosmarinic and caffeic acid were found in basil at low N treatments (Nguyen and Niemeyer, 2008). Flavonoid yield of *Scutellaria lateriflora* increased in response to P (Shiwakoti et al., 2016), although total flavonoids decrease with increasing N fertilization in the medicinal plants *Chrysanthemum morifolium* Ramat. and *Labisia pumila* Benth (Liu et al., 2010; Ibrahim et al., 2011). There are no reports of the effects of nutrient addition on ellagitannin content of medicinal plant species, but N fertilization does not affect levels of hydrolysable tannins in woody plant species (Haukioja *et al.* 1998).

The existing literature on fireweed provides strong support of phytochemicals of interest present in fireweed, but little on production practices that may enhance the yield of important bioactive compounds in fireweed. To pave the way for future field experiments and targeted analyses of phytochemical compounds, fireweed was established in a greenhouse hydroponic system to examine the effects of varying mineral nutrient solution concentration on fireweed biomass and phytochemical production.

MATERIALS AND METHODS

Preparation of hydroponic solutions and experimental design

Treatments consisted of six strengths of Hoagland's nutrient solutions (Hoagland and Arnon, 1950): full-strength, half-strength, quarter-strength, eighth-strength, sixteenth-strength, and full-strength with no N. Stock solutions were first prepared (Table 3.1) and

then combined for the final nutrient solutions (Table 3.2). Each experimental unit consisted of a 1-liter amber Nalgene HDPE Plastic Wide Mouth Leakproof Bottle (Berlin Packaging) filled with nutrient solution. Hydroponic bottles were organized in a randomized complete block design across the greenhouse bench and replicated eight times. Each bottle contained a single plant.

Preparation of plant materials and greenhouse growth conditions

Fireweed seeds collected from northwestern Wisconsin were purchased from Prairie Moon Nursery in Winona, MN. Seeds were germinated and grown for three weeks on the moistened surface of general potting soil. Seedlings were then transferred to 1.5" x 1.5" x 2.25" plug trays and grown for an additional six weeks. Seedlings with a two-centimeter rosette diameter and maximum root length of five centimeters were carefully extracted from the soil using forceps and soil was rinsed away from the roots using distilled water. Seedlings were transplanted into hydroponic net pots (Growneer) filled with sterilized perlite and placed in nutrient solutions. Nutrient solutions were maintained at constant levels below the net pot and completely renewed weekly.

Fireweed plants were grown April to August 2017 in an east-west oriented greenhouse at the University of Minnesota Plant Growth Facilities in St. Paul, MN. Minimal temperature set points controlling air heating were 15/18°C night/day, and maximal temperature set points were 18/20°C night/day. Vent opening temperatures were 20/25°C night/day. Plants were grown in hydroponic conditions in the greenhouse until flowering

or the termination of the experiment. The experimental period was June to August 2017, where natural sunlight was supplemented by artificial lighting for a 16-h photoperiod.

Biomass and metabolomics harvest

Fireweed plants were mainly harvested at flowering throughout the experiment, as the flowering period is reportedly the best source of bioactive compounds (Agnieszka et al., 2018). The experiment was terminated at 18 weeks and all remaining plants were harvested when no further plants showed signs of flowering (e.g. development of buds). For harvest, individual plants in net pots were removed from their hydroponic bottles, perlite was rinsed away, and roots were teased out of the net pot. Five tissue types were harvested separately: anther, flower, leaf, stem, and root. Tissue-specific harvest details are described in Table 3.3. Approximately 200 milligrams of fresh biomass of each tissue type was harvested, placed in a 1.5-milliliter microcentrifuge tube, and immediately placed on dry ice. All samples were stored at -80°C in the dark pending extractions. Following the metabolomic tissue harvest, the remaining aerial and root tissues were separated and placed in paper envelopes, dried in forced-air ovens for 72 hours at 60°C, and weighed.

Phytochemical extraction and analyses

Extraction of plant samples were completed at the Plant Metabolomics laboratory, University of Minnesota using standard extraction procedures (Martin et al., 2014). Harvested samples in microcentrifuge tubes were removed from the freezer, weighed,

and a 2.5-milliliter tungsten bead was added to each sample along with 70% isopropanol at a rate of one milliliter per 200 milligrams of frozen sample. HPLC-grade isopropanol and HPLC-grade water were purchased from Sigma Aldrich (St. Louis, MO, USA). Samples were ground and homogenized using a SPEX SamplePrep model 2010 Geno/Grinder® for five minutes at 1500 rpm. Microcentrifuge tubes containing homogenized sample tissues were centrifuged for four minutes at 14000 rpm. Following centrifugation, the supernatant (extract) was removed from the original tube and transferred to a sterile tube. Extractions were carried out at room temperature (approximately 25°C). Extracts were stored at -80°C in the dark prior to LC/MS analysis.

Metabolomic profiles were obtained using C_{18}-reversed-phase ultra-performance liquid chromatography-electrospray ionization-hybrid quadrupole-orbitrap mass spectrometer (Ultimate® 3000 HPLC, Q Exactive™, Thermo Scientific) with an autosampler and with a sample vial block maintained at 4°C. Chromatographic separations were carried out on an Acquity reversed-phase C_{18} HSS T_3 1.8 μm particle size, 2.1 × 100 mm column (Waters) with column temperature 40°C, flow rate 0.40 ml/min, and 0.5 μL injected. A 19.5 minute gradient using mobile phases A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile was run according to the following gradient elution profile: initial 2% B, 2 min 2% B, 0.5 min 4% B, 1 min 20% B, 4 min 29% B, 1 min 55% B, 9 min 98% B, 1 min 2% B, 1 min 2% B. The MS conditions used were full scan mass range 115-1000 *m*/*z*, resolution 75,000, desolvation temperature 350°C, spray voltage 3800 V, auxiliary gas flow rate 20, sheath gas flow rate 50, sweep gas flow rate 1, S-Lens RF level 50, and auxiliary gas heater temperature 300°C. Xcalibur™ software version 2.1

(Thermo Scientific) was used for data collection and chromatogram visualization. Sample analysis order was randomized across the entire sample set.

Biomass yield statistical analyses

Biomass data were analyzed using the R programming language (R Core Team, 2014). Non-linear regression was used to determine the relationship between shoot biomass and nutrient solution strength, and root:shoot ratio and nutrient solution strength. Growth curve parameters for shoot biomass production in relation to nutrient solution strength were estimated with a logistic growth function (SSlogis). The relationship of the root:shoot ratio in relation to nutrient solution strength was estimated using exponential decay and the SSasymp function. All fireweed individuals in the no N treatment withered and developed little beyond the transplanted seedling stage, therefore this treatment was omitted from biomass and metabolomics analyses.

Metabolomics data processing and statistical analyses

The ProteoWizard tool msconvert (Chambers et al., 2012) was used to convert raw format files to the open mzML format. Due to excessive noise overwhelming the signal, data from the first four minutes and the last nine minutes was eliminated. Thermo Scientific Xcalibur software was used for centroid fitting. All mzML files were compressed into ZIP format using the 7-Zip software prior to uploading to Galaxy.

The Galaxy platform Workflow4metabolomics (W4m) was used to preprocess, annotate, and perform statistical analyses of the metabolomic data collected from the Q Exactive™. All of the following processes were performed for each ionization mode up until Join +/- Ions tool (Eschenlauer et al., 2018) was used to combine features from the positive and negative ionization modes. Features were picked from the centroided data using the W4m XCMS Set tool (Guitton et al., 2017) with the following parameters: "centWave" method, maximum ppm *m/z* deviation 3 ppm, minimum peak width 3 seconds, maximum peak width 5 seconds, signal/noise threshold 3, minimum difference in *m/z* for peaks with overlapping retention times -0.001, peak limits based on second derivative, prefilter "3,1e5", and noise filter 0. Following feature picking, the W4m XCMS Set Merger tool (Guitton et al., 2017) was used to merge all resultant files from the W4m XCMS Set.

To correct for differences in chromatographic retention time across samples, the W4m XCMS Group tool (Guitton et al., 2017) was run before and after the retention correction with the following parameters: "density" grouping method, minimum fraction of samples 0 and width of overlapping *m/z* slices 0.006, with a maximum of 50 groups to identify in a single *m/z* slice. Prior to retention correction the bandwidth was six seconds, and following retention correction the bandwidth was one second. The W4m XCMS Retcor tool (Guitton et al., 2017) was used for retention correction with the following parameters: the "peakgroups" method, 50 missing samples allowed in retention-time correction groups, 1 extra peak allowed in retention time correction groups, the LOESS smoothing method with a degree of smoothing for local polynomial regression fitting of 2 and gaussian fitting.

Low-intensity features in some samples were reconciled using the “chrom” method in the W4m XCMS FillPeaks tool (Guitton et al., 2017). Feature lists were annotated using the W4m CAMERA tool (Guitton et al., 2017) using default parameters with the exception of matching the ionization mode parameter to that of the samples. Features were eliminated from further analyses using the W4m Quality Metrics tool (Guitton et al., 2017). Eliminated features were of the following classes: features with intensities threefold greater in blanks than in samples, features with no variance across samples, samples with no variance across features, data for blanks, and data for pools.

Features not annotated by CAMERA as the most abundant mass in a given isotopic envelope were eliminated from future analyses (Eschenlauer et al., 2019). This was accomplished using the “silent” operation mode with the following SED program: 1 {p; d}; /[[]M[]]/ {p; d} Resultant peak lists for data collected in the positive and negative ionization modes were combined using the Join +/- ions tool (Eschenlauer et al., 2018). Data values in the dataMatrix were log base 2 transformed so that a fold change of two corresponded to a difference of one (Kohl et al., 2012).

Principal components analyses (PCA) using the W4m Multivariate tool (Guitton et al., 2017) were conducted to determine differences in metabolomic fingerprints of the different tissues. To identify features that changed over the nutrient treatments, the W4m Univariate tool (Guitton et al., 2017) with Benjamini-Yekutieli-corrected significance *p*-

value less than 0.01 was used. Features were provisionally identified by comparing UV absorption maxima and *m/z* values of ions and fragments with previously published datasets.

RESULTS

Biomass production

The strength of the Hoaglund nutrient solution influenced fireweed shoot biomass yield and root:shoot ratio. Mean shoot biomass production increased as the nutrient solution strength increased, and there was greater variation in the data at higher nutrient levels (Figure 3.1). Due to the large variation in the data at higher nutrient treatments, a second model was developed to examine the maximum biomass growth, with data points based on the largest measurements from each nutrient treatment. A non-linear logistic growth model (Equation C.1) was used to estimate the relationship between nutrient solution strength and shoot biomass production for the mean and maximum values (Figure 3.1). Growth curve parameters were estimated for both models using the SSlogis function, where *asym* is the asymptote, *xmid* is the x-value at the inflection point, and *scal* is the angular coefficient of the tangent line at the point of inflection. All parameters were significant at the 0.05 level. For the mean shoot biomass values, *xmid* was estimated to be 0.4, *scal* was 0.1, and *asym* was 1.1. For the maximum shoot biomass values, *xmid* was estimated to be 0.3, *scal* was 0.1, and *asym* was 2.4.

Root biomass increased from eighth-strength to full strength, although it initially decreased from sixteenth-strength (Supplementary Figure C.1). There was little difference between the half- and full-strength treatments. As nutrient solution strength increased, the root:shoot ratio and variation around the mean decreased (Figure 3.2). For the root:shoot ratio data, a non-linear exponential decay model (Equation C.2) was used to fit an exponential decay curve to the data. Model parameters were estimated using the SSasymp function, in which the measured value y value starts at y_0 and decays towards y_f at a rate of α. Residuals were evenly distributed and all parameters were significant at the 0.001 level. y_0 was estimated to be 2.2, y_f was 0.2, and α was 1.9. The root:shoot ratio of fireweed in a basic hydroponic system standardizes near 0.2.

Metabolomic profiles of different tissues

To investigate metabolite differences among tissues, samples of five tissue types were harvested for LC-MS analyses. Subsequent data processing with Galaxy-M detected 1129 features from positive- and negative ionization mode together. Unsupervised multivariate statistical analyses by PCA was performed to visualize the changes in features among tissues (Figure 3.3). The first principal components (PC1) explained 61% of the total variability, whereas the second principal components (PC2) accounted for only 8%. PCA plots showed a separation of root tissue from the other tissues mainly along PC1. The metabolomic profiles of anther and flower tissues appear similar and are separated from leaf and stem tissue along PC2.

Presence of bioactive natural products

Seventeen compounds with of commercial interest due to medicinal and therapeutic effects were provisionally identified from hydroponic fireweed samples through literature and MS/MS library searches, as well as UV absorbance data (Table 3.4). Oenothein B was detected in all tissues. Leaf and stem tissue had, on average, the greatest relative intensities of oenothein B (Figure 3.4A). Quercetin-3-*O*-glucuronide (miquelianin) was also detected in all tissues analyzed and was a major component of flower, leaf and stem tissue (Figure 3.4B).

Nutrient solution strength treatment effects

Mineral nutrient solution concentration had no effect on the provisionally identified compounds in flower or anther tissue. Oenothein B and quercetin-3-*O*-glucuronide were not found to vary in response to nutrient solution strength in any tissue. Additional unidentified features were found to vary significantly over the nutrient treatments in three tissue types: 136 in leaf tissue, 11 in root tissue, and 192 in stem tissue. Of these features, 103 were shared in common between stem and leaf, 1 between root and stem, and 1 shared among all three tissues (Supplemental Table C.1). Overall, only 8% of the features determined to vary significantly in response to increasing nutrient solution concentration increased, and the majority of these features were in the root tissue. When responses to nutrient solution concentration were significant for a specific feature in more than one tissue, it showed the same trend in those tissues. For example, compounds 12 and 16

(provisionally identified as caftaric acid and tellimagrandin II) decreased in intensity in leaf and stem tissue as the strength of the nutrient solution increased (Figure 3.5).

Comparison of tissue differences within a nutrient strength treatment

A heat map was used to visualize the extent of metabolite differences across the five tissue types across five of the nutrient strength treatments (Figure 3.6). The relative abundances of ions detected in each sample (log_2 of the intensity) were averaged across replicates for each tissue and nutrient treatment. The dark blue color of the tile indicates a high abundance of the metabolite and white indicates low abundance. Oenothein B was one of the most abundant features among the tissues. Kaempferol and kaempferol 3-rhamnoside were abundant in anther and flower tissues, while phenolic acids were more abundant in leaf and stem tissues. Root tissue had low abundances of most of the identified features.

DISCUSSION

Fireweed is a widely known medicinal plant with an extensive history of use in traditional medicine (Schepetkin et al., 2016). Recent work on fireweed has focused on mainly on phytochemical content (Baert et al., 2017; Agnieszka et al., 2018) and fitness, growth, and resource allocation strategies in relation to ploidy level (Bales et al., 2019; Walczyk et al., 2019). In this research, fireweed biomass yield and metabolomic response to nutrient availability in a hydroponic system were examined. Examination of the effects

of nutrients on biomass and phytochemical production will aid future work on the production of fireweed for beneficial phytochemicals.

Effect of nutrient solution strength on biomass production and implications for phytochemical production

The strength of Hoagland's nutrient solution, and consequently nutrient availability, significantly affected the shoot biomass yield of fireweed. A logistic growth model was established for mean fireweed shoot growth in hydroponic culture, and fireweed biomass production would be maximized at a Hoagland's nutrient solution strength near the full-strength utilized in this research (approximately 0.9-strength). There is potential of fireweed to produce more than twice as much shoot biomass than the value reported for the mean, as the *asym* value was 2.4. The mean and maximum models determined similar values for both the *xmid* and *scal* parameters, indicating that the optimal Hoagland solution strength for biomass production is similar to the full-strength solution. Previous work on the response of fireweed to nutrient amendment has shown that fireweed has a strong affinity for taking up N fertilizer in soil and hydroponic systems (Hangs et al., 2002; Hangs et al., 2003), although N addition alone does not affect biomass production (Bennett et al., 2004). Maximum biomass accumulation in fireweed has resulted from fertilizer with added N and P, or N, P and K, and is has been suggested that fertilization with P and K help to stimulate the uptake of N in fireweed, therefore decreasing the need for excess N fertilization (Pinno et al., 2013). Although nutrient addition can benefit biomass production in fireweed, previous research has shown that N fertilization may limit the production of certain phenolic compounds of interest in other medicinal plants

(Nguyen and Niemeyer, 2008; Kováčik and Klejdus, 2014; Guillén-Román et al., 2018), so further work is needed on determining appropriate levels of certain nutrients in the hydroponic solution to optimize biomass as well as phytochemical production.

Although variation in shoot biomass accumulation among individual plants increased as nutrient solution strength increased, the variation in root:shoot ratio decreased, indicating a more consistent response to nutrient allocation to root and shoot tissue under higher nutrient treatments. Many biotic and abiotic factors can affect the root:shoot ratio, although the most widely addressed factor is nutrient availability (Mokany et al., 2006). It is generally assumed that, under conditions of high nutrient availability, plants will allocate more to shoot growth rather than root growth (Agrin and Franklin, 2003). The findings of this research are consistent with this theory: as the strength of the solution, and thus the availability of nutrients, increased, the root:shoot ratio decreased. It is also important to note that reduced root growth is common in hydroponic systems, potentially due to inadequate aeration (Trolldenier and Hecht-Buchholz, 1984). Fireweed leaf and flower tissue are typically targeted for medicinal purposes (Kosalec et al., 2013), so a lower root:shoot ratio would be desirable for maximum production. However, in hydroponic systems, the harvest of root tissue is much less labor intensive than plants grown in soil and root tissue may be a novel source of desirable compounds, although no previous reports of phytochemical production in fireweed roots could be found.

Most tissues possess distinct metabolomic fingerprints and contain commercially relevant compounds

Previous work on phytochemical production of fireweed has identified numerous compounds with medicinal value (Schepetkin et al., 2016). Several of these compounds were provisionally identified in hydroponically-cultivated fireweed (Table 3.3) and tissue comparisons of phytochemical content were visualized through a PCA plot (Figure 3.3). PCA analysis separated the tissues into distinguishable clusters, although some tissues overlapped. Not surprisingly, flower and anther tissue, both reproductive tissues, were found to have similar metabolomic fingerprints. Leaf and stem tissue also shared similar metabolomic fingerprints, likely due to the sharing of similar key functions such as photosynthesis leading to the presence of similar metabolites.

A comparison of intensity across tissue types and nutrient treatments were visualized in heat map (Figure 3.6). Previous work on tissue specificity of phytochemical compounds in fireweed is limited to an analysis in which polyphenolic compounds from leaf, flower, and stem parts were identified and quantified (Baert et al., 2017). Ellagitannins constituted the majority of the dry weight of leaves and flowers. In hydroponically-grown fireweed, the relative abundance of oenothein B (feature 16) was the highest in leaf and stem tissue, and it was the third most abundant feature in flower and anther tissue. Three flavonoids (kaempferol-3-*O*-rhamnoside, myricetin-3-*O*-rhamnoside, and quercetin-3-*O*-rhamnoside) were previously found to be unique to flower tissue (Baert et al., 2017). Quercetin-3-*O*-rhamnoside (feature 4) and kaempferol 3-rhamnoside (feature 3) were provisionally identified in hydroponically-cultivated fireweed samples in tissues

other than the flower, although levels were over three-fold greater in flower tissue than in leaf, root and stem tissue.

Belowground tissues of many medicinal plants are typically rich sources of bioactive phytochemicals (Briskin et al., 2000). In this experiment, root tissue was distinctly different from all other tissues and possessed the lowest relative abundances of most provisionally identified features. The reduced content of bioactive compounds in root tissue may have several explanations. First, root growth was limited in this hydroponic system (under 0.3 g root mass per plant) and the architecture of developing roots suggested a strong role in nutrient acquisition rather than storage (Chen et al., 2011). Second, hydroponic systems lack soil, and thus soil microbes which are believed to play a strong role in the production of bioactive compounds in medicinal plants (Solaiman and Anawar, 2014; Wu et al., 2009). Finally, there is a bias in fireweed research, as attention has historically been given to leaf and flower tissue and there is a massive gap in knowledge on bioactive compounds that may reside in the root tissue. Future analyses and comparisons of commercially-relevant phenolic compounds from hydroponically-produced fireweed should include confirmation of feature identification with standards and quantification of these compounds to determine yield per biomass yield.

Nutrient availability affects abundance of features in leaf, root, and stem tissue

Nutrient solution strength affected various metabolites in leaf, stem, and root tissue. The mineral nutrient solution concentration did not affect the metabolites in flower or anther

tissue, potentially due to small sample sizes and lack of representation across the nutrient treatments as not all individual plants flowered in this experiment. Environmental conditions, especially nutrient availability or stress, influence the plant metabolome, as there is a strong relationship between elemental stoichiometry and the plant metabolome (Rivas-Ubach et al., 2012). Nutrient availability and subsequent allocation within the plant have been shown to affect the production of storage (Martin et al., 2002), defensive (Coviella et al., 2002; de Lange et al., 2019), and stress avoidance compounds (Hale et al., 2005; Galieni et al., 2015) in other plant species. Available published research on environmental drivers of phytochemical production in fireweed is limited to the effects of elevation on phenolic compound content (Monschein et al., 2015). Increasing elevation was correlated with increased concentrations of flavonols, potentially due to increased UV-B radiation or resource limitation. This research serves as a first step in examining nutrient-related changes to the metabolome of fireweed.

Phenolic compounds are the main components of fireweed extracts and include phenolic acids, flavonoids, and ellagitannins. Similar responses to nutrient addition have been reported across these chemical classes. Organic and mineral fertilizers tend to decrease the content of phenolic acids (Nørbæk et al., 2003; Nguyen and Niemeyer, 2008; Sinkovič et al., 2015). In general, nutrient fertilization negatively affects the concentration of flavonoids in plants (Ibrahim et al., 2011; Deng et al., 2012; Nybakken et al., 2018), although increases in flavonoid content have been reported as well (Naguib et al., 2012). Much of the work on ellagitannin content in response to nutrient availability has been conducted on long-lived perennial woody species, not on herbaceous species

like fireweed, and has found no evidence for fertilization influencing levels of hydrolysable tannins (Haukioja et al., 1998). Most research on the production of phenolic compounds in response to nutrient availability have focused on fertilization with a single nutrient (most commonly N), or include additional alterations to environmental conditions that may confound interpretations of results. In this research, the majority of features determined to change significantly across nutrient treatments were found to decrease in intensity/abundance as nutrient solution strength increased. MS/MS library searches of varying features other than the provisionally identified features (in Supplementary Table C.1) indicate that many are potentially phenolic compounds. The decrease in abundance suggests that increasing nutrient solution strength negatively affects phenolic compound content of fireweed.

Eight of the provisionally identified compounds (2, 3, 8, 9, 12, 13, 15 and 16) were found to vary in intensity across nutrient treatments in leaf, stem and root tissue. Those flavonoids determined to vary in root tissue (kaempferol-3-rhamnoside (3), naringenin (8), and kaempferol 3-O-arabinoside (2)) increased in response to increasing nutrient availability, as did the majority of features detected in root tissue (8 out of 11). The majority of the remaining identified and unidentified features in leaf and stem tissue decreased in response to nutrient availability. Nutrient stress has previously been shown to increase production of phenolic compounds in aboveground tissues of other plant species (Muzika, 1993; Caretto et al., 2015; Galieni et al., 2015). This “opposite metabolic response” in root and shoot tissue has been reported from studies on drought and temperature stress (Gargallo-Garriga et al., 2014; Gargallo-Garriga et al., 2015).

When under environmental stress, there is an up-regulation of not only root growth (Berendse and Moller, 2008), but also metabolism in the root system (Gargallo-Garriga et al., 2014), which may account for the increases in root tissue shown here. Many popular botanical-based medicines are derived primarily from the root tissue (e.g. *Echinacea*, licorice, and turmeric). Fireweed root tissue is easily harvestable in a hydroponic system, but further work on the presence of phytochemicals of potential commercial value must be conducted to determine the usefulness of the root tissue.

Importantly, nutrient solution strength did not appear to affect levels of the two main phytochemicals of commercial interest in fireweed. Surprisingly, quercetin-3-*O*-glucuronide, a flavonoid, was not influenced by nutrient availability, whereas other flavonoids were affected. Oenothein B, an ellagitannin, also was not affected by the strength of the nutrient solution. Unlike the synthesis of phenylpropanoids, such as phenolic acids, which competes with the synthesis of proteins due to the common precursor phenylalanine, the biosynthesis of hydrolysable tannins such as oenothein B proceeds without direct competition (Haukioja et al., 1998; Jones and Hartley, 1999). Therefore, at least in the case of N availability, oenothein B production should not be influenced by nutrient status.

A need to reconcile phytochemical production with biomass production

Whether in the field or in hydroponic culture, there is a need to optimize phytochemical production in medicinal plants such as fireweed if the beneficial compounds are to be

commercialized. In the case of phenolic compounds, such as those prevalent in fireweed, a trade-off between investment in plant defense versus growth has been proposed and studied through the lenses of the carbon-nutrient balance and growth-differentiation balance hypotheses (Massad et al., 2012). These hypotheses state that plant growth and phenolic compound production are competing processes. Although not directly addressed in this research, there is a hint of this phenomenon in the production of fireweed, as biomass production was positively correlated with increasing nutrient availability whereas many phytochemical features decreased in intensity.

Application of environmental stress has been utilized to influence phenolic compound production in medicinal plants (Fonseca et al., 2006; Reilly et al., 2008; Chrysargyris et al., 2016). However, nutrient deficiencies often negatively affect yield, and there must be an awareness of the effect of an applied environmental stress on biomass production. Nutrient starvation, specifically N, has been recommended for the production of phenolic compounds in medicinal plants (Giorgi et al., 2009). Instead of strictly reducing nutrient availability and risking reductions in yield, recently there has been interest in short-term limitation of nutrients (Zhou et al., 2018) and balancing nutrient supply with yield (Radusiene et al., 2019) in plant production to optimize both biomass and phytochemical yields. This could be easily accomplished in a hydroponic system by simply switching nutrient solutions at a critical time, but would be difficult in a field setting. The trade-off between the production of biomass and phytochemical compounds with commercial value must be acknowledged and explored in medicinal plant species prior to large-scale production operations.

CONCLUSION

Fireweed was successfully established in hydroponic culture under a range of nutrient treatments. Fireweed grown with full strength Hoagland's nutrient solution produced the most harvestable biomass, and logistic modeling suggests that biomass production is maximized near the full-strength solution. Fireweed grown under hydroponic conditions produced several therapeutic compounds, and an untargeted metabolomics approach allowed for detection of the effects of nutrient solution strength on metabolite abundance. Oenothein B and quercetin-3-*O*-glucuronide, two phytochemicals of potential commercial interest, were detected in fireweed under hydroponic conditions, and their abundances were not affected by the level of minerals in the nutrient solution. Tissues varied in abundances of provisionally identified features, with higher abundances of features found in leaf tissue for most compounds, followed by flower and stem tissue. Abundance of several features decreased when nutrient strength increased, suggesting that nutrient stress may elicit the production of these phytochemicals. Based on these results, future research should address the trade-off between nutrient availability and biomass production to optimize growing conditions for fireweed as a medicinal crop. This work paves the way for further metabolomic studies to improve fireweed yield and address phytochemical production. In future studies, a combination of targeted and untargeted metabolomics approaches should be utilized to examine known compounds of importance, but also to address the possibility of synergistic or additive actions of co-occurring compounds. Compounds of interest, such as oenothein B and several flavonoids, should be quantified from samples and linked to biomass production in order

to provide recommendations for fireweed production. Studies are currently underway to examine the production of beneficial compounds in field cultivated fireweed under N fertilization treatments.

ACKNOWLEDGEMENTS

Thank you to Dr. Dana Freund, Lindsay Wilson, Abigail Diering, Andrea Loudenback, and Titania Markland for assistance in the greenhouse and laboratory. Thank you to Dr. Arthur Eschenlauer and Mark Esler for technical support with Galaxy M. Funding for this research was provided by the AGRI Crop Research Grant Program from the Minnesota Department of Agriculture, the Hueg-Harrison Fellowship, and private funding from Leanna Forcier through the Minnesota Institute for Sustainable Agriculture.

Table 3.1. Stock solution requirements for Hoagland nutrient solutions.

Stock solution	Chemical constituents	Chemical Formula	Amount per L of solution
A) Nitrogen	Potassium nitrate	KNO_3	82.15 g
	Calcium nitrate tetrahydrate	$Ca(NO_3)_2 \cdot 4H_2O$	118.08 g
B) Phosphate and sulfate	Ammonium phosphate monobasic	$NH_4H_2PO_4$	1.44 g
	Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	61.62 g
C) Micronutrient	Boric acid	H_3BO_3	2.86 g
	Manganese chloride tetrahydrate	$MnCl_2 \cdot 4H_2O$	1.81 g
	Zinc sulfate heptahydrate	$ZnSO_4 \cdot 7H_2O$	0.22 g
	Molybdic acid monohydrate	$H_2MoO_4 \cdot H_2O$	0.02 g
	Copper (II) sulfate pentahydrate	$CuSO_4 \cdot 5H_2O$	0.08 g
D) Iron chelate	Sodium Hydroxide	NaOH	5 g
	Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	32.2 g
	Ferric Chloride (40%) w/v	$FeCl_3$	24 mL
E) Magnesium (no N)	Magnesium Sulfate	$MgSO_4 \cdot 7H_2O$	246 g
F) Potassium (no N)	Potassium sulfate	K_2SO_4	87 g
G) Calcium sulfate (no N)	Calcium sulfate dihydrate	$CaSO_4 \cdot 2H_2O$	1.72 g
H) Calcium phosphate (no N)	Calcium phosphate monobasic monohydrate	$Ca(H_2PO_4)_2 \cdot H_2O$	12.6 g

Table 3.2. Recipes for the six different Hoagland nutrient solutions.

Stock solution	mL of stock solution needed per L of distilled water					
	Full	**Half**	**Quarter**	**Eighth**	**Sixteenth**	**No N**
A) Nitrogen	8	4	2	1	0.5	-
B) Phosphate and sulfate	8	4	2	1	0.5	-
C) Micronutrient	1	0.5	0.25	0.125	0.0625	1
D) Iron chelate	1	0.5	0.25	0.125	0.0625	1
E) Magnesium (no N)	-	-	-	-	-	2
F) Potassium (no N)	-	-	-	-	-	5
G) Calcium sulfate (no N)	-	-	-	-	-	200
H) Calcium phosphate (no N)	-	-	-	-	-	10

Table 3.3. Tissue-specific metabolomics harvest details for hydroponic fireweed.

Tissue type	**Harvest procedure**
Anther	Anthers producing pollen were cut away from their filaments from two flowers (for a total of 16 anthers) using sterile scissors.
Flower	Two flowers, not yet producing pollen or possessing a receptive stigma, were cut from the plant at the base of the ovary using sterile scissors.
Leaf	The seventh through twelvth leaves, from top to bottom, were cut from the stem using a sterile razor blade. A sterile 8-mm diameter leaf punch was used to sample from directly the middle of the leaves and through the midvein, for a total of six leaf discs.
Stem	Using a sterile razor blade, stem pieces four millimeters in length were cut from directly below where the leaves were sampled, for a total of six stem pieces.
Root	Using sterile scissors, a one-centimeter crosssection of roots three centimeters from the base of stem was cut after roots were thoroughly rinsed to remove all perlite.

Table 3.4. Provisionally identified features in hydroponically-cultivated fireweed.

	Feature	Feature assignment	Observed ion	Molecular formula	Monoisotopic mass	Retention time (sec)	Observed *m/z*	Adduct *m/z*	Accuracy (ppm)	Trend[a]	Reference(s) for identification
Flavonoids											
	1	Epicatechin or catechin	M-H	$C_{15}H_{14}O_6$	290.0790	298	289.0705	289.0718	4.5		Agnieszka et al., 2018; Bark et al., 2011; Chen et al., 2001
	2	Kaempferol 3-*O*-arabinoside	M+Na	$C_{20}H_{18}O_{10}$	418.0900	441	441.0790	441.0792	0.5	Increase in root	Cai et al., 2005; Schepetkin et al., 2016
	3	Kaempferol 3-rhamnoside	M-H	$C_{21}H_{20}O_{10}$	432.1056	457	431.0962	431.0978	3.7	Increase in root	Baert et al., 2017; Cai et al., 2005; Schepetkin et al., 2016
	4	Quercetin 3-rhamnoside	M-H	$C_{21}H_{20}O_{11}$	448.1006	401	447.0915	447.0933	4.0		Regos et al., 2009; Schepetkin et al., 2016
	5	Kaempferol 3-glucuronide	M-H	$C_{21}H_{18}O_{12}$	462.0798	401	461.0706	461.0726	4.3		Baert et al., 2017; Schepetkin et al., 2016
	6	Hyperoside or isoquercetin	M-H	$C_{21}H_{20}O_{12}$	464.0955	366	463.0866	463.0877	2.4		Schepetkin et al., 2016; Sukito and Tachibana 2014
	7	Quercetin 3-*O*-glucuronide	M-H	$C_{21}H_{18}O_{13}$	478.0747	364	477.0652	477.0675	4.8		Agnieszka et al., 2018; Baert et al., 2017; Vasco et al., 2009
	8	Naringenin	M+H	$C_{15}H_{12}O_5$	272.0685	415	273.0755	273.0757	0.7	Decrease in leaf	Constantin et al., 2013; Schepetkin et al., 2016; Vallverd-Queralt et al., 2010
	9	Kaempferol	M+H	$C_{15}H_{10}O_6$	286.0477	457	287.0547	287.055	1.0	Increase in root	Schepetkin et al., 2016; Szostek et al., 2003
	10	Quercetin	M+H	$C_{15}H_{10}O_7$	302.0427	344	303.0498	303.0499	0.3		Cai et al., 2005; Chen et al., 2001; Schepetkin et al., 2016
Phenolic acids											
	11	Chlorogenic acid	M-H	$C_{16}H_{18}O_9$	354.0951	277	353.0862	353.0878	4.5		Baert et al., 2017; Vasco et al., 2009
	12	Caftaric acid	M+H-2H2O	$C_{13}H_{12}O_9$	312.0481	319	277.0341	277.0354	4.7	Decrease in leaf and stem	Lee and Scagel 2009
	13	Ellagic acid	M-H	$C_{14}H_6O_8$	302.0063	361	300.9975	300.999	5.0	Decrease in stem	Schepetkin et al., 2016; Szostek et al., 2003

Table 3.4 continued

	Feature	Feature assignment	Observed ion	Molecular formula	Monoisotopic mass	Retention time (sec)	Observed *m/z*	Adduct *m/z*	Accuracy (ppm)	Trend	Reference(s) for identification
Ellagitannins											
	14	Oenothein B	M-2H	$C_{68}H_{50}O_{44}$	1570.1670	284	783.0657	783.0675	2.3		Agnieszka et al., 2018; Baert et al., 2015; Boulekbache-Makhlou et al., 2013; Granica et al., 2012
	15	Tellimagrandin II (-)	M-H	$C_{41}H_{30}O_{26}$	938.1025	332	937.0922	937.0953	3.3	Decrease in leaf and stem	Boulekbache-Makhlou et al., 2013; Yoshida et al., 2018
	16	Tellimagrandin II (+)	M+Na	$C_{41}H_{30}O_{26}$	938.1025	331	961.0911	961.0917	0.6	Decrease in leaf and stem	Boulekbache-Makhlou et al., 2013; Yoshida et al., 2018
Other											
	17	Coumarin	M+ACN+H	$C_9H_6O_2$	146.0368	279	188.0705	188.0706	0.5		Sayik et al., 2017; Schepetkin et al., 2016

[a]Trend in tissue in response to increasing nutrient strength

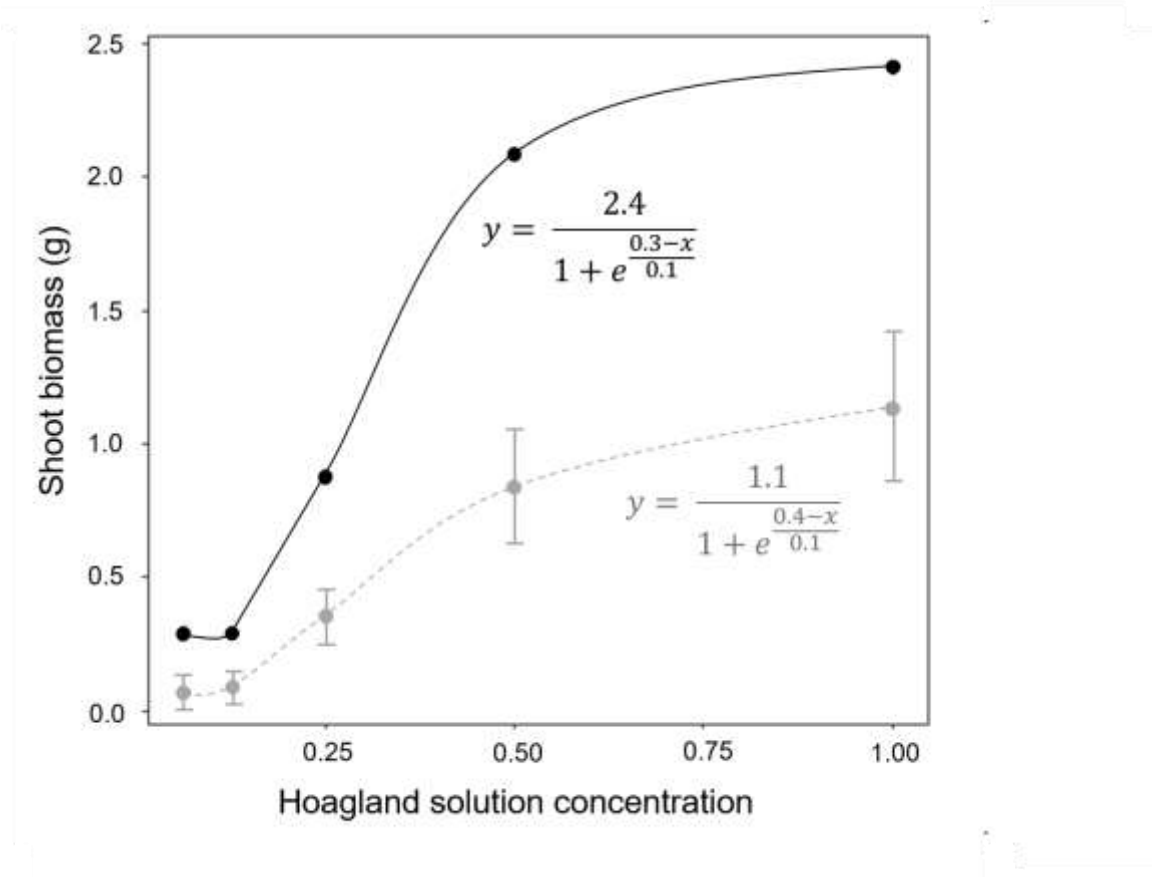

Figure 3.1. Logistic growth curves for maximum (solid black line) and mean (dashed gray line) biomass production of hydroponic fireweed under nutrient treatments, with equations for the determined non-linear logistic growth models. Standard error bars are shown for the mean biomass production.

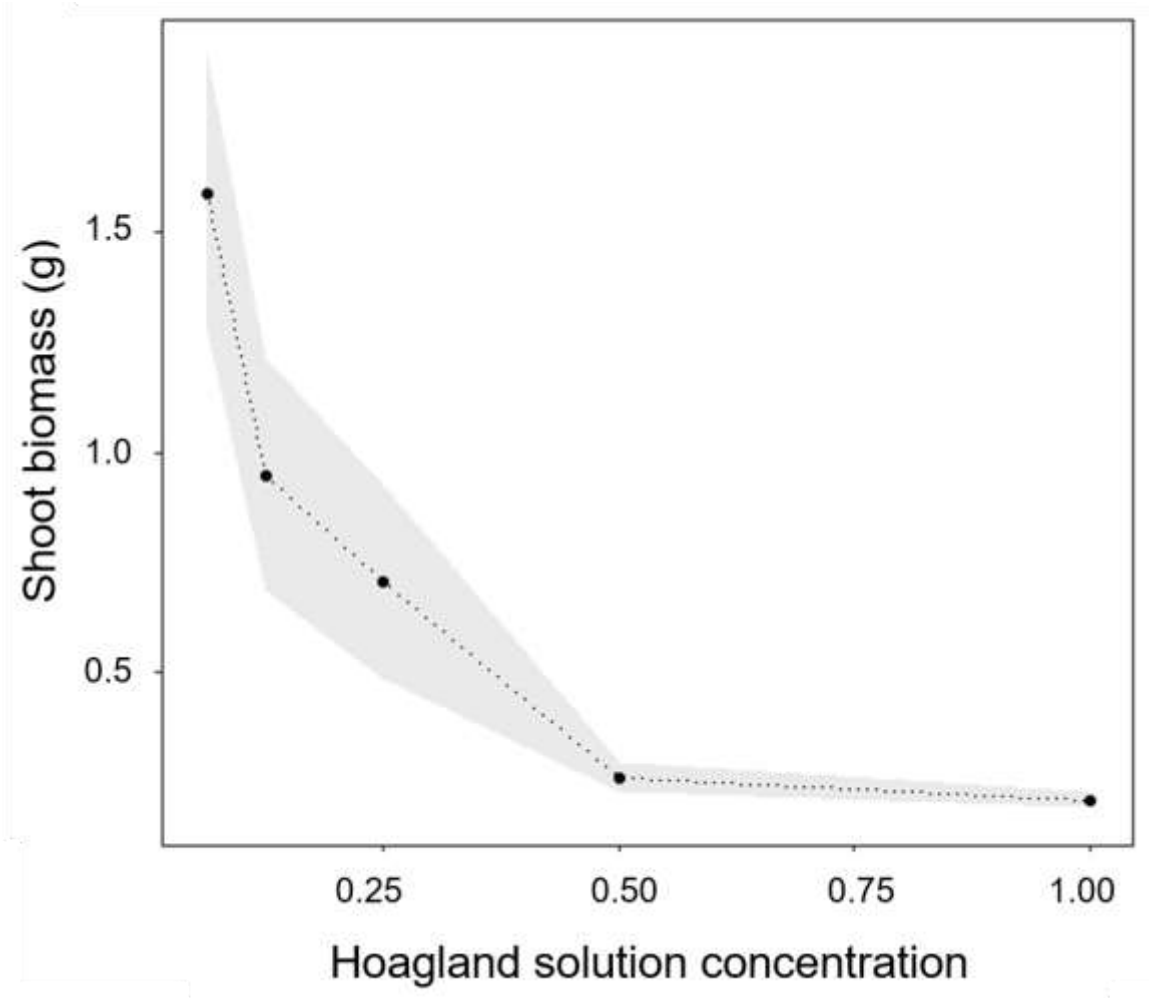

Figure 3.2. Root:shoot ratio of hydroponically-cultivated fireweed in response to strength of Hoagland solution. Shaded cone around mean values indicates standard error.

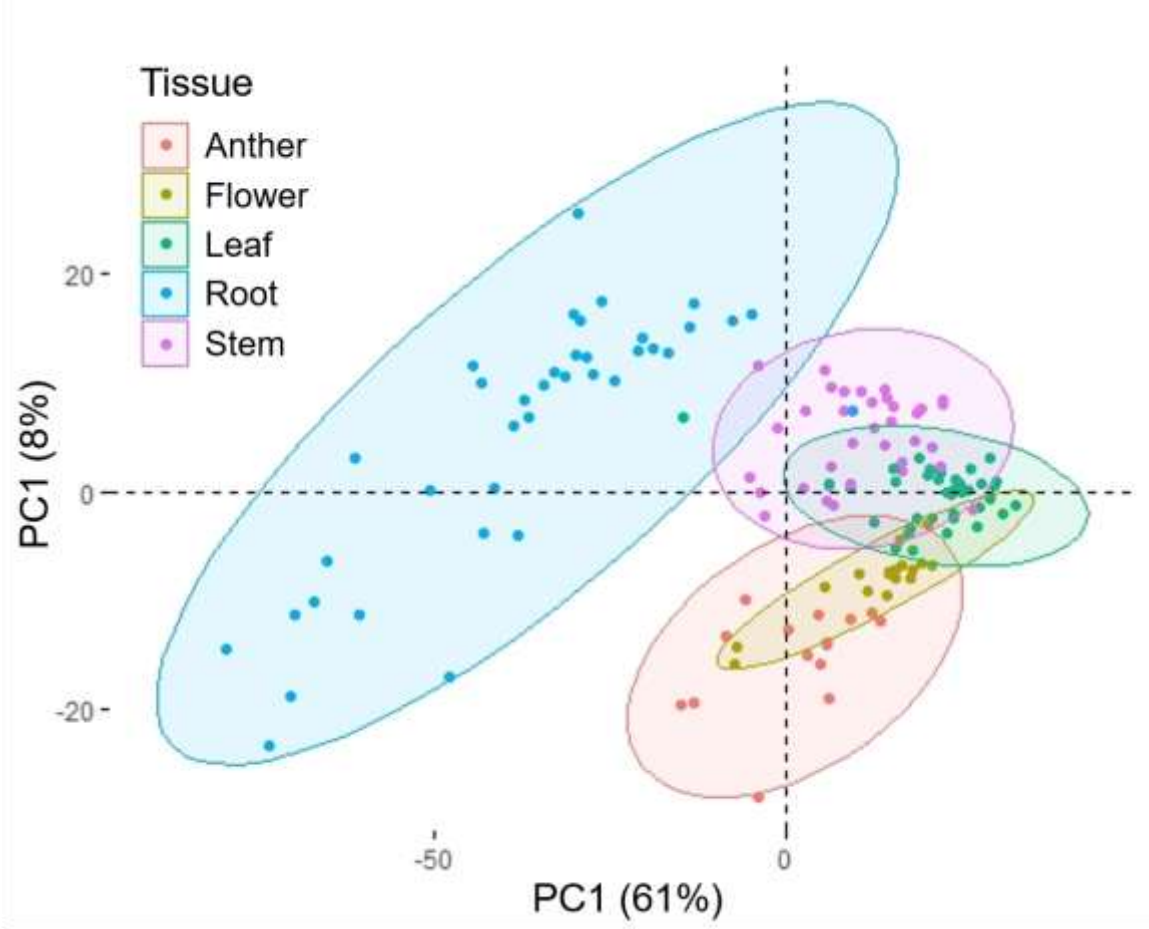

Figure 3.3. PCA score plot for five tissues harvested from hydroponically-cultivated fireweed. Data were log (base 2)-transformed, centered, and pareto-scaled. Colored ovals correspond to 95% confidence region for each tissue type.

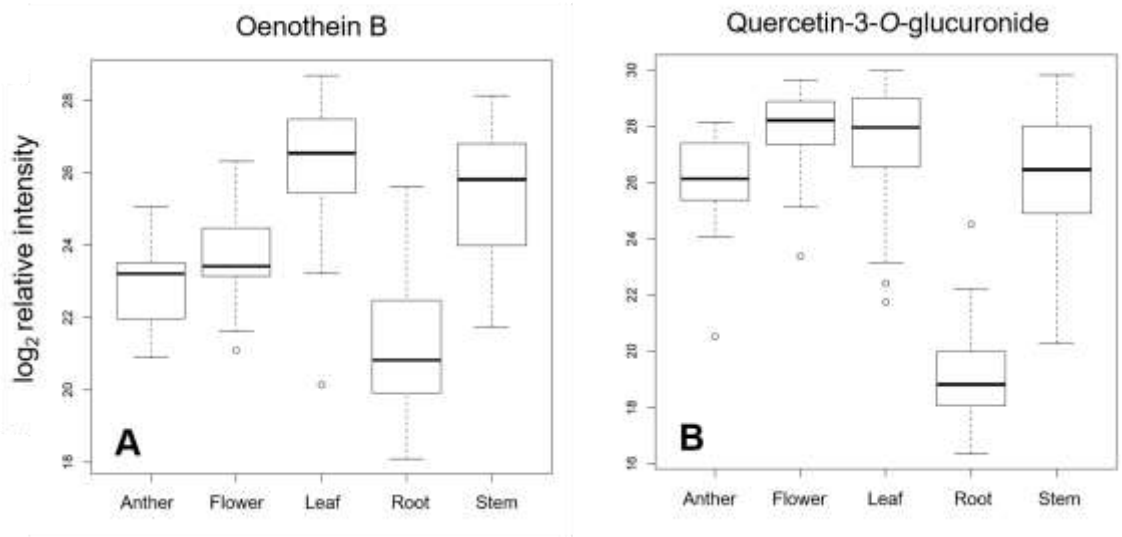

Figure 3.4. Boxplots showing the distribution of oenothein B (A) and quercetin-3-O-glucuronide (B) in five fireweed tissues. Means displayed include all nutrient treatments other than no nitrogen treatment.

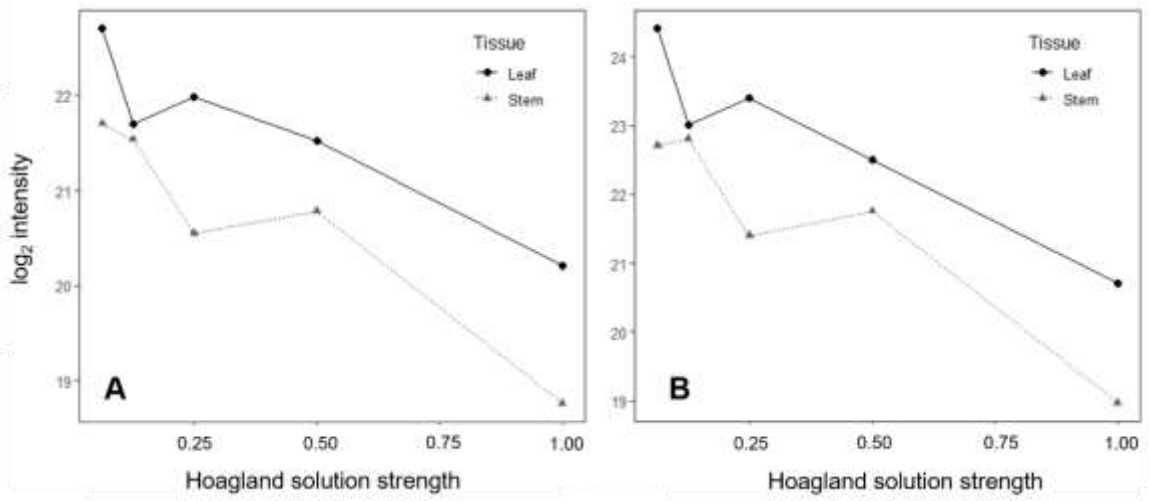

Figure 3.5. Mean intensity of features 12 (A) and 17 (B), putatively identified as caftaric acid and tellimagrandin II, in leaf (solid line) and stem (dashed line) tissue in response to strength of nutrient solution.

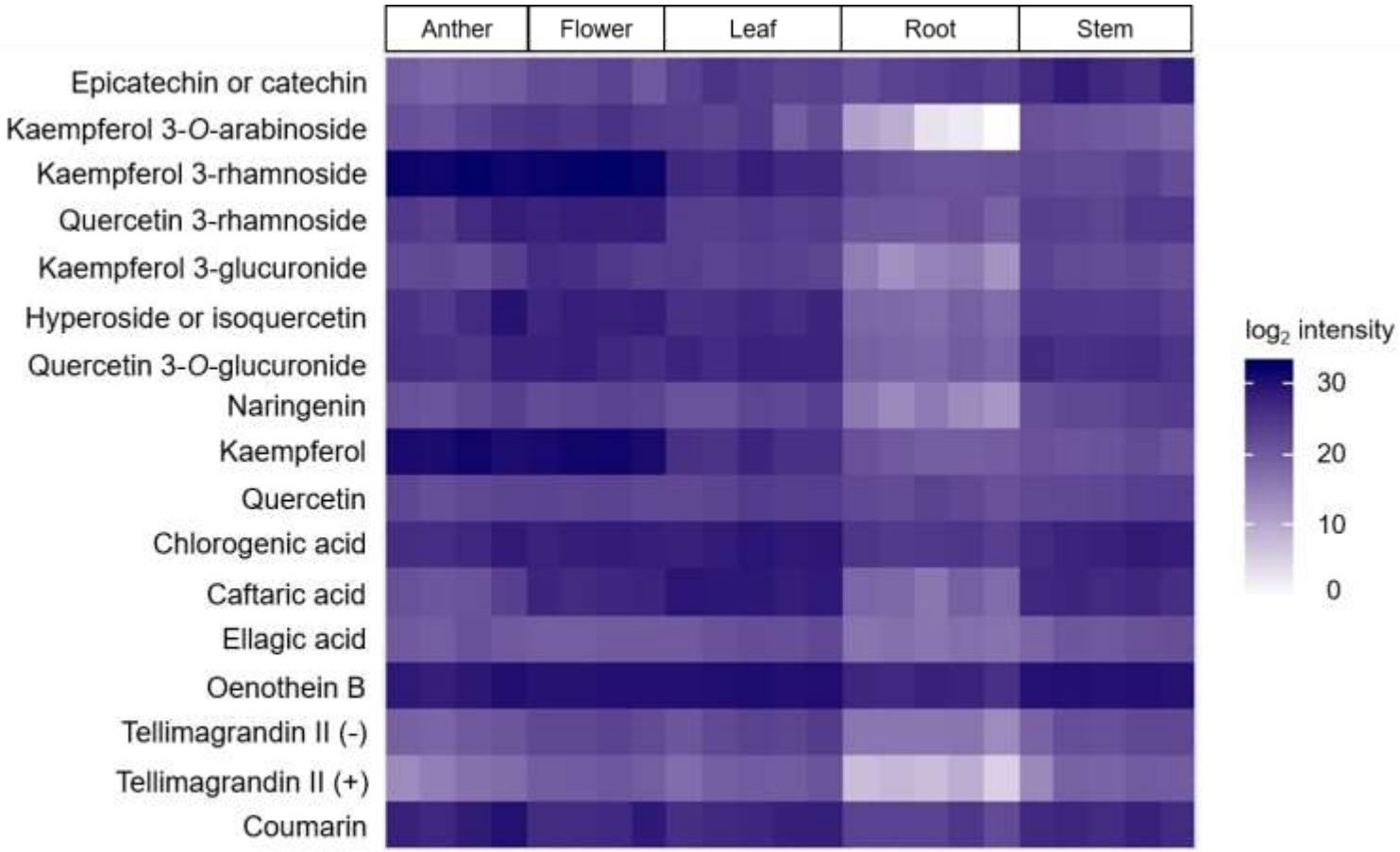

Figure 3.6. Heat map of the mean log_2-transformed intensity values for anther, flower, leaf, root and stem tissue. Within tissue columns nutrient treatments are, from left to right, full strength, half, quarter, eighth, and sixteenth. There were no sixteenth-strength anther or flower samples.

Conclusion

Prior to largescale, widespread establishment of native and naturalized perennial species for marketable products, there must evaluations of yields across space and time, as well as in response to production practices. Four native and naturalized Minnesota species – Canada milk vetch (*Astragalus canadensis* L.), purple coneflower (*Echinacea purpurea* Moench [L.]), showy tick trefoil (*Desmodium canadense* L.), and fireweed (*Chamaenerion angustifolium* (L.) scop.) – were selected for initial examination due to their potential for providing marketable seed, biomass, and natural products (phytochemicals).

A field experiment was conducted to evaluate seed and biomass production in three species of potential economic and ecological interest: Canada milk vetch, purple coneflower, and showy tick trefoil (Chapter 2). Experimental plots were established at two locations and harvested for seed and biomass over three consecutive years. It is rare to have three years of data from multiple locations for native prairie seed and biomass production. Seed yields for all three species declined over the three years, potentially due to the high frequency of seed harvest. Biomass yield, however, varied by species. Similar to previous reports, Canada milk vetch stands declined over time which limits long-term yield potential. In contrast, purple coneflower and showy tick trefoil may have the potential for long-term biomass production, as yields were stable and increased, respectively, over the course of the experiment. Biomass yield results cannot be

generalized across different species, and this warrants further investigation on a species-basis, as different mechanisms are likely at play.

Increased biodiversity in native plantings can provide further ecosystem services, but the greater plant community may affect seed and biomass yield of native perennial species. Therefore, Canada milk vetch, purple coneflower, and showy tick trefoil were established in six different agronomic designs, ranging from single monoculture rows to larger polycultures to examine the effects of plant community structure on seed and biomass yield (Chapter 2). Although continuous spans of the focal species (community designs) exhibited higher yields than rows separated by native grasses (row designs), there were no significant differences detected among the community designs. Plant community diversity and richness did not affect seed or biomass yields in this experimental system. The planting configurations implemented on the landscape should thus be determined by the individual grower's preferences, although factors such as ease of harvest must be considered.

Many native and naturalized perennial species are considered medicinal plant species and are highly valued for the bioactive phytochemicals they produce. Historically, whole plant and tissue extracts including wide arrays of phytochemicals were used medicinally. Plant-based medicines have taken a reductionist approach over the past few decades in that select bioactive compounds are isolated for medicinal use. Purple coneflower is widely used as a dietary supplement for its purported medicinal properties, and recent research has shown that several compounds may synergistically contribute to its reported

bioactivity. The surrounding plant community likely influences phytochemical production, and there is little information on the effects of cultivation on the phytochemical profile, as medicinal plants are commonly harvested from wild stands. In cultivation, purple coneflower produced a wide variety of medicinal compounds, and production was not affected by site of cultivation (Chapter 3). Untargeted metabolomics approaches highlighted caffeic acid derivatives and alkamides, both important bioactive chemical classes in purple coneflower, that were positively and negatively correlated. Examination of the relationships between beneficial compounds and chemical classes is an important step in developing higher-quality purple coneflower products. The plant community structure ("agronomic design") and location did not affect the overall phytochemical profile of purple coneflower tissues. Agronomic design and location did, however, affect levels of specific bioactive compounds in leaf, stem and root tissue, emphasizing the need to address specific compounds as well as overall chemical profiles when examining the effects of various experimental and agronomic conditions on phytochemical production.

Due to the nature of the field experiment – several experimental treatments, locations, and replicates – the number of samples was much greater than in a typical metabolomics experiment. High-throughput methods were established to efficiently process and analyze thousands of samples. Similar methods were applied to and developed for exploring phytochemical production in hydroponically-cultivated fireweed in response to mineral nutrient concentration. Relative abundance of oenothein B, the main phytochemical of commercial interest due to its immunomodulatory activity, did not vary with nutrient

availability. However, the abundance of several provisionally identified compounds decreased as mineral nutrient concentration increased. Biomass production increased with increasing nutrient mineral concentration, presenting a dilemma for optimized production of fireweed for medicinal phytochemicals beyond oenothein B. Future work must address this apparent trade-off between nutrient availability and biomass production if compounds beyond oenothein B are of interest.

Overall, the research presented in this dissertation provides a foundation for examining the biomass, seed and phytochemical production of native and naturalized plant species. Native and naturalized plants can play important roles on agricultural landscapes including marketable products for growers (Chapter 1). Canada milk vetch, purple coneflower, and showy tick trefoil had similar seed yield trajectories but different biomass yield trajectories through time, further emphasizing the need to address native and naturalized species individually, and to examine specific mechanisms affecting yields over time (Chapter 2). Purple coneflower established in agronomic designs produced phytochemicals of medicinal value, and findings presented here emphasize the need to examine the overall phytochemical profile as well as individual compounds (Chapter 3). Most phytochemicals of interest in fireweed were influenced by mineral nutrient availability, but phytochemical production must be reconciled with biomass production if aims are to optimize both (Chapter 4). Ultimately, the research presented in this dissertation provides valuable information on the establishment and sustainability of four native and naturalized species for the production of seed, biomass, and phytochemicals.

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Appendix A: Chapter 1 Supporting Information

Supplementary Table A.1. Seed and biomass harvest dates for the three focal species at two locations.

	Becker			Rosemount		
	2016	**2017**	**2018**	**2016**	**2017**	**2018**
Seed	----------Date(s) of harvest----------					
Canada milk vetch	24 Aug	13 Sept	11 Sept	25 Aug	5 Sept	6 Sept
Purple coneflower	19 Sept	20 Sept	11 Oct[a]	20 Sept	19 Sept	6 Sept
	30 Sept	5 Oct		1 Oct	4 Oct	21 Sept
	7 Oct	12 Oct		14 Oct	11 Oct	4 Oct
Showy tick trefoil	24 Aug	6 Sept	11 Sept	25 Aug	8 Sept	6 Sept
Biomass	----------Date(s) of harvest----------					
Canada milk vetch	24 Sept	14 Sept	14 Sept	25 Sept	8 Sept	7 Sept
Purple coneflower	10 Oct	12 Oct	11 Oct	14 Oct	11 Oct	4 Oct
Showy tick trefoil	16 Sept	14 Sept	14 Sept	17 Sept	8 Sept	7 Sept

[a] The harvest of purple coneflower seed in Becker 2018 was the only purple coneflower harvest to occur on one day due to low seed yield

Supplementary Table A.2. Mean focal seed yield (kg ha^{-1}) of the six designs and three focal species in Becker and Rosemount 2016-2018. Letters are for comparison of designs within each species column and year, with similar letters denoting no significant difference at the 0.05 probability level.

	Becker			Rosemount		
	Canada milk vetch	**Purple coneflower**	**Showy tick trefoil**	**Canada milk vetch**	**Purple coneflower**	**Showy tick trefoil**
Design			2016			
1-row	496 c	49 c	484 b	439 b	256 c	89 c
3-row	1266 b	110 bc	585 b	495 b	451 bc	157 bc
6-row	1416 b	90 bc	569 b	605 b	727 b	224 abc
Monoculture	7804 a	772 b	2324 a	3745 a	3120 a	1697 a
Low-diversity polyculture	6368 a	351 ab	2810 a	3680 a	2201 a	673 abc
High-diversity polyculture	6362 a	362 ab	3262 a	3268 a	2911 a	1006 ab
			2017			
1-row	194 c	114 b	378 bc	58 c	137 b	3 ab
3-row	495 bc	100 b	368 c	97 bc	237 b	5 a
6-row	355 b	83 b	384 bc	117 b	313 b	1 b
Monoculture	1889 a	678 a	1300 a	951 a	1304 a	22 a
Low-diversity polyculture	2647 a	554 a	1327 ab	681 a	1233 a	11 a
High-diversity polyculture	1864 a	599 a	1844 a	734 a	1277 a	8 a
			2018			
1-row	0 a	3 a	81 b	0 a	121 b	5 a
3-row	0 a	4 a	92 b	0 a	262 b	2 ab
6-row	0 a	4 a	196 b	0 a	292 b	1 b
Monoculture	0 a	46 a	1478 a	0 a	1514 a	8 a
Low-diversity polyculture	0 a	77 a	1085 a	0 a	1543 a	12 a
High-diversity polyculture	0 a	54 a	1830 a	0 a	1665 a	9 a

Supplementary Table A.3. Mean focal biomass yield (kg ha^{-1}) of the six designs and three focal species in Becker and Rosemount 2016-2018. Letters are for comparison of designs within each species column and year, with similar letters denoting no significant difference at the 0.05 probability level.

	Becker			Rosemount		
	Canada milk vetch	**Purple coneflower**	**Showy tick trefoil**	**Canada milk vetch**	**Purple coneflower**	**Showy tick trefoil**
Design			2016			
1-row	1623 c	455 b	1400 b	3448 b	1275 c	502. a
3-row	3512 bc	720 b	1916 b	5156 b	2095 bc	1217 a
6-row	3578 bc	874 b	2359 b	5476 b	3591 bc	1691 a
Monoculture	8465 a	2863 a	5882 a	13289 a	6520 a	3547 a
Low-diversity polyculture	6598 ab	1774 ab	5101 a	11541 a	4133 ab	1893 a
High-diversity polyculture	5877 ab	1770 ab	5403 a	12358 a	6632 a	3519 a
			2017			
1-row	1016 b	870 b	2173 c	352 c	1710 b	1559 c
3-row	1818 ab	803 b	3365 bc	1003 bc	2790 ab	3527 bc
6-row	1914 ab	625 b	3071 c	811 bc	3071 ab	2515 b
Monoculture	2244 ab	2549 a	6347 a	3243 a	4092 ab	6393 ab
Low-diversity polyculture	4179 a	1793 ab	5410 ab	1753 ab	2872 ab	7941 a
High-diversity polyculture	1578 ab	1915 ab	5898 a	1930 ab	5042 b	7015 a
			2018			
1-row	23 a	198 b	1953 b	0 a	1315 b	2507 b
3-row	64 a	336 b	3388 b	0 a	1807 b	3874 b
6-row	45 a	421 ab	4010 b	0 a	2563 ab	2221 b
Monoculture	123 a	1990 a	7431 a	0 a	3716 ab	5353 ab
Low-diversity polyculture	269 a	1717 ab	7417 a	25 a	2975 ab	7154 a
High-diversity polyculture	104 a	1550 ab	6721 a	117 a	4399 a	3624 b

Supplementary Table A.4. Mean total biomass yield (kg ha^{-1}) of the six designs and three focal species in Becker and Rosemount 2016-2018. Letters are for comparison of designs within each species column and year, with similar letters denoting no significant difference at the 0.05 probability level.

	Becker			Rosemount		
	Canada milk vetch	**Purple coneflower**	**Showy tick trefoil**	**Canada milk vetch**	**Purple coneflower**	**Showy tick trefoil**
Design			2016			
1-row	3722 b	2278 a	3454 a	6572 b	4732 a	3373 a
3-row	5715 ab	2426 a	3634 a	7528 b	3952 a	4030 a
6-row	5076 ab	2059 a	3612 a	7351 b	6025 a	4693 a
Monoculture	8465 a	2863 a	5882 a	13289 a	6520 a	3547 a
Low-diversity polyculture	7272 ab	2554 a	5220 a	13387 a	4705 a	3969 a
High-diversity polyculture	6437 ab	2397 a	5651 a	14214 a	6810 a	5764 a
			2017			
1-row	3068 a	2382 a	3711 c	3732 a	4680 a	4858 c
3-row	3192 a	2431 a	4846 abc	3842 a	5137 a	5961 bc
6-row	3394 a	1443 a	4118 bc	2999 a	4776 a	5510 bc
Monoculture	2244 a	2549 a	6347 ab	3247 a	4092 a	6393 abc
Low-diversity polyculture	4604 a	2600 a	5441 abc	3398 a	2977 a	9558 a
High-diversity polyculture	2984 a	2693 a	6874 a	3970 a	5131 a	8495 ab
			2018			
1-row	2777 a	3172 ab	5428 b	1963 a	2618 a	4208 ab
3-row	2985 a	2559 b	5650 b	1645 ab	3062 a	5632 ab
6-row	2265 a	2309 b	5696 b	1185 ab	3331 a	3349 a
Monoculture	123 b	1990 b	7431 ab	0 c	3716 a	5353 ab
Low-diversity polyculture	1087 ab	4167 a	7450 ab	1427 ab	3311 a	7166 a
High-diversity polyculture	1691 a	3315 ab	9251 b	638 bc	4427 a	4122 ab

Supplementary Table A.5. F-value and significance for the main effects of design and year on species richness and Shannon diversity of the low- and high-richness polyculture designs.

	Becker						Rosemount					
	Canada milk vetch		Purple coneflower		Showy tick trefoil		Canada milk vetch		Purple coneflower		Showy tick trefoil	
	F-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*
Species richness												
Design	14.06	0.0038	63	<0.0001	0.2	NS	1.07	NS	0.75	NS	1.67	NS
Year	1.56	NS†	1.86	NS	4.55	NS	3.8	NS	12.58	0.0019	11.27	0.0027
Design x Year	2.44	NS	0.43	NS	0.35	NS	0.067	NS	0.25	NS	1.27	NS
Shannon diversity												
Design	13.31	0.0045	43.93	0.0001	0.55	NS	7.99	0.018	0.95	NS	0.001	NS
Year	3.43	NS	3.49	NS	1.47	NS	1.44	NS	6.13	0.018	6.79	0.014
Design x Year	2.82	NS	0.39	NS	0.71	NS	0.13	NS	0.69	NS	0.55	NS

† NS, not significant at the 0.05 probability level.

Appendix B: Chapter 2 Supporting Information

Supplementary Table B.1. F-value and significance for the main effects of site and design on mean log_2 relative intensity for the top five phenolic compounds in five different tissues.

	Flower		**Leaf**		**Root**		**Seed**		**Stem**	
	F-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*
Caffeic acid										
Site	3.220	0.074	119.845	<0.0001	15.302	<0.0001	0.002	0.963	214.612	<0.0001
Design	0.370	0.870	5.413	0.000	2.657	0.024	1.494	0.196	1.681	0.141
Site x Design	1.790	0.117	3.644	0.004	0.865	0.506	1.482	0.200	1.319	0.258
Caftaric acid										
Site	2.320	0.129	109.194	<0.0001	11.409	0.001	0.068	0.795	215.261	<0.0001
Design	0.340	0.891	4.836	0.000	2.075	0.070	1.387	0.233	2.028	0.077
Site x Design	1.690	0.138	3.747	0.003	0.966	0.440	1.339	0.252	1.637	0.153
Cichoric acid										
Site	3.710	0.055	103.437	<0.0001	8.370	0.004	0.135	0.714	166.107	<0.0001
Design	0.310	0.905	3.808	0.003	2.300	0.046	1.406	0.226	166.107	0.123
Site x Design	2.100	0.067	2.874	0.016	0.990	0.423	0.814	0.542	2.045	0.075
Chlorogenic acid										
Site	0.458	0.499	53.049	<0.0001	15.624	<0.0001	8.435	0.004	25.158	<0.0001
Design	0.709	0.618	0.715	0.613	2.835	0.017	0.669	0.647	1.039	0.396
Site x Design	1.923	0.092	0.528	0.755	1.204	0.309	0.712	0.615	2.240	0.052
Echinacoside										
Site	4.051	0.046	12.070	0.001	1.031	0.311	0.001	0.974	9.117	0.003
Design	2.830	0.017	0.449	0.814	3.819	0.003	0.302	0.911	3.020	0.012
Site x Design	2.663	0.024	1.069	0.379	0.661	0.654	0.757	0.583	1.198	0.312

Supplementary Table 2. Mean log_2 relative intensity and standard error for the top five phenolic compounds in purple coneflower from five different tissues harvested in Becker and Rosemount.

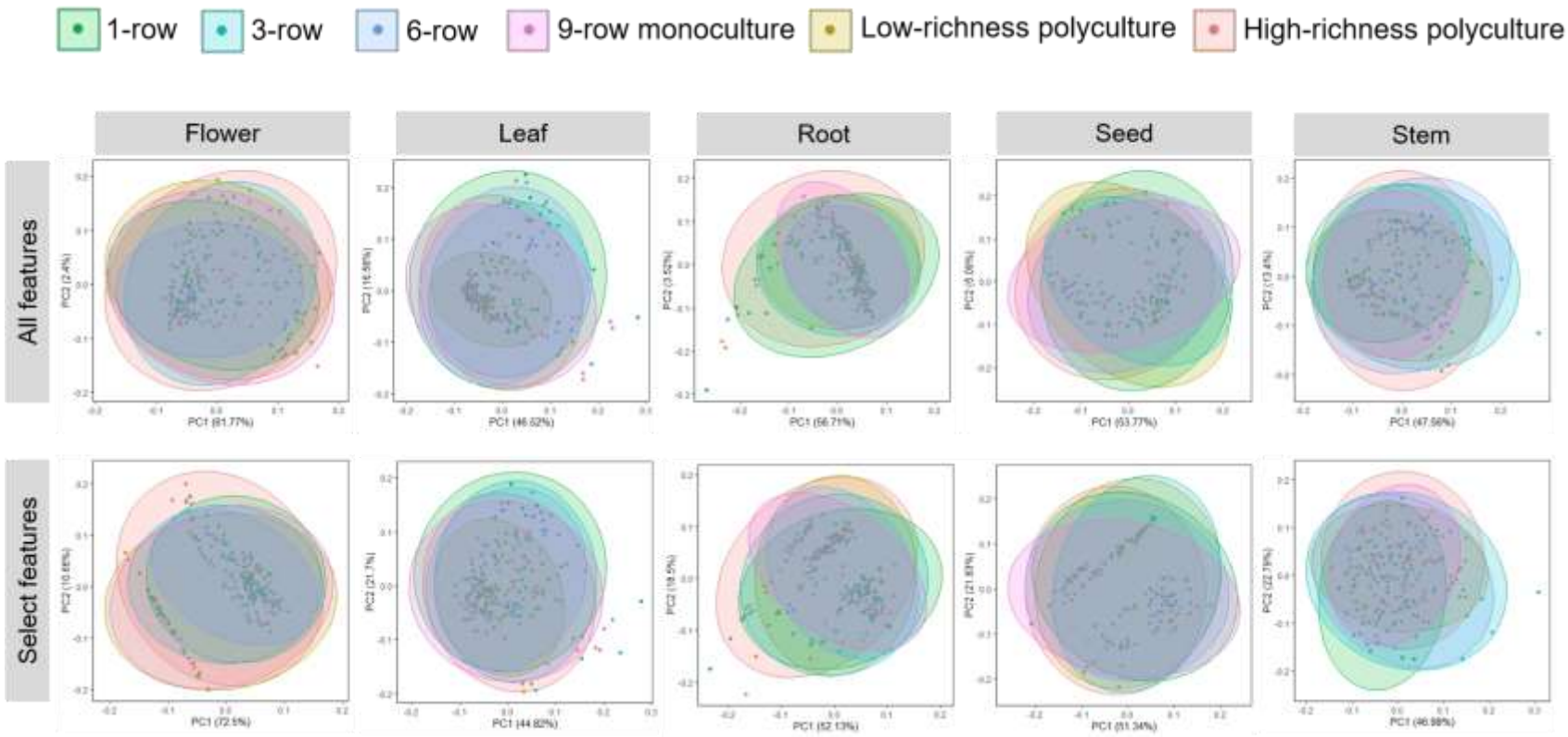
	Flower		Leaf		Root		Seed		Stem	
	Becker	**Rosemount**	**Becker**	**Rosemount**	**Becker**	**Rosemount**	**Becker**	**Rosemount**	**Becker**	**Rosemount**
Caffeic acid										
Mean	26.85	26.48	28.20	25.49	25.30	24.51	21.52	21.54	25.46	21.85
Standard Error	0.15	0.15	0.11	0.25	0.13	0.16	0.22	0.25	0.18	0.17
Caftaric acid										
Mean	28.91	28.56	30.27	27.29	28.02	27.26	23.82	23.92	27.84	23.21
Standard Error	0.16	0.17	0.12	0.29	0.14	0.18	0.26	0.31	0.19	0.26
Chlorogenic acid										
Mean	26.87	27.04	21.03	19.06	22.21	21.12	28.93	27.86	18.24	15.52
Standard Error	0.19	0.18	0.22	0.15	0.19	0.21	0.21	0.32	0.30	0.48
Cichoric acid										
Mean	31.32	30.95	32.07	29.37	31.05	30.45	25.63	25.48	29.30	24.92
Standard Error	0.13	0.14	0.10	0.27	0.13	0.17	0.28	0.33	0.20	0.29
Echinacoside										
Mean	13.50	11.68	14.94	17.24	20.01	20.27	8.51	8.53	18.19	19.71
Standard Error	0.63	0.70	0.52	0.40	0.19	0.18	0.93	1.07	0.40	0.32

Supplementary Table B.3. Mean log_2 relative intensity and standard error for caffeic acid, caftaric acid, and cichoric acid in leaf tissue from Rosemount.

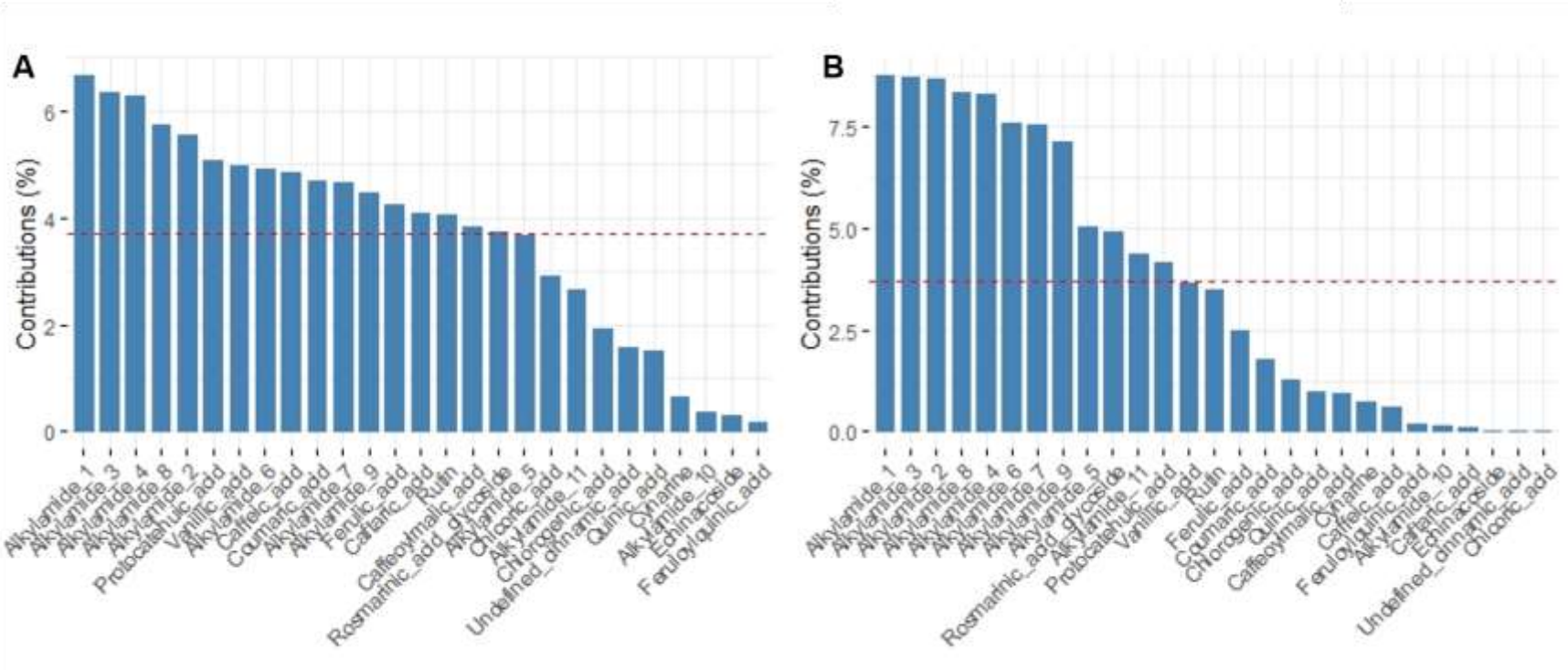
	Caffeic Acid	Caftaric acid	Cichoric acid
1-row			
Mean	23.90	25.60	27.90
Standard Error	0.46	0.52	0.48
3-row			
Mean	24.50	25.90	28.40
Standard Error	0.46	0.52	0.48
6-row			
Mean	24.80	26.70	28.80
Standard Error	0.49	0.55	0.51
9-row monoculture			
Mean	26.30	28.20	30.30
Standard Error	0.45	0.50	0.47
Low-richness polyculture			
Mean	27.30	29.30	31.00
Standard Error	0.45	0.50	0.47
High-richness polyculture			
Mean	25.90	27.70	29.70
Standard Error	0.46	0.52	0.48

Supplementary Table B.4. Mean $\log_2$ relative intensity values for the 27 select features in five different tissue types corresponding to the heat map depicted in Figure 2.3.

Feature	Feature assignment	Flower	Leaf	Root	Seed	Stem
P01	Protocatechuic acid	18.59	20.47	16.91	16.52	21.45
P02	Coumaric acid	23.15	24.79	23.21	16.33	24.76
P03	Caffeic acid	26.66	26.88	24.90	21.53	23.74
P04	Quinic acid	25.19	18.46	19.29	25.79	15.63
P05	Caffeoylmalic acid	25.94	27.32	26.70	20.14	28.07
P06	Caftaric acid	28.74	28.82	27.64	23.86	25.63
P07	Vanillic acid hexoside	21.08	23.71	19.44	19.85	23.85
P08	Chlorogenic acid	26.95	20.07	21.67	28.47	16.95
P09	Ferulic acid hexoside	19.42	20.57	15.05	12.45	19.35
P10	Feruloylquinic acid	21.43	17.89	13.88	18.39	13.96
P11	Chicoric acid	31.13	30.76	30.75	25.56	27.21
P12	Undefined cinnamic acid	27.15	25.44	27.47	17.66	20.65
P13	Cynarin	24.79	18.52	19.50	23.69	15.79
P14	Rosmarinic acid glycoside	20.44	24.98	6.74	16.63	20.54
P15	Rutin	26.73	25.94	13.05	18.99	23.24
P16	Echinacoside	12.59	16.06	20.14	8.52	18.91
A01	Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide	27.27	20.07	30.59	31.36	23.55
A02	Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	25.00	19.91	31.89	28.61	24.09
A03	Dodeca-2E/Z,4E,10E-triene-8-ynoic acid isobutylamide	23.83	18.78	29.72	27.88	23.26
A04	Dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide	30.62	21.42	33.17	33.76	29.53
A05	Dodeca-2E,4E,8Z-trienoic acid isobutlyamide	23.25	16.06	21.77	26.63	22.59
A06	Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide	21.93	18.33	31.19	25.41	23.17
A07	Dodeca-2E-ene-8,10-diynoic acid 2-methylbutylamide	21.24	17.11	29.24	24.17	23.01
A08	Dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide	25.98	19.87	30.07	29.37	26.01
A09	Dodeca-2E,4E-dienoic acid 2-methylbutylamide	21.20	19.06	25.58	25.49	22.45
A10	Pentadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide	28.11	24.86	25.85	24.52	29.58
A11	Pentadeca-2E,9Z-diene-12,14-diynoic acid 2-methylbutylamide	21.35	22.06	16.90	15.39	16.66



Supplementary Figure B.1. Principal components analysis of experimental designs from all five tissues for all features (A-E) and the 27 select features (F-J).



Supplementary Figure B.2. Percent contributions of select features from Becker (A) and Rosemount (B) to the PCA plots from Figure 1. Red reference line corresponds to the expected value if the contribution for each feature were uniform. For the first principal component, features with a contribution above the reference line is considered as important in contributing to the dimension.

Appendix C: Chapter 3 Supporting Information

Equation C.1. Equation for a non-linear logistic growth model to estimate the relationship between nutrient solution strength and shoot biomass production for the mean and maximum values

$$y = \frac{asym}{1 + e^{\frac{xmid - x}{scal}}}$$

Equation C.2. Equation for a non-linear exponential decay model to fit an exponential decay curve to the root:shoot ratio data

$$y(x) = y_f + (y_0 - y_f)\, e^{-\exp(\log \propto) x}$$

Supplementary Table C.1. Features that changed in response to nutrient solution strength. ↓ indicates a decrease in relative intensity in response to increasing nutrient solution strength, ↑ indicates and increase in relative intensity

Galaxy ID	Observed *m/z*	Retention time	Benjamini-Yekutieli-corrected *p*-value	R^2	Trend in tissue		
					Root	Stem	Leaf
NM271T414	271.0599	414	0.0075	0.28			↓
NM273T443	273.1694	443	0.0099	0.17	↓	↓	
NM301T361	300.9975	361	0.0061	0.28		↓	
NM301T422	300.9978	422	0.0059	0.28		↓	↓
NM317T306	317.0393	306	0.0012	0.39		↑	↓
NM317T315	317.0394	315	0.0011	0.40		↓	↓
NM321T430	321.1538	430	0.0035	0.31		↑	
NM331T265	331.1019	265	0.0056	0.28		↓	↓
NM377T425	377.0499	425	0.0061	0.28		↓	↓
NM389T302	389.0824	302	0.007	0.27		↑	
NM392T290	392.0369	290	0.0017	0.37		↓	↓
NM392T303	392.0369	303	0.0017	0.36		↓	↓
NM392T319	392.0371	319	0.0019	0.35		↓	↓
NM393T316	393.0452	316	0.0023	0.34		↓	↓
NM393T436	393.1751	346	0.00098	0.26	↑		
NM394T334_2	394.0474	334	0.0017	0.37		↓	↓
NM394T342_2	394.0474	342	0.0011	0.40		↓	↓
NM395T384	395.0027	384	0.0066	0.29			↓
NM399T316	399.0267	316	0.0055	0.29		↑	
NM404T343	404.0356	343	0.00078	0.48		↓	↓
NM425T270_1	425.0690	270	0.0027	0.33		↓	
NM431T457	431.0963	457	0.0071	0.18	↑		
NM433T415	433.1123	415	0.0087	0.27		↓	
NM439T384	438.9924	384	0.008	0.28		↓	
NM454T469_2	454.0581	469	0.006	0.28		↓	↓
NM461T471	461.1080	471	0.0053	0.29		↓	↓
NM461T476	461.1074	476	0.0084	0.26		↓	↓
NM464T469	464.0458	469	0.0046	0.30		↓	↓
NM468T307	468.0416	307	0.00097	0.42		↓	
NM468T315	468.0416	315	0.00078	0.45		↓	↓
NM469T315_2	469.0027	315	0.0019	0.36		↓	↓
NM469T321_2	469.0453	322	0.0035	0.31		↓	↓
NM469T331	469.0449	331	0.00087	0.43		↓	↓
NM476T300	476.0393	300	0.0056	0.28		↓	↓
NM476T311	476.0394	311	0.00081	0.44		↓	↓
NM479T321	479.0329	321	0.00081	0.44		↓	
NM479T325_1	479.0331	325	0.0041	0.30		↓	
NM481T267	481.0967	267	0.0049	0.29		↓	

Supplementary Table C.1 continued

Galaxy ID	Observed *m/z*	Retention time	Benjamini-Yekutieli-corrected *p*-value	R^2	Root	Stem	Leaf
NM481T306	481.0968	306	0.0097	0.26		↓	↓
NM481T364_2	481.0436	364	0.0041	0.30		↓	↓
NM483T288	483.0759	288	0.0017	0.36		↓	
NM485T441	485.0677	441	0.0076	0.18	↑		
NM491T364	491.0317	364	0.003	0.32		↓	
NM496T364	496.0092	364	0.0062	0.28		↓	
NM517T446	517.1332	446	0.0069	0.19	↑		
NM542T305	542.0317	305	0.0019	0.35		↓	↓
NM542T332	542.0316	332	0.00097	0.42		↓	↓
NM544T355	544.0472	355	0.0019	0.35		↓	↓
NM544T363	544.0469	363	0.0011	0.41		↓	↓
NM545T402	545.0553	402	0.0017	0.37		↓	
NM546T402_1	545.5567	402	0.0019	0.35		↓	↓
NM552T330	552.0445	330	0.0017	0.37		↓	↓
NM556T395	556.0460	395	0.0019	0.35		↓	↓
NM556T401	556.0459	401	0.00078	0.45		↓	↓
NM557T266	557.0658	266	0.0046	0.30		↓	↓
NM557T280	557.0656	280	0.0041	0.30		↓	↓
NM558T270_1	557.5675	270	0.006	0.32			↓
NM561T457	561.0542	457	0.0012	0.26	↑		
NM568T264	568.0566	264	0.0063	0.31			↓
NM568T270	568.0564	270	0.0063	0.30			↓
NM583T400	583.0669	400	0.006	0.32			↓
NM595T425	595.2006	426	0.0027	0.40			↓
NM609T389	609.1433	389	0.0081	0.27		↓	
NM609T424	609.0864	424	0.0039	0.36			↓
NM611T484	611.1380	484	0.0083	0.26		↓	
NM617T365_1	617.0272	365	0.0029	0.32		↓	↓
NM618T316	618.0374	316	0.0019	0.35		↓	↓
NM618T326	618.0370	326	0.0087	0.26		↓	↓
NM619T334	619.0919	334	0.0055	0.29		↓	
NM625T375	625.0809	375	0.0041	0.34			↓
NM626T309	626.0347	309	0.0015	0.38		↓	↓
NM627T303_2	627.0387	303	0.0028	0.33		↓	
NM627T309_1	626.5359	309	0.0017	0.36		↓	↓
NM629T415_1	629.1132	415	0.0085	0.27			↓
NM631T296	631.0548	296	0.0082	0.26		↓	↓
NM635T292_1	635.0844	292	0.0035	0.31		↓	↓
NM635T300	635.0858	300	0.0031	0.32		↓	
NM635T307	635.0842	307	0.0018	0.36		↓	
NM635T315_1	635.0842	315	0.0011	0.41		↓	↓

Supplementary Table C.1 continued

Galaxy ID	Observed *m/z*	Retention time	Benjamini-Yekutieli-corrected *p*-value	R2	Root	Stem	Leaf
NM637T309	637.0254	309	0.0024	0.33		↓	
NM639T440	639.0968	440	0.0017	0.37		↓	
NM647T289	647.0499	289	0.0019	0.35		↑	
NM647T298_1	647.0497	298	0.0041	0.30		↑	
NM708T290	708.0690	290	0.0087	0.27			↓
NM709T283	709.0717	283	0.0039	0.36			↓
NM709T290_1	708.5704	290	0.009	0.27			↓
NM755T425	755.1084	425	0.0051	0.29		↓	↓
NM768T297_1	768.0606	297	0.0019	0.35		↑	
NM777T299	777.0669	299	0.0038	0.31		↑	
NM783T324_2	783.0665	324	0.0082	0.26		↓	
NM783T338	783.0663	338	0.0069	0.27		↓	
NM784T308	784.0739	308	0.0035	0.31		↓	
NM785T290_1	785.0782	290	0.0023	0.34		↓	↓
NM785T303_1	785.0783	303	0.0019	0.35		↓	↓
NM785T308_1	784.5753	308	0.0017	0.38		↓	
NM785T337_2	785.0739	337	0.0077	0.28			↓
NM787T334	787.0979	334	0.0017	0.37		↓	↓
NM787T340_1	787.0942	340	0.00078	0.45		↓	
NM792T287_3	792.0678	287	0.0011	0.40		↓	↓
NM794T324_1	794.0542	324	0.0047	0.30		↓	
NM794T338_1	794.0542	338	0.0044	0.30		↓	↓
NM795T308_1	795.0622	308	0.0017	0.36		↓	
NM795T317	795.0647	317	0.0018	0.36		↓	↓
NM799T281_1	799.0582	281	0.00087	0.43		↑	
NM800T281_2	800.0644	281	0.0018	0.36		↑	
NM801T237	801.0780	237	0.0032	0.32		↓	↓
NM801T252	801.0772	252	0.0012	0.39		↓	↓
NM801T258	801.0777	258	0.0019	0.35		↓	↓
NM805T312	805.0224	312	0.0082	0.26		↓	
NM805T478	805.2906	478	0.0027	0.40			↓
NM806T308_1	806.0535	308	0.0019	0.35		↓	
NM806T321_2	806.0263	321	0.0051	0.29		↓	
NM807T303	807.0630	303	0.0022	0.34		↓	
NM807T318_1	807.0625	318	0.003	0.32		↓	
NM809T334_1	809.0795	334	0.0012	0.39		↓	↓
NM809T340_1	809.0795	340	0.00078	0.46		↓	
NM810T285_3	810.0240	285	0.0075	0.28			↑
NM810T324_2	810.0221	324	0.0056	0.28		↓	
NM811T308_2	811.0323	308	0.0019	0.35		↓	
NM855T340	855.0843	340	0.00078	0.46		↓	

Supplementary Table C.1 continued

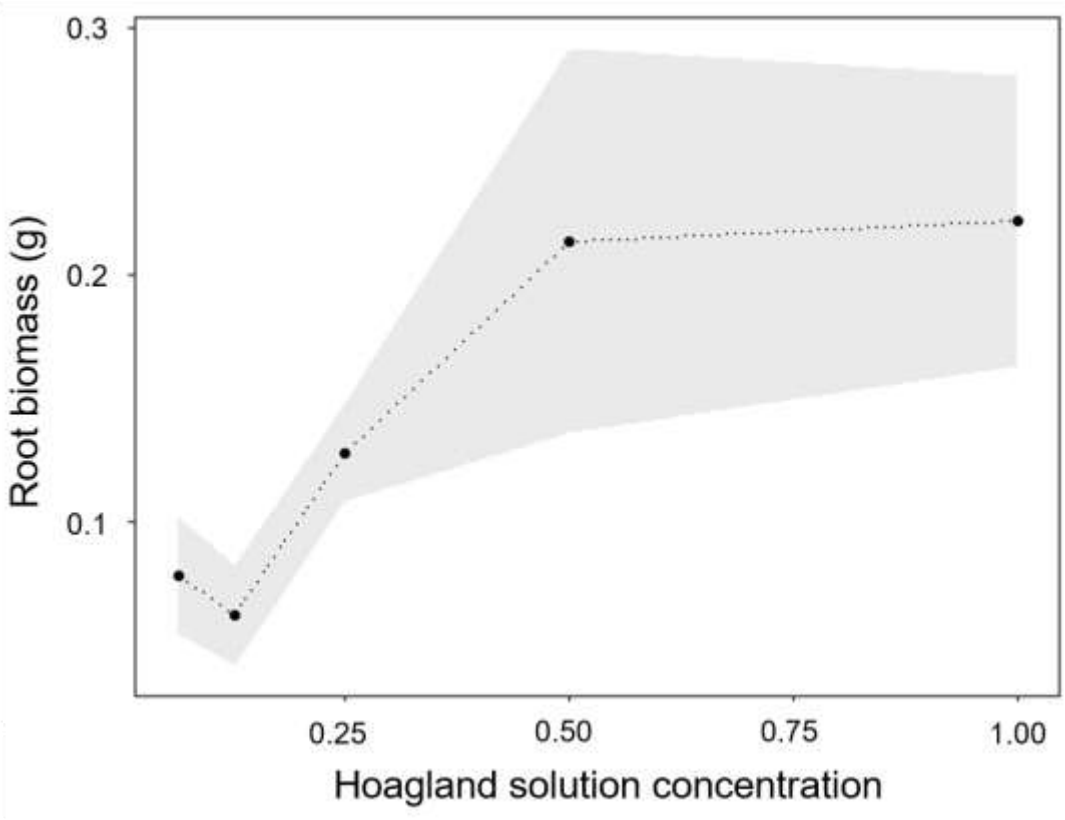
Galaxy ID	Observed *m/z*	Retention time	Benjamini-Yekutieli-corrected *p*-value	R2	Root	Stem	Leaf
NM859T341	859.0715	341	0.0049	0.29		↓	
NM860T324_1	860.0789	324	0.009	0.26		↓	
NM861T332_2	861.0832	332	0.0043	0.30		↓	
NM867T287	867.0680	287	0.0023	0.34		↓	
NM868T298_1	868.0767	298	0.0011	0.40		↓	↓
NM868T309_1	868.0769	309	0.00087	0.43		↓	↓
NM907T469_1	907.1185	469	0.0037	0.31		↓	↓
NM928T314	928.1064	314	0.0027	0.45			↓
NM934T315_1	934.0688	315	0.0034	0.31		↓	↓
NM934T327	934.0685	327	0.0041	0.30		↓	↓
NM937T308	937.0920	308	0.0019	0.35		↓	↓
NM937T332_1	937.0922	332	0.0012	0.39		↓	↓
NM939T364_1	939.1079	364	0.0041	0.30		↓	
NM939T381	939.1086	381	0.0011	0.41		↓	
NM961T364	961.0895	364	0.0049	0.29		↓	
PM225T299_2	225.1484	299	0.009	0.27			↓
PM225T361_1	224.5348	361	0.0019	0.35		↓	
PM226T483	226.1661	483	0.00068	0.28	↓	↓	↓
PM229T361	229.0017	361	0.0034	0.32		↓	
PM241T361_1	240.5097	361	0.0023	0.34		↓	
PM273T415	273.0755	421	0.0064	0.30			↓
PM277T319	277.0341	319	0.0018	0.36		↓	↓
PM287T457	287.0547	457	0.0056	0.19		↑	
PM303T303	303.0133	303	0.0087	0.26		↓	
PM303T310	303.0133	310	0.0041	0.30		↓	↓
PM303T315	303.0132	315	0.0084	0.27			↓
PM303T319	303.0133	319	0.0087	0.26		↓	↓
PM303T361	303.0133	361	0.0046	0.30		↓	
PM305T319_1	305.0652	319	0.0056	0.28		↓	
PM315T288	315.0708	288	0.0019	0.36		↓	
PM315T318	315.0708	318	0.0077	0.27		↓	
PM367T300	367.5282	300	0.0022	0.34		↓	↓
PM371T401	371.2061	401	0.0042	0.21	↓		
PM414T340	414.0340	340	0.00081	0.44		↓	
PM422T340	422.0204	340	0.00052	0.52		↓	
PM427T334_1	427.5587	334	0.0058	0.28		↓	↓
PM427T340_1	426.5588	340	0.00052	0.51		↓	
PM433T287	433.1678	287	0.0063	0.30			↓
PM433T457	433.1127	457	0.0091	0.17	↑		
PM435T415	435.1285	415	0.0066	0.29			↓
PM441T441	441.0790	441	0.00022	0.32	↑		
PM442T291	441.5260	291	0.0011	0.41		↓	↓

Supplementary Table C.1 continued

Galaxy ID	Observed *m/z*	Retention time	Benjamini-Yekutieli-corrected *p*-value	R2	Root	Stem	Leaf
PM442T303_1	441.5258	303	0.0015	0.38		↓	
PM447T322	447.1285	322	0.0019	0.35		↓	↓
PM449T306	449.0712	306	0.0035	0.31		↓	
PM453T304	453.0087	304	0.0087	0.27			↓
PM467T291	467.0820	291	0.0017	0.36		↓	↓
PM467T303	467.0819	303	0.0017	0.37		↓	
PM467T306	467.0819	306	0.0019	0.35		↓	
PM467T315	467.0819	315	0.0017	0.37		↓	↓
PM471T315	471.0193	315	0.0022	0.34		↓	↓
PM471T332	471.0193	332	0.003	0.32		↓	↓
PM483T364	483.0443	364	0.0087	0.26		↓	↓
PM485T261_1	485.1266	261	0.003	0.32		↓	
PM485T303	485.0922	303	0.0018	0.36		↓	↓
PM490T364	490.0391	364	0.0019	0.35		↓	
PM498T364	498.0254	364	0.0017	0.37		↓	
PM578T265	578.0562	265	0.0096	0.26			↓
PM578T270	578.0559	270	0.0029	0.39			↓
PM586T270	586.0422	270	0.0063	0.30			↓
PM592T302_1	591.5213	302	0.0052	0.29		↓	↓
PM615T293	615.0616	293	0.00078	0.46		↓	↓
PM617T303	617.0771	303	0.0019	0.35		↓	↓
PM617T319	617.0772	319	0.0017	0.36		↓	↓
PM619T300	619.0929	300	0.003	0.32		↓	↓
PM619T341	619.0929	341	0.00078	0.44		↓	
PM619T373	619.1369	373	0.0059	0.28		↓	
PM620T312	620.0963	312	0.00081	0.43		↓	↓
PM637T311_1	637.1036	311	0.00097	0.42		↓	↓
PM659T306	659.0852	306	0.0011	0.40		↓	
PM659T315	659.0852	315	0.00078	0.45		↓	↓
PM662T309	662.0466	309	0.0039	0.35			↓
PM737T282	737.0456	290	0.0065	0.30			↓
PM739T469	739.1141	469	0.0049	0.29		↓	↓
PM767T304	767.0725	304	0.0037	0.31		↓	↓
PM767T324	767.0725	324	0.0049	0.29		↓	↓
PM767T339	767.0725	339	0.0061	0.28		↓	↓
PM767T364	767.0720	364	0.008	0.27		↓	
PM769T303	769.0877	303	0.0032	0.32		↓	↓
PM769T319	769.0877	319	0.0053	0.29		↓	
PM769T331	769.0878	331	0.0013	0.39		↓	↓
PM771T334	771.1031	334	0.0022	0.34		↓	↓
PM771T364	771.1031	364	0.0031	0.32		↓	
PM771T381	771.1037	381	0.00087	0.43		↓	

Supplementary Table C.1 continued

Galaxy ID	Observed *m/z*	Retention time	Benjamini-Yekutieli-corrected *p*-value	R2	Root	Stem	Leaf
PM771T394	771.1037	394	0.0017	0.36		↓	↓
PM776T278_1	775.5857	278	0.0019	0.35		↓	
PM785T293	785.0830	293	0.0015	0.38		↓	↓
PM785T305	785.0832	305	0.0023	0.34		↓	↓
PM785T310	785.0829	310	0.00097	0.42		↓	↓
PM787T290	787.0986	290	0.0019	0.35		↓	↓
PM787T303	787.0981	303	0.0019	0.35		↓	↓
PM789T307	789.0648	307	0.0011	0.41		↓	↓
PM796T324	796.0677	324	0.003	0.32		↓	
PM797T308	797.0756	308	0.0017	0.37		↓	
PM797T339	796.5667	339	0.0058	0.28		↓	
PM798T316	798.0743	316	0.0075	0.28			↓
PM804T302	804.1251	302	0.0024	0.33		↓	
PM806T308_2	806.0677	308	0.0012	0.39		↓	↓
PM806T334	806.1405	334	0.0041	0.30		↓	↓
PM806T340	806.1410	340	0.00078	0.47		↓	
PM809T290	809.0801	290	0.0017	0.37		↓	↓
PM809T303	809.0798	303	0.0017	0.37		↓	
PM811T334	811.0962	334	0.0011	0.40		↓	↓
PM811T340	811.0961	340	0.0011	0.41		↓	
PM812T338	812.0426	338	0.0057	0.28		↓	
PM813T317	813.0501	317	0.0061	0.31			↓
PM833T340_1	832.5564	340	0.0043	0.30		↓	↓
PM923T401	923.1144	401	0.00064	0.49		↓	
PM931T468	931.1178	468	0.0046	0.30		↓	
PM958T363	958.1510	363	0.0017	0.37		↓	
PM961T331	961.0911	331	0.0035	0.31		↓	↓
PM963T364	963.1063	364	0.0029	0.32		↓	



Supplementary Figure C.1. Mean root biomass of hydroponically-cultivated fireweed in response to strength of Hoagland solution. Shaded cone around mean values indicates standard error.