Effects of the Enzyme Inhibitor Prohexadione-calcium on Hops Determined by LC-TOF-MS

By

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Abstract

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Humulus lupulus L. (hops) is an agricultural crop valued for its inflorescences, commonly known as hop cones, which produce a diverse collection of secondary metabolites. Hop cones are most valued for their terpenophenolic contents, which are essential to beer production, and the subject of biomedical research. We studied two hop cultivars, Willamette and Zeus, over five stages of development, which were characterized by detailed flower morphology, gross cone measurements, and phytochemical quantitation. By combining morphological observations with phytochemical quantitation we produced an index to inform our developmentally dependent experiments. In order to understand these developmental processes and in an attempt to induce agronomically positive effects, we perturbed hop morphological and phytochemical development using enzyme inhibition.

Prohexadione-calcium (Pro-Ca) is a known inhibitor of 2-oxoglutaric acid dependent dioxygenases present in the flavonoid, gibberellic acid, and ethylene biosynthetic pathways. We treated hops with Pro-Ca at each of the five time-points over two seasons; these time-points were later characterized as five developmental stages. Pro-Ca treatment induced significant increases in terpenophenolic content by 9.1-87.3%; however some treatments also induced significant decreases. Increases in cone biomass production by 1.5-19.6% were also measured in response to treatment in both seasons.

Induced changes in cone biomass production and terpenophenolic accumulation were most dependent on cultivar and the developmental stage at which plants were treated.

In a second series of experiments we conducted a targeted analysis of phenolic acids, flavonoids, and terpenophenolics over 22 days following a single Pro-Ca treatment conducted during early flowering. Terpenophenolics significantly increased following treatment, and coincided with changes in the flavonoid biosynthetic pathway including accumulation of metabolic precursors upstream from flavanone-3-hydroxylase, and decreases in flavonoid products downstream from flavanone-3-hydroxylase. In addition to changes in known compounds, marker analysis revealed the presence of two markers in treated samples not previously reported from hops. One of these markers has been tentatively identified as the antimicrobial compound luteoliflavan.

This research provides insight into the relationship between secondary metabolic pathways in hops and indicates targets for future research into perturbation of metabolic pathways to increase medicinal and flavor compounds in hops.

Acknowledgements

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I would like to thank my dissertation committee members including Drs. Amy Litt, Akira Kawamura, and Dwight Kincaid. Dr. Litt gave me extensive help with the morphological observations and descriptions presented in this work; I also thank her for reminding us in the phytochemical lab to take a step back to remember that we work on plant material that contains complex tissues which get more intricate the closer you observe. Dr. Kawamura has given significant insight into the phytochemical methods and provided critical feedback in several phases of this project. Dr. Kincaid taught me valuable statistical tools, advised me on different ways of analyzing data, and helped with experimental designs. Dr. Kincaid is a seasoned professor and it is always fun and informative to sit and talk about science and the real world.

It has been a pleasure to be part of the phytochemistry laboratory at Lehman College and I look forward to life long collaborations. A diverse group of scientists have worked in the lab over the years and their collaborative effort has created a productive

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I am indebted to my parents, Sandy and Mort, who have never yielded in their unconditional love and support during the challenging moments that have arisen in this pursuit. I am also grateful to my loving family, Jonathan, Nathalie, Dana, Jordan, Emily, and Justin, who have cheered me on the entire way. I also am very thankful to my best friend and partner, Kristen, for her balanced insight and encouragement, not to mention her training in the use of pivot tables, which were a major time- and life-saver.

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This work has been presented in poster form at the 50th Annual Meeting of the American Society of Pharmacognosy in Waikiki, HI and the 2nd Annual *Humulus* Symposium in Ghent, Belgium. I have had several opportunities to present the results of this work to the scientific community by giving oral presentations at The 51st Annual meeting of the American Society of Pharmacognosy in St. Petersberg, FL., Fairleigh Dickinson University in Teaneck, NJ, Minzu University in Beijing, China, and The Institute of Chinese Medicine in Hong Kong.

Preliminary studies that have been used to inform the experiments presented in this work have been published in *Acta Horticulturae*; this preliminary work also resulted in a patent application submitted to the World Intellectual Property Organization. **Chapter 2** of this dissertation has been accepted for publication in the *Journal of Agricultural and Food Chemistry*. **Chapter 3** has also been submitted for publication, and **Chapter 4** is in preparation to be submitted for publication.

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Chapter 1: Introduction

1.1 Purpose

The purpose of this work was two-fold: (1) To understand the morphological and phytochemical progression of hop cones over late stages of development, when biomedically- and industrially- relevant compounds accumulate, and (2) to gain insight into these processes using prohexadione-calcium (Pro-Ca) to inhibit enzymes in pathways fundamental to phytochemical and morphological development with hopes of inducing agronomically favorable outcomes. Pro-Ca treatment has had agronomically positive effects on the morphology and biochemistry of a number of crops, which made Pro-Ca studies on hops desirable.

Three data-driven chapters are preceded by an introduction and followed by a conclusion. In **Chapter 1** I provide background information on the phytochemistry and morphology of hops, and the effects of Pro-Ca on the biochemistry and morphology of other agricultural species. **Chapter 2** describes our morphological and phytochemical characterization of five developmental stages of hop cones from two varieties. In **Chapter 3** I discuss our more agronomically focused experiments in which we treated hop plants with Pro-Ca at each of the five developmental stages outlined in **Chapter 2**. Then in **Chapter 4** I discuss experiments we conducted to assess the developmental effects of Pro-Ca treatment on hop polyphenolic and terpenophenolic pathways. Finally, in **Chapter 5** I present future directions indicated by these results.

1.2 *Humulus lupulus* L.

Humulus lupulus (L.), the common hop, is a dioecious perennial vine native to the temperate zone of the northern hemisphere. The genus *Humulus* is a member of the Cannabaceae family and contains three species. The other genus in the family, *Cannabis*, is well known for the psychoactive properties of its flowers, and has been used as a source of fiber in several cultures. Although the Cannabaceae form a small group, containing up to six species, plants in this family have been used for food, medicine, and other daily needs for thousands of years.

1.2.1 History

The hop plant has long been valued for its inflorescences, which form a strobilus, commonly referred to as a "hop cone." First documented evidence describing the cultivation of hops dates back to 736 AD in reference to a Slovenian man tending to his hop garden (*1*). This proposed origin of hop farming is supported by additional documentation of cultivation from the 9th-12th centuries when hops were grown in Bohemia, Slovenia, and Bavaria (*2*). By the 1100s small-scale hop farming was common throughout Eastern Europe. Hop cultivation continued to grow as it moved further west where farmers bred some of the first aroma varieties still popular today. These quality lines were bred in order to supply the expanding commercial production that was in full swing by the mid 1800's (*3*).

Hops were originally cultivated as a medicine for the treatment of fever, jaundice, ringworm, and insomnia (*4-6*). Hops were used as a diuretic, and considered to cleanse the blood of toxins, specifically the liver and spleen (*2*). The most common historical medicinal use of hops was to treat insomnia. Plants were often grown outside of bedroom

windows, and pillows were filled with hops cones to aid in sleeping, although the efficacy of the sedative effects of hops remains questionable (*6*).

Hops were originally used in beer brewing in the 12th century by German monks (*7*). When hop cones were added to the brewing kettle, conditions remained aseptic and beer batches did not spoil. In the fermentation stage the mixture contains low alcohol content in conjunction with high sugar levels; this environment is highly susceptible to microbial proliferation. Prior to the use of hops, various combinations of herbs, collectively known as gruit, were used to control contamination (*2, 7*). The addition of hop cones proved far superior in keeping the beer kettle sanitary, and the use of gruit was soon discontinued. Initially the addition of hops to beer was ill received due to the bitter flavors the new ingredient imparted on the beverage. Over the past hundred years, hops became an essential ingredient in beer making and, the advance of knowledge in hop chemistry and traditional breeding produced cultivars with more favorable flavor characteristics (*8*).

1.2.2 Morphology

Most herbaceous vines climb by means of tendrils whereas hops climb by sharp bifid trichomes present on the stem and abaxial surface of the leaf. Hops are vigorous climbing plants, twining around any available trellis in a clockwise rotation. While ill in bed, Darwin noted that the tip of a hop vine made an entire revolution around its trellis in just a few hours (*2*).

In the early spring, young shoots break ground and grow long and slim, containing only few nodes, twining in search of structure to climb. Young shoots sprout from modified underground stems, which store carbohydrates through the temperate

winter (*9*). Leaves emerge from large protective stipules often purple in the early spring, as they absorb UV light in protection of young leaves. The morphology of the leaves is highly variable. Leaf shapes range from chordate to palmate, containing 3-7 lobes in which the degree of dissection is highly variable even among branches of the same plant; leaves are found in an opposite arrangement to the shoot.

Hops are short day plants that begin to flower by middle to late summer. Floral development begins with the production of small swollen bracts that arise in the axils of leaves towards the apical portions of each stem (*9*). These bracts subtend an inflorescence stalk bearing several partial inflorescences. Each partial inflorescence, referred to as a strobilus, contains multiple flowers arranged on a central axis, commonly referred to as a strig. Each node of the strig produces a single bract that subtends a pair of bracteoles each enclosing a reduced perianth and a gynecium composed of two fused carpels with bifid stigmas (*10*). The ovary is encased in a single cup-shaped vestigial perianth that is likely composed of modified sepals, as sepals are opposite the stamens in male flowers. The perianth originates at the base of the ovary, then grows to envelop the ovary at the base of the flower and remains persistent on the developed achene in the pollinated female flower (*11*).

The bracts and bracteoles of each strobilus open and a plume of white stigmas emerge. Hops are valued for their unpollinated female flowers. The pollination and resulting production of an achene can produce additional oils that are disliked among brewers; therefore hop fields consist of female clones. Unpollinated stigmas senesce as the bracts and subtending bracteoles become conspicuous, elongating to produce a mature

hop cone used for beer production. The strobilus, or hop cone, contains 36-48 flowers (*11*). A mature cone is green in color as bracts and bracteoles are photosynthetic.

Peltate sessile glandular trichomes, commonly known as lupulin glands, are present on the perianth and the abaxial surface of the base of the bracts. Lupulin glands contain an abundance of terpenophenolics and essential oils. These terpenophenolics have become a focus in biomedical research and are an essential source of bittering compounds in hops used as a flavor ingredient for the brewing industry.

1.2.3 Chemistry

A diverse collection of secondary metabolites has been identified from hop cones (*4*). The most biologically and economically interesting hop compounds are the polyketide-derived constituents highly valued for their medicinal and flavor properties. These compounds include terpenophenolics, which are produced in the glandular trichomes, and polyphenols, including flavonoids and phenolic acids, produced in vegetative tissues including leaves, bracts, and bracteoles.

Polyketide-based secondary metabolites from hops: Polyketides are a structurally diverse group of secondary metabolites derived from a few basic building blocks. These basic building blocks are small carbon chains with one or more ketone groups, and contain a coenzyme-A functional group attached to a terminal carbon. Polyketides are synthesized by three stepwise de-carboxylation condensation reactions between these units, which are catalyzed by large enzyme complexes collectively known as type three polyketide synthases (PKS IIIs) (*12*). The characterization of two hop PKS III enzymes has provided an understanding of the proximal relationship among diverse secondary metabolites, some of which are unique to hops. Valerophenone synthase (VPS) and chalcone synthase I (*chs*-

H1) catalyze the production of polyketides that provides the building blocks for the synthesis of terpenophenolics and polyphenols (*13, 14*).

Terpenophenolics: Terpenophenolics are a biologically active class of compounds present in a small number of unrelated plant families (*15*). Hops contain two main classes of terpenophenolics, both of which are produced via a combination of the polyketide and dimethylallyl pyrophosphate (DMAPP) biosynthetic pathways (*16*). Initially, terpenophenolics are synthesized via PKS IIIs forming a base molecule, which is then modified by the addition of five-carbon isoprenoid-derived prenyl groups. The addition of a prenyl group is commonly referred to as "prenylation" and is catalyzed by specialized prenyl-transferase enzymes (*17*). The two main classes of terpenophenolics found in hops, prenylated acylphloroglucinols and prenylflavonoids, each originate from distinct polyketide precursors.

Prenylated acylphloroglucinols: Two main classes of prenylated acylphloroglucinols are found in hops: humulones, commonly referred to as α-acids, and lupulones, commonly known as β-acids (**Figure 1.1**). The α-acids are di-prenylated compounds well known for their bittering flavor properties. The β-acids are tri-prenylated

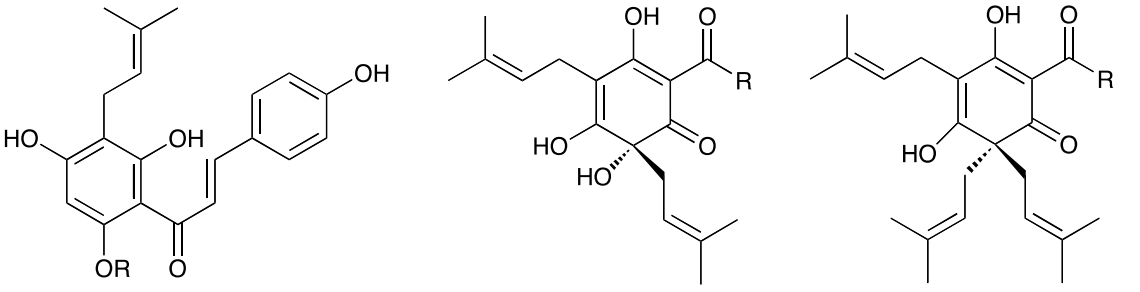


Figure 1.1 Prenylflavonoids and prenylated acylphloroglucinols from hops

and have significant antimicrobial activity. These compounds are synthesized from the catabolic products of the branched chain amino acids valine and leucine. These catabolic pathways produce isovaleryl-coenzyme A and isobutyryl-coenzyme A which are converted to phloroisovalerophenone and phloroisobutyrophenone, respectively, by three condensation reactions catalyzed by VPS (**Figure 1.2**) (*13*). Phloroisovalerophenone and phloroisobutyrophenone are then either di-prenylated to produce cohumulone and humulone, or tri-prenylated to produce colupulone and lupulone, respectively. *In situ* experiments have shown VPS activity in glandular trichomes (*18*). It is unknown whether the amino acid degradation products, substrates of VPS, are synthesized in the glandular trichomes or if they are imported via the phloem or plasmodesmata, which is a common occurrence in some plant tissues (*19*). In other glandular trichome systems, such as *Mentha* and tobacco, it is suggested that glandular trichome metabolism is isolated and independent from other tissues (*20*). Localization of amino acid degradation products, substrates of VPS, is relevant to our work and is a topic of future investigations.

Biomedical applications of prenylated acylphloroglucinols are a more recent topic of interest (*21, 22*). *In vitro* experiments have shown humulone to be effective at inducing apoptosis in the human leukemia cell line HL-60 and the human metastatic colon carcinoma-derived cell line SW620 at low μM concentrations (*23, 24*). Lupulones have also displayed biological activity against gingivitis and plaque buildup; hops have been patented for use in toothpaste and a hop-based mouthwash has been proposed (*25-28*). Rho-iso-α-acids, and tetrahydro-iso-α-acids, products of isomerization and hydrogenation of naturally occurring α-acids, have shown strong anti-inflamatory activity *in vitro*. These compounds were found to inhibit cyclooxygenase-2 (COX-2) protein expression in macrophages and NF-$_K$B nuclear translocation and abundance, and have shown selective inhibition of inflammatory signal transduction (*21*).

Prenylflavonoids: A second class of biomedically active terpenophenolics found in hops are the prenylflavonoids (**Figure 1.1**). These compounds are produced by the prenylation of chalcone naringenin, which is a product of the phenylpropanoid and

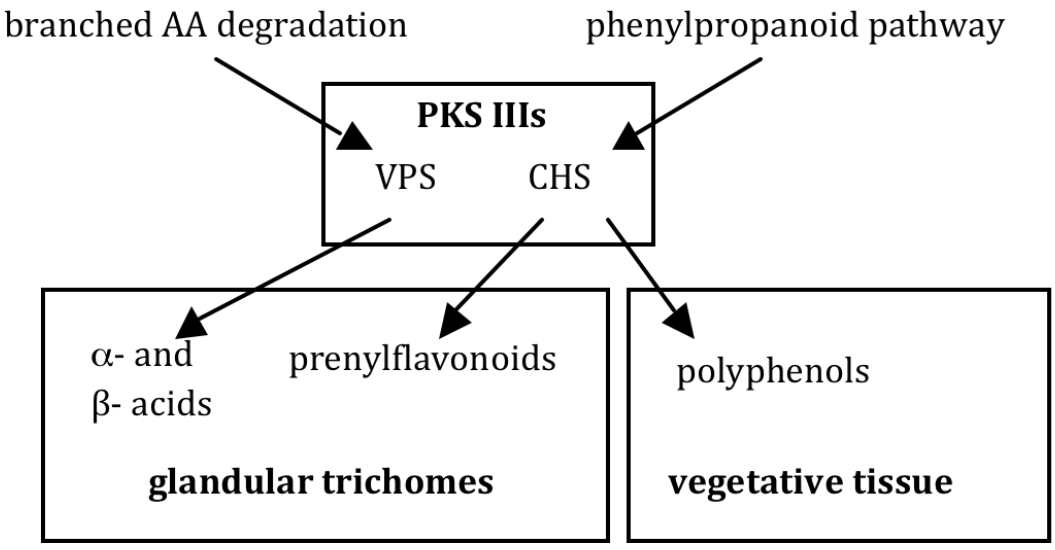


Figure 1.2 PKS IIIs and localization of terpenophenolic and polyphenolic products

phenolic acid pathways. In the phenylpropanoid pathway, phenylalanine is converted to *p*-coumaroyl-CoA over a series of three reactions. *chs*-H1 then catalyzes the three-step condensation of three malonyl-coenzyme A molecules with one *p*-coumaroyl-CoA, to produce chalcone-naringenin; this represents the first committed step in prenylflavonoid biosynthesis in glandular trichomes (**Figure 1.2 and 1.3**) (*17, 29*). Chalcone-naringenin is then prenylated by an aromatic prenyltransferase enzyme, which catalyzes the addition of a 5-carbon prenyl group to carbon 6, producing desmethylxanthohumol. Desmethylxanthohumol is then methylated by *O*-methyl transferase I (OMT-1) to produce xanthohumol (*16*). A series of isomers are also found in small amounts in hops including 8-prenylnaringenin and 6-prenylnaringenin. Additionally several isomers of desmethylxanthohumol and xanthohumol have been found in hops, although they are present at very low concentrations (*4*).

Prenylflavonoids are a biologically active group of compounds and have been a focus of *in vitro* and *in vivo* experiments; some prenylflavonoids have advanced to nutraceutical development and human clinical trials. Xanthohumol is the principle prenylflavonoids in hop cones, where it is present at 1-2% by dry weight; this compound has been characterized as a broad range anti-carcinogenic agent with potent anti-inflammatory properties (*30, 31*). Xanthohumol was found to inhibit proliferation at each of the three cellular stages of carcinogenesis known as initiation, promotion, and progression. Phase 1- and 2- enzymes that play key roles in carcinogen metabolism are inhibited by xanthohumol, as are cyclooxygenases Cox-1 and Cox-2 (*32-34*). In addition, xanthohumol has inhibited human DNA polymerase *in vitro*; these results sparked interest

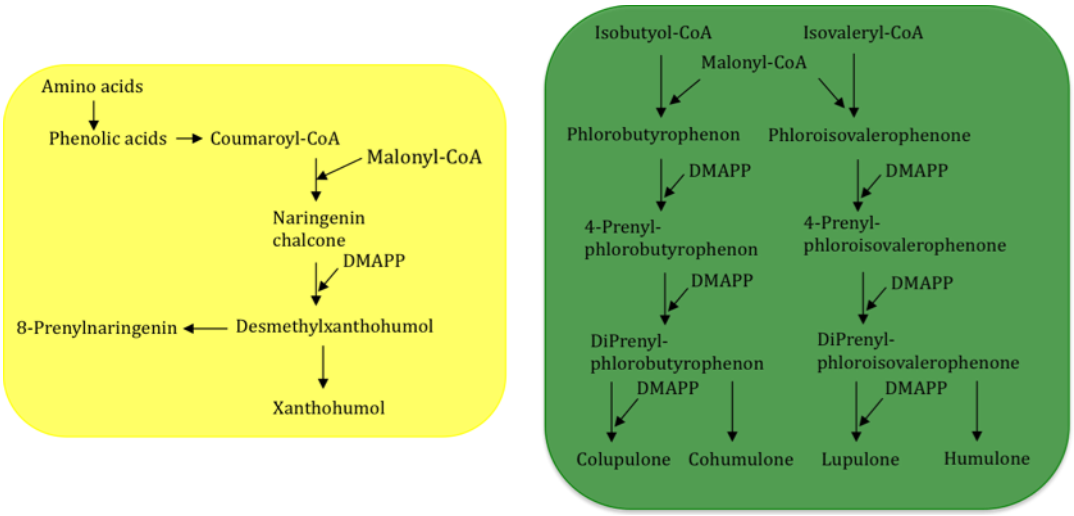


Figure 1.3 Biosynthetic pathways for terpenophenolics in hop glandular trichomes, including prenylflavonoids (yellow) and prenylated acylphloroglucinols (green)

in additional research investigating effects on human cancer cell lines (*33*, *34*). It has also recently been shown that xanthohumol induces apoptosis in human prostate cell lines (*32*), although the precise mechanisms by which xanthohumol works on these systems are only beginning to be elucidated (*30*).

Estrogen receptor-positive breast cancer cell line MCF-7, ovarian cancer cell line A-2780, and HCT116-derived colon cancer cell line 40-16 were exposed to *in vitro* xanthohumol treatments for 2-4 days. All cancer lines either underwent apoptosis or cell cycle arrest when xanthohumol was added, with IC_{50} values of less than 6 µM (*33*). Additional *in vitro* experiments have displayed xanthohumol anti-malarial and anti-HIV1 activities (*35*, *36*).

Present in trace amounts (0.1% dry hop cone weight), 8-prenylnaringenin is a prenylated chalcone unique to hops, and is the most potent phytoestrogen known to date (*17*). 8-Prenylnaringenin, and related compounds, have gained interest for use in treating menopausal symptoms, as current hormone replacement therapy treatments are unpopular

due to medical complications. 8-Prenylnaringenin, and related prenylflavonoids are currently in phase-2 clinical trials for their effects on a variety of menopausal symptoms including hot flashes, depression, and insomnia (*37*, *38*).

In vitro studies have focused on the metabolism of isoxanthohumol, the isomer of xanthohumol, and the most abundant prenylflavonoid found in beer. Using the simulator of the human intestinal microbial ecosystem (SHIME) assay, prenylflavonoid metabolism was studied in human-replicate systems (*39*). Further *in vivo* human clinical studies confirmed isoxanthohumol to be metabolized by a P450 enzyme in the human digestive pathway (**Figure 1.4**) (*39-41*). Due to human intestinal conversion, up to 80% of isoxanthohumol is converted to 8-prenylnaringenin; the functionally-equivalent 8-

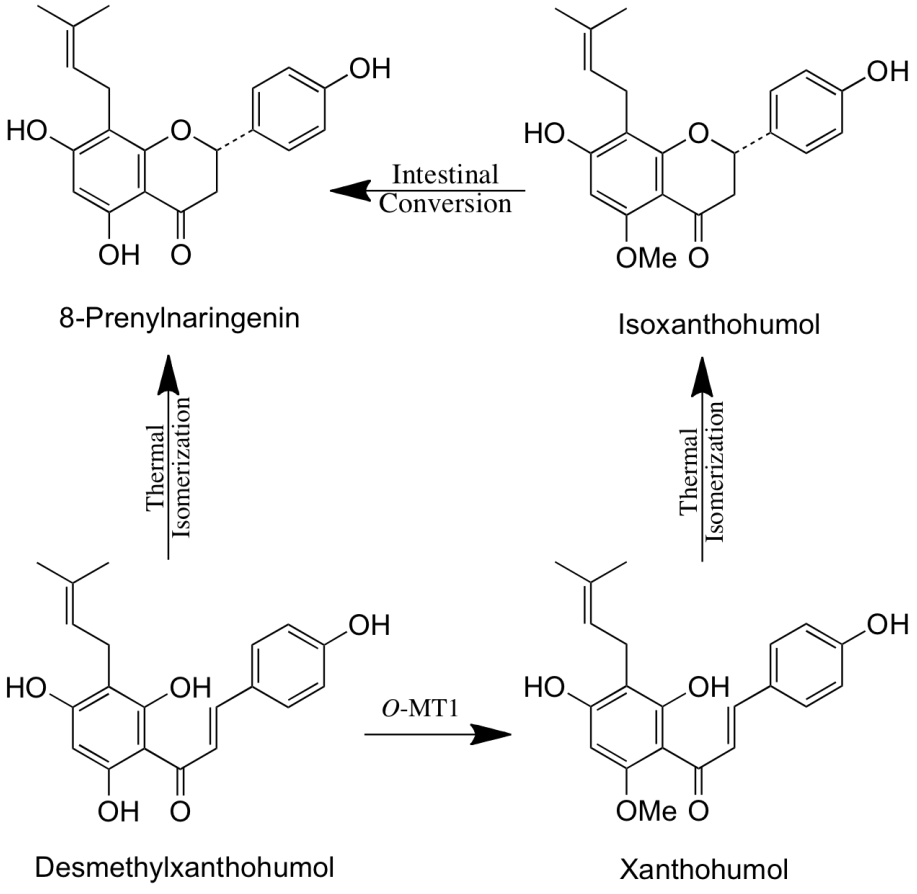


Figure 1.4 Interconversion of hop prenylflavonoids

prenylnaringenin dosage from beer is over 10-fold that from 8-prenylnaringenin alone. Clinical trials are underway to further investigate a potential 8-prenylnaringenin and isoxanthohumol product for use in women's health (*37, 40*).

Polyphenols: Polyphenols are a group of hydroxylated aromatic compounds that have received significant interest from phytochemical, nutraceutical, and nutritional industries for their antioxidant and anti-inflamatory properties (*42*). These compounds are also produced by PKS IIIs, and are produced from chalcone precursors. In polyphenol biosynthesis, chalcones are isomerized to form flavanones by a group of enzymes known as chalcone isomerases (CHI). Further modification of these compounds most commonly includes reduction of carbonyls to form hydroxyl functional groups, glycosylation, and polymerization (**Figure 1.5**). The most common polyphenols found in hops are flavan-3-ols, flavonols, flavonol glycosides, and polymeric procyanidins (**Figure 1.6**) (*43, 44*). The biosynthesis of these compounds is localized in the leaves, and the bracts and bracteoles of the inflorescence; polyphenols have not been reported in hop glandular trichomes (*18*).

Polyphenols are of growing interest to brewers as they impart unique and complex flavors to beer (*45*). The antioxidant properties of polyphenols also keep beer from accumulating carbonyls, which impart a stale flavor; polyphenol antioxidant activity increases beer stability, which increases shelf life. However, excessive concentrations of polyphenols can form a colloidal haze in beer as these compounds adhere to proteins and form insoluble complexes; this also results in a reduction of the flavor stability of beer (*46*). Hop polyphenol extracts are also of interest for their bioactivity and have shown antiallergic effects *in vivo (47, 48)*. To further investigate the relationships between terpenophenolic and polyphenolic biosynthetic pathways in hops, and investigate the agronomic effects of enzyme inhibition, we treated hop plants with prohexadione-

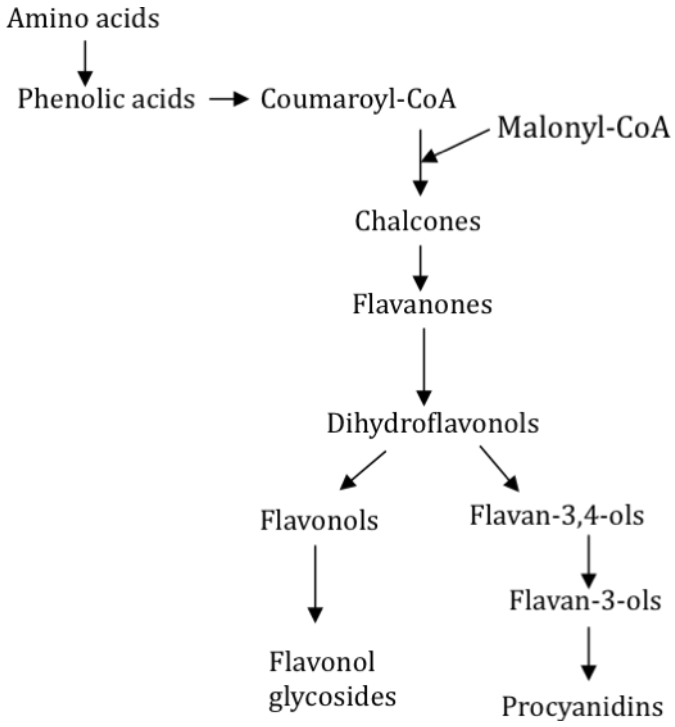


Figure 1.5 General phenolic acid and flavonoid biosynthetic pathways.

calcium and measured the morphological and phytochemical responses of total plants and cones.

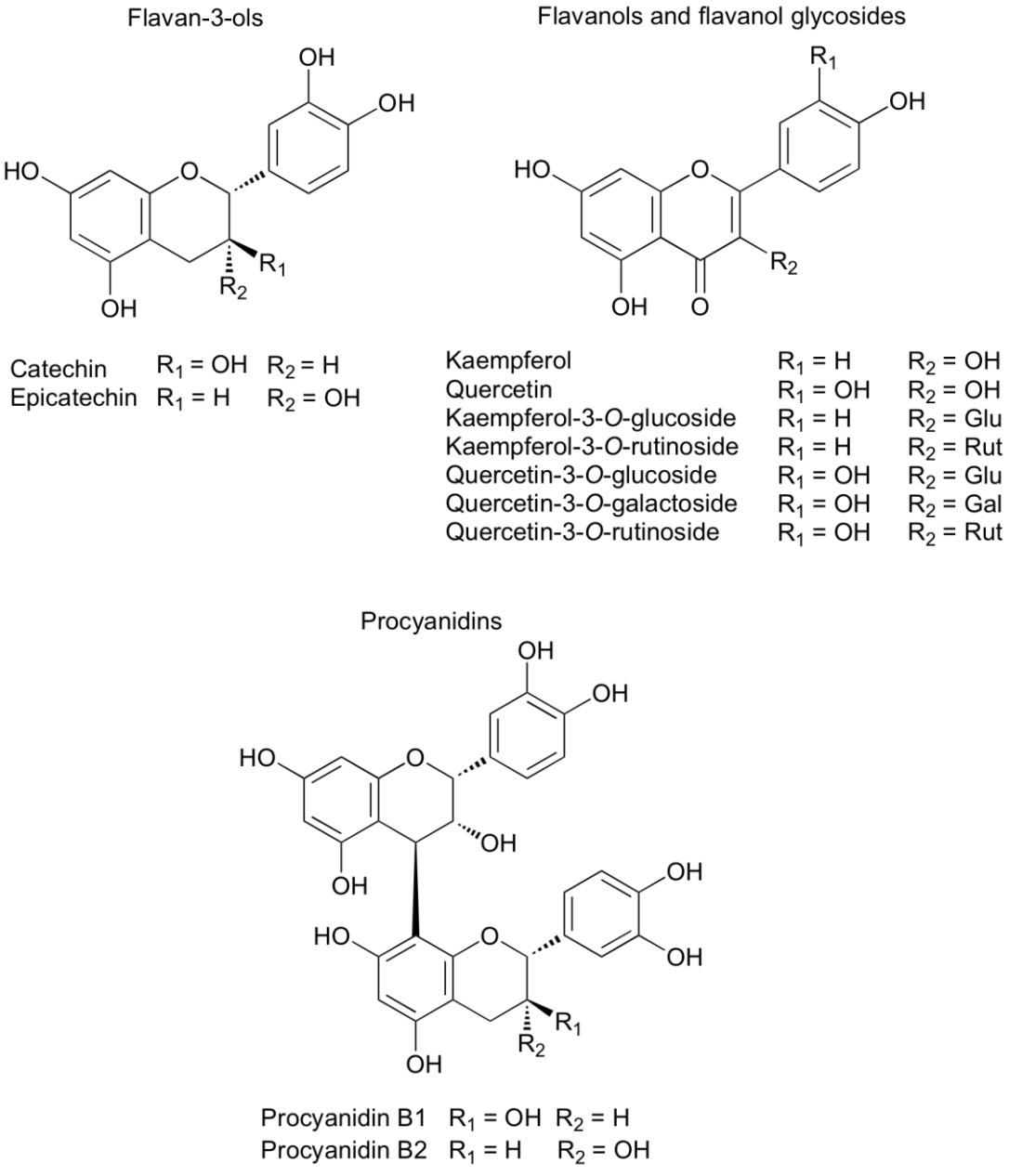


Figure 1.6 Common polyphenols in hops

1.3 Prohexadione-calcium

Prohexadione-calcium (calcium 3-hydroxy-5-oxo-4-propionyl-cyclohex-3-enecarboxylate, Pro-Ca) is one of a number of growth inhibitors in the class of acylcyclohexanediones. Acylcyclohexanediones are most recognized for their ability to mimic ascorbic and 2-oxoglutaric acids, two co-substrates of 2-oxoglutaric acid dependent dioxygenases (2-ODDs) (**Figure 1.7**); this co-substrate mimicry has shown inhibitory effects on enzymes critical for the production of active gibberellins, ethylene, and several classes of flavonoids (**Table 1.1**) (*49*).

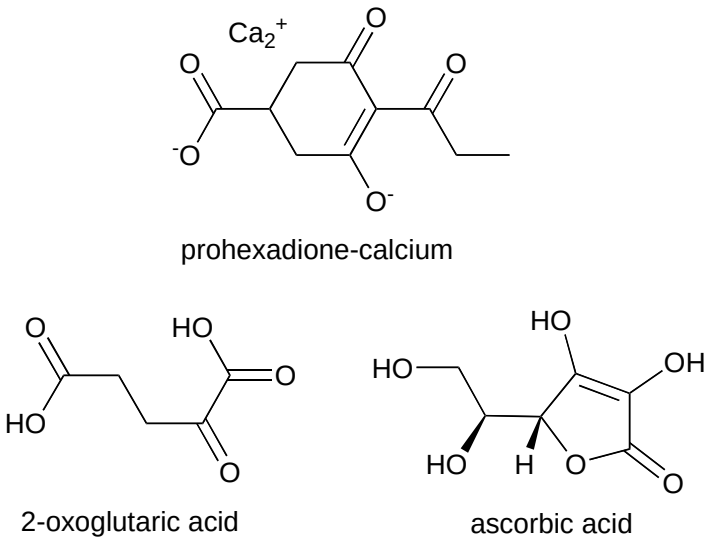


Figure 1.7 Prohexadione-calcium mimics 2-oxoglutaric and ascorbic acids

Table 1.1 Effects of Pro-Ca on several enzymes in the flavanoid, gibberellic acid, and ethylene biosynthetic pathways

Enzyme	Abr.	Catalyzed reaction	Effects of Pro-Ca
flavanone-3β-hydroxylase	F3H	flavanones → dihydroflavanones	changes in flavonoid profiles (52,*62*)
gibberellin 3β-hydroxylase	GA3ox	$GA_{20} \rightarrow GA_1$	decrease in active gibberellins and stem elongation (*49, 59*)
gibberellin 2β-hydroxylase	GA2ox	$GA_1 \rightarrow GA_8$	increase in active gibberellins and stem elongation (*66*)
1-aminocyclopropane-1-carboxylate oxidase	ACCO	ACC → ethylene	decreases production of ethylene and increase in fruit yield. (*51*)

Pro-Ca was first tested on fruit trees as a growth inhibitor by BASF in the early 1990s. In 2000 BASF released two wettable granular forms of Pro-Ca for application on pome fruits, these products were sold as APOGEE® and REGALIS® and contain 27.5% and 10% Pro-Ca, respectively (*50*).

The active ingredient in APOGEE® and REGALIS® is the free acid prohexadione, which is an unstable form of the compound. A stable calcium salt is used and the free acid form of prohexadione is generated when the product is mixed in aqueous solution. Each product contains approximately 60% ammonium sulfate ($H_8N_2SO_4$). Upon the addition of water the calcium from the Pro-Ca salt binds to sulfate as calcium sulfate ($CaSO_4$) and the free acid prohexadione is released; this non-dissociated form of prohexadione penetrates the cell membrane. The pKa of prohexadione is 5.15; therefore the non-dissociated free acid is present in a higher concentration at a pH lower than this value. After entering the cell the free acid dissociates in the slightly alkaline environment of the cytosol. This dissociation hinders the ability of prohexadione to leave the cell, forcing the cell to metabolize the compound (*49*).

Pro-Ca is considered a "reduced risk" pesticide by the Environmental Protection Agency due to its low mammalian toxicity, benign ecotoxicological profile, and low potential for crop residues (*50*). Due to rapid photodegradation and efficient microbial and plant metabolism, the biological half life of Pro-Ca in bacteria is 12 hours, and in plants 10-14 days (*50*). The short half-life necessitates additional treatments in order to provide effective growth inhibition. Pro-Ca is usually applied at two time points during early growth; it is most effective when applied during the first 30 days after flower production during which time 75% of vegetative growth occurs in pome fruit trees.

Therefore, Pro-Ca is most effective when applied to apples when less than 10 cm of new shoot growth has occurred; this usually correlates with flower petal senescence (*51*).

In 1999 and 2000, 57 European studies were conducted on 14 apple cultivars to assess the efficacy and safety of Pro-Ca use. Several of these studies also looked at the differences between single and double treatments. A single treatment of 250 g ha^{-1} provided the same results as two treatments of 125 g ha^{-1}. Both treatments led to a 40% reduction in shoot growth (*51*). While the results of these studies suggest a similarity between single and split treatments it is often the preference of farmers to conduct two treatments in order to control late season flushes of shoot growth, a possible outcome of climactic variation (*49*).

1.3.1 Phytochemical effects of Pro-Ca

In response to Pro-Ca treatment, apple leaves produced novel antimicrobial and antiherbivory compounds; luteoforol, a flavan-4-ol, and the 3-deoxycatechin luteoliflavan (*52*). Both compounds are previously unknown to occur in the Roseaceae family, of which apples are a member. In addition to antimicrobials, the flavanone glycoside eriodictyol-7-*O*-glucoside and upstream phenylpropanoid derivative 6″-*O*-trans-p-coumaroyl-eriodictyol-3′-*O*-glucoside, previously unknown in apples, accumulated in response to treatment (**Figure 1.8**) (*53-55*). The production of these compounds coincided with a decrease in the production of flavonols glycosides of quercetin and kaempferol, and procyanidins B1, B3, and B5, following treatment with Pro-Ca (*55*). Concurrently, metabolites found biosynthetically upstream from the flavonoid pathway, including chlorogenic and *p*-coumaric acids, accumulated in treated plants (**Figure 1.8**) (*54, 55*). Similar chemical effects were induced by Pro-Ca in several agronomic species

including grapevine (*Vitis vinifera*), strawberry (*Fragaria x ananassa*), cranberry (*Vaccinium macrocarpon*), peach (*Prunus persica*), and kiwi fruit (*Actinidia* sp.) (*56*).

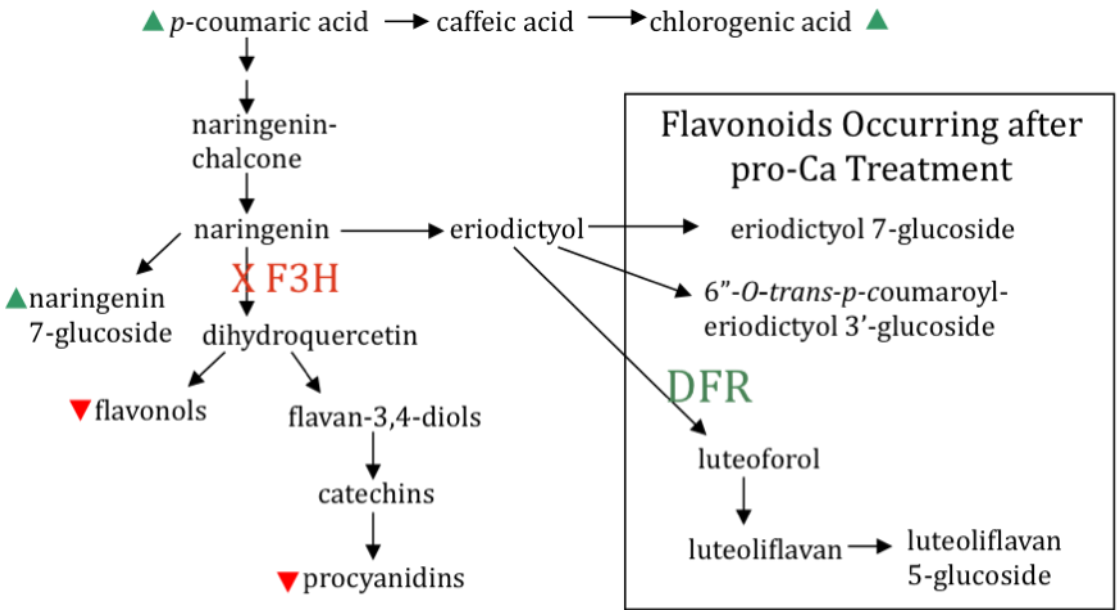


Figure 1.8 Pro-Ca effects on flavonoids and phenolic acids. Adapted from Roemmelt *et. al.* (*52*) ▲-increase in level of compound, ▼- decrease in level of compound

Following Pro-Ca treatment, several agronomic species displayed significant increases in resistance to common and sometimes devastating microbial diseases (**Table 1.2**) (*54*). Pro-Ca treatment also reduced the occurrence of predatory insects, including several species of aphid, common pests of apples and hops, by 40-60% (*51*). Additional experiments confirmed luteoforol to be the source of fungal and bacterial resistance; *in vitro* assays displayed the inhibition of a variety of microbial diseases using luteoforol at concentrations as low as 0.1-1 mM (*57*). Luteoliflavan, a stable product of luteoforol produced *in planta* following Pro-Ca treatment, was later found to be a potent insect growth inhibitor (*58*). Luteoforol was also shown to significantly inhibit growth of the

human pathogenic bacteria *Streptococcus sorbinus* and may have future therapeutic applications (*55*, *57*).

Table 1.2 Disease resistance induced by Pro-Ca treatment (*54*)

Host Plant	Pathogen
Malus x *domestica* (Apple)	*Erwinia amylovora* (Fire blight)
Pyrus communis (Pear)	*Erwinia amylovora* (Fire blight)
Malus x *domestica* (Apple)	*Venturia inaequalis* (Scab)
Malus x *domestica* (Apple)	*Podosphaera leucotricha* (Apple powdery mildew)
Prunus persica (Peach)	*Sphaerotheca pannosa* (Peach powdery mildew)
Rosa sp. (Rose)	*Diplocarpon rosae* (Black spot)
Vitis vinifera (Grape vine)	*Botrytis cinerea* (Grey mould)
Vitis vinifera (Grape vine)	*Plasmopara viticola* (Downy mildew)
Solanum lycopersicum (tomato)	*Ralstonia solanacearum* (Bacterial wilt)
Solanum lycopersicum (tomato)	*Xanthomonas vesicatoria* (Bacterial spot)
Solanum lycopersicum (tomato)	*Pseudomonas syringae* pv. *Tomato* (Bacterial speck)

1.3.2 Morphological effects of Pro-Ca

Pro-Ca was originally developed as a growth inhibitor and has been primarily used to control shoot elongation in fruit trees. Pro-Ca treatment replaces extensive labor requirements of tree pruning and avoids the loss of assimilates from the plant, an

inevitable effect of pruning. Treatment with Pro-Ca decreases shoot internode length and creates a tree of favorable architecture for fruit production and development (*51, 59*).

In addition to effective growth inhibition Pro-Ca treatment also increases fruit yield in specific genotypes; the Golden Delicious apple variety produced a 15% increase in yield when treated with a single application of 175 ppm Pro-Ca (*49*). An increase in fruit quality has also been observed in response to treatment of apple trees with Pro-Ca; this is due to the increased light exposure from decreased canopy growth, which can help favorably ripen and color the fruit (*49*). Yield increases, however, were found to be highly genotypically specific and seasonably variable (*49*).

1.3.3 Biochemical effects of Pro-Ca treatment

Pro-Ca is a plant dioxygenase inhibitor. Plant dioxygenases are nonheme, iron-containing enzymes, and are co-substrate dependent (*60, 61*). This family of enzymes has the capacity to catalyze diverse types of reactions by incorporating both oxygen atoms from O_2 in the reduction of the substrate. Through this mechanism dioxygenases can catalyze hydroxylation, epoxidation, and desaturation reactions, depending on the substrate (*60*). 2-Oxoglutaric acid-dependent dioxygenases (2-ODDs) are found in several locations throughout primary and secondary metabolic pathways. The widespread presence, and promiscuous character of this family of enzymes, presents a challenge to study the effects of a 2-ODD specific inhibitor as each 2-ODD differs in sensitivity to Pro-Ca treatment.

1.3.4 Pro-Ca effects on flavonoids

Flavonone-3-hydroxylase (F3H) is a 2-ODD located at a major branching point in the flavonoid pathway (**Figure 1.8**). F3H catalyzes the stereospecific 3β-hydroxylation

of flavanones to produce dihydroflavonols. Common substrates of F3H include naringenin and eriodictyol, which are converted to dihydrokaempferol and dihydroquercetin, respectively. These dihydroflavonols are critical substrates for the production of catechins, procyanidins, and anthocyanins. In response to Pro-Ca treatment, F3H inhibition occurred and caused a decrease in levels of flavonols and proanthocyanidins; concurrently F3H inhibition resulted in the accumulation of metabolites biosynthetically upstream from F3H, including phenolic acids chlorogenic and *p*-coumaric acids, as well as flavanones eriodictyol, eriodictyol 7-*O*-glycoside, and naringenin 7-*O*-glycosides (**Figure 1.8**) (*52, 55*). *In vitro* experiments using purified enzymes from both pear and apple leaves confirmed Pro-Ca to be a strong inhibitor of F3H, with an IC_{50} value of 23 mM (*62*).

1.3.5 DFR and substrate specificity

Dihydroflavonol 4-reductase, (DFR) catalyzes the NADPH-mediated stereospecific reduction of dihydroflavonols to leucoanthocyanidins, precursors of anthocyanins, catechins, and proanthocyanidins. DFR enzymes are known to display distinct substrate specificity; depending on the identity of the 134^{th} amino acid, DFR will reduce one of the three common dihydroflavonols, dihydrokaempferol, dihydroquercetin, or dihydromyricetin, each containing one, two or three hydroxyl groups attached to the flavonoid B-ring, respectively (*63*). DFR forms hydrogen bonds with hydroxyl groups on the B ring in order to twist the molecule into line with NADPH for hydroxylation. DFR is ecologically necessary in that it produces the precursors to anthocyanins, which provide protection from harmful UV radiation, and proanthocyanidins which provide biological protection from pathogens (*45, 64*).

1.3.6 DFR response to Pro-Ca inhibition of F3H

In response to Pro-Ca treatment the inhibition of F3H resulted in a decrease in dihydroflavonol levels while upstream metabolites eriodictyol and naringenin glycosides increased. In such an environment it has been shown that DFR reduces flavanones to flavan-4-ols, a reaction which has previously been shown to be catalyzed by flavanone 4-reductase (FNR) in *Sorghum* (*65*). *In vitro* assays have confirmed that DFR has the ability to catalyze the reduction of eriodictyol to produce luteoforol (**Figure 1.9**) (*66*). The only difference in DFR's usual substrates, the dihydroflavonols, and its induced substrates, the flavanones, is the presence of a hydroxyl group attached at carbon-3.

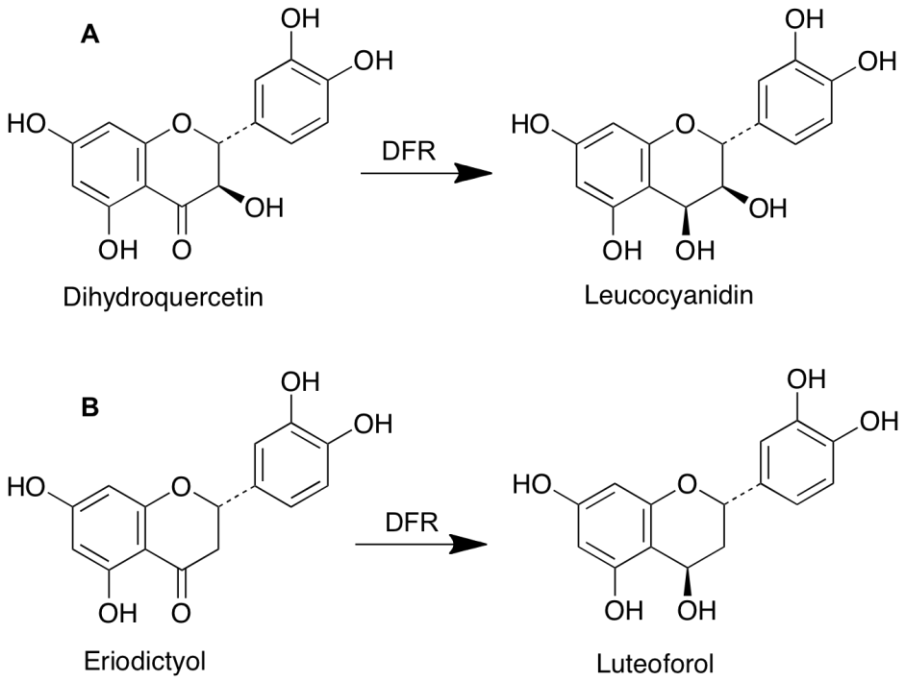


Figure 1.9 Effects of Pro-Ca treatment on DFR substrate specificity
(A) DFR normally reduces dihydroquercetin
(B) Following Pro-Ca treatment DFR reduces eriodictyol

1.3.7 Pro-Ca effects on gibberellic acids

Two main dioxygenases found in the gibberelic acid (GA) pathway display sensitivity to Pro-Ca treatment; GA_{20}3β-hydroxylase (GA3ox) and $GA_1$2β-hydroxylase (GA2ox) have been shown to be inhibited by Pro-Ca treatment (*49, 59, 66*). GA3ox catalyzes the conversion of GA_{20}, a metabolically inactive form of GA, into GA_1, a metabolically active form of GA. The application of Pro-Ca inhibits the GA3ox 3β-hydroxylation of the inactive gibberellin GA_{20} and thereby impedes the production of the active gibberellic acid GA_1, causing a dwarfing of shoot internodes (*49, 51, 59*). The decrease in levels of GA_1 is paralleled by an accumulation of upstream inactive GAs including GA_{19}, GA_{20}, and GA_{29}.

Pro-Ca is most effectively used early in the agricultural season when inactive GAs are dominant. As the season progresses, and vegetative growth increases, inactive GAs are converted to their metabolically active forms; Pro-Ca treatment has no dwarfing effect on vegetative growth when applied at middle or late season timepoints. $GA_1$2β-hydroxylase is responsible for the breakdown of biologically active GA_1 into biologically inactive GA_8. Therefore late season Pro-Ca treatment may increase growth vigor once metabolically active GAs have been produced as catabolism of active GA_1 is inhibited (*66*).

1.3.8 Pro-Ca effects on ethylene

Most dioxygenases require 2-oxoglutaric acid as a co-substrate except in a few circumstances where similar compounds are used. 1-Aminocyclopropane-1-carboxylate oxidase (ACCO) requires ascorbic acid as a co-substrate in the final step of ethylene

biosynthesis (*67*). The mechanism by which Pro-Ca produces yield increases in fruit trees is associated with its effects on ACCO.

Pro-Ca treatment has been shown to competitively inhibit ACCO and decrease ethylene production in treated plants. *In vitro* experiments using purified enzymes from ripe pear fruits have verified Pro-Ca as a strong inhibitor of ACCO where 50% inhibition was reached at a concentration of 10^{-5}M (*51*). 2-Oxoglutaric acid also inhibits this reaction which explains why Pro-Ca, a 2-oxoglutaric acid mimic, produces the same results (*51*). A decrease in ethylene production induced by Pro-Ca treatment decreased fruit drop in apples and increased total fruit yield in some varieties (*51*).

1.4 Pro-Ca treatment of hops

The morphological and biochemical effects of Pro-Ca have produced agronomically positive results in several crops as described above. The effects induced in secondary metabolism and vegetative and reproductive morphology suggest Pro-Ca treatment could be of use in hop agriculture. The effects seen in secondary metabolism may have implications in the production of polyphenols and terpenophenolics in hops as these are closely related pathways and share some substrates such as phenolic acids and chalcones. Inhibition of active gibberellic acids and ethylene synthesis may also prove interesting to hop agriculture, as the inflorescences are the product of hop agriculture. The potential implications of gibberellic acid and ethylene alterations may effect hop cone production or change plant stature. Hops are a very attractive model for Pro-Ca treatment because of the large production of secondary metabolites and flower biomass upon which Pro-Ca may induce effects. In order to measure the effects of Pro-Ca on

hops, exogenous treatment of Pro-Ca was conducted on greenhouse and field grown plants, following which we measure agronomically and scientifically interesting phytochemical and morphological characters to assess the effects induced by Pro-Ca.

1.5 Preliminary results

Two seasons of pilot studies were conducted which helped focus the three seasons of larger-scale experiments described in this thesis. Preliminary results from pilot studies indicated positive agronomic effects of Pro-Ca treatment of hops; this work has been published in *Acta Horticulturae*, and was submitted as a patent application (*68*, *69*). Field treatments were conducted with Pro-Ca over a wide range of concentrations and throughout the development of the plants, starting with shoots in the early spring. Multiple treatment regimes and cumulative weekly treatments over a range of concentrations from 100 to 500 ppm Pro-Ca were conducted. These trials lead to obvious stunting and reduced yield of bines from early treatments at high doses. We also noted significant decreases in polyphenol content in the leaves of plants treated with high doses of Pro-Ca.

After two seasons of trials, we had a good indication that middle to late season treatments of nascent hop cones would yield overall positive agronomic effects. We had also realized that application of Pro-Ca in late season with experimental-scale sprayers was difficult at best. To better our application protocol, we decided a spray-down approach application from the top of the canopy was best, and constructed a special gang walk truck to put workers at the top of the canopy. The gang-plank was long enough to

visit 7 hills simultaneously, and allowed not only for safe and efficient treatment, but also for a platform for careful and deliberate sampling of cones from the apex of the bines.

In our pilot studies we measured significant increases in total cone biomass production and terpenophenolic contents in plants treated with low doses of Pro-Ca (50 and 100 ppm). Effects of Pro-Ca on hops appeared to be specific to cultivar and dependent upon dosage level. These pilot studies helped focus additional experiments outlined in this work where we treated hops at middle and late stages of cone development with low doses of Pro-Ca; results also clarified the need for developmental accuracy in treatment time-points of our experiments.

1.6 Outline of studies

Hop cone development is a rapid and complex process that occurs over the final two months of the agricultural season. Pro-Ca is a transient enzyme inhibitor that has a half-life of a few weeks within the plant. In order to maximize the transient effects of Pro-Ca on the rapid process of cone development we conducted an agronomically focused set of experiments varying the timing and dosage of treatments and measuring the cone yield, total biomass, and terpenophenolic production. We later characterized the different treatment time-points using morphological and phytochemical characters to define the five developmental stages during which we conducted our treatments. We found significant increases in both cone yield and terpenophenolic content in response to some treatments; our results from these experiments are discussed in **Chapter 3**. To further our understanding of the effects of Pro-Ca on polyphenols and terpenophenolics over time we quantitated the responses of 28 compounds over 22 days following a single

treatment with Pro-Ca. In this experiment we also found significant increases in terpenophenolic levels, which coincided with significant changes in several polyphenols. This final study, which is outlined in **Chapter 4**, provides us with insight into the potential relationships between different secondary metabolic pathways and allows us to form hypothesis on transport and primary metabolic responses to Pro-Ca treatment. In **Chapter 5** I outline hypotheses based on our results and suggest future experiments to further investigate secondary metabolism in hop cones and assess the industrial utility for Pro-Ca treatment of hops.

Chapter 2: Phytochemical and morphological characterization of hop (*Humulus lupulus* L.) cones over five developmental stages using high performance liquid chromatography coupled to time-of-flight mass spectrometry, ultra-high performance liquid chromatography photodiode array detection, and light microscopy techniques[1]

2.1 Introduction

Humulus lupulus (L.), commonly known as hops, is an agricultural crop valued for its rich terpenophenolic, polyphenolic, and essential oil contents, used primarily in the brewing industry but also in biomedical research (*4, 70, 71*). Hops contain two main classes of terpenophenolics, namely prenylflavonoids and prenylated acylphloroglucinols, the latter of which are typically known as α- and β-acids (**Figure 2.1)**. Recently, prenylflavonoids xanthohumol and 8-prenylnaringenin have been the focus of biomedical (*4, 22, 72*) and molecular genetics (*16, 17*) research, because of their antiprolific and proestrogenic activities. Furthermore, in addition to a traditional focus on terpene essential oils as flavor components of beer, major brewers have become interested in hop polyphenolic contributions to foam stability, reduction-oxidation effects, and bitterness intensity as well as in marketing of the functionality of beverages, i.e., the health and nutritive effects of ingredients (*73, 74*). Nevertheless, hops are produced primarily for α-acids, or humulones, which impart a bitter flavor to beer (*2*). Finally, new-use interest in the antimicrobial activity of β-acids, or lupulones, in fodder and fermentation of biofuels, merits careful analytical attention to terpenophenolics (*75,*

[1] This chapter was published in the *Journal of Agricultural and Food Chemistry*, in March, 2011.

76). Since only the female inflorescence is used in beverage production and unfertilized cones are preferred in brewing because seed fatty acids can impart undesired flavors to beers, we describe the development of female hop cones that have not been exposed to pollen.

Terpenophenolics and essential oils accumulate in sessile glandular trichomes which are highly metabolically active structures most abundant in the inflorescence, but also present on vegetative leaves (*77*). Two types of glandular trichomes have been described in hops; peltate glands, which are large and contain 100-200 cells, and bulbous glands, which are much smaller, containing eight cells at maturity (**Figure 2.2**) (*18*). Trichome development can be classified into two main stages, the growth stage and the biosynthetic-secretory stage (*18, 78*). The growth stage includes the growth and development of gland stalk cells which support a layer of gland head cells, the main site of biosecretory production. In peltate trichomes, the cuticle thickens at the apical side of head cells and the number of plastids significantly increases just prior to secretion (*78, 79*). During the secretory stage the apical cell wall of the head cells splits to form an intrawall cavity; terpenophenolics secreted by the head cells accumulate in this secretory cavity. The resulting mature gland is a biconal structure, filled with secretions contained by the cuticle and subcuticular wall (*78, 79*). Developmental stages of bulbous glands are difficult to identify due to their small size and rapid development, and have not been as well studied (*18*).

Coinciding with the development of the glandular trichomes, terpenophenolics accumulate over the progression of cone development (*80, 81*). Terpenophenolic accumulation has been studied over three developmental stages which were distinguished

on the basis of cone length (*81*). It was shown that terpenophenolics accumulate as cones increase in length, which corresponds to the elongation of bracts and bracteoles. In contrast to terpenophenolic-rich glandular trichomes, the green tissues of the bracts and bracteoles contain a diverse set of polyphenolic constituents.

The most common polyphenols found in hops are catechins, phenolic acids, flavonol glycosides, namely glycosides of quercetin and kaempferol, and procyanidins (**Figure 2.1**) (*45*). In addition to their ability to protect the plant from pests, polyphenols are important to brewers as they impart unique and complex flavors to beer (*44, 73, 82*). Some of these polyphenols have been shown to decrease in content at late stages of hop cone development (*82*).

Hop cone development is a rapid and dynamic process, and in-depth phytochemical profiles mapped to morphological assessment of developmental stages are useful to experimental, agronomic, and industrial applications. In this context, here we report a targeted metabolic approach to characterize changes in agronomically relevant compounds from hops over five developmental stages in two cultivars. We accomplished morphological and phytochemical characterization for two commercially prevalent hop cultivars, Willamette and Zeus, over five morphologically defined developmental stages. Willamette and Zeus cultivars were selected to represent the two classes of commercially grown hops: low-α-acid-content (2-7% w/w), "aroma" hops; and high-α-acid-content (10-20% w/w), "alpha" hops, respectively. Aroma hops, like Willamette, are added to the brewing process as whole hops for complex flavoring arising from infusion of polyphenols, terpenes, and terpenophenolics into the wort; while alpha hops are mainly used for the production of α-acid-enriched hop extracts. Polyphenols, terpenes, and

lupulones are byproducts of the production of α-acid extracts, used for bittering of large production-scale beers, but these byproducts are nevertheless botanicals used in cosmetics, functional beverages, and medicines. Over the last three years in the U.S.A., Willamette and Zeus have occupied the most acreage within their respective aroma and alpha classes (*83*).

Using LC-TOF-MS and UHPLC-PDA we accomplished the simultaneous quantitation of 21 polyphenolic and seven terpenophenolic constituents, and correlated the phytochemical accumulation with detailed morphology of each cone stage using light microscopy. Seasonal, climatic, edaphic and biotic stress-induced variation in both terpenophenolic and polyphenolic content of hop cones can be large; therefore, an index relating morphological and phytochemical development can be useful in providing developmental reference points for experimental research, for harvest-ripeness determination, and for product quality (*80, 82*).

2.2 Materials and Methods

2.2.1 Plant material

Two hop (*H. lupulus)* cultivars, Willamette and Zeus, were grown under standard agronomic conditions at Golden Gate Ranches, Hopsteiner, S.S. Steiner, Inc., near Prosser, WA. A total of 150 samples were collected from the upper third section of plants at five developmental stages on the following dates in 2008: Willamette (28 July, 2 August, 7 August, 14 August, and 21 August); and Zeus (30 July, 5 August, 12 August, 17 August, and 21 August). Cones collected on these five dates were characterized as stages I-V, each collection date corresponds to a single developmental stage and each

stage was sequentially collected in order of developmental stage (I-V). Willamette cones ripen earlier than Zeus cones, so we started the collection of Willamette cones earlier than that of Zeus. Cones were collected from each of fifteen hop vines for each sample group.

2.2.2 Digital-morphometrics

A high-throughput method was developed to assess the approximate volumes of cones in each sample. Silhouettes of 10-20 cones for each cultivar from each sampling time point were produced in multiplex by transillumination and photography with a C-type 1.3 megapixel video camera driven by iREZ-iNSPECTX capture software (Global Media LLC, Canada). Silhouettes were analyzed using SigmaScan Pro 5.0 margin recognition software (Systat Software Inc., San Jose, CA). Cone perimeters were measured, and the average radius was used to calculate approximate cone volumes based on radial symmetry. Cones were also weighed, and cone density (in mg/cm^3) was calculated.

2.2.3 Microscopy

Hop cones were preserved in formalin-propionic acid-ethanol 50% (FPA, 1:1:18 v/v) under vacuum. Five cone stages were studied using a Nikon SMZ1500 stereoscopic dissecting microscope. Images were recorded using a Nikon DXM1200F digital camera and Nikon ACT-1 software. Flowers develop acropetally, so basal flowers were characterized for each cone developmental stage. While detailed observations of basal flowers were used to characterize cone stages, information from apical flowers was included when informative.

2.2.4 Chemicals

Authentic phytochemical standards catechin, epicatechin, 4-coumaric acid, 4-hydroxybenzoic acid, chlorogenic acid, ferulic acid, naringenin, phenylalanine, procyanidins B-1 and B-2, quercetin, kaempferol, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-rutinoside were purchased from Extrasynthese (Genay, France), and leucine, naringenin chalcone, and valine were purchased from Chromadex (Irvine, CA). Prenylflavonoids xanthohumol and desmethylxanthohumol, and prenylated acylphloroglucinols, adhumulone, cohumulone, humulone, colupulone, lupulone, and co-multifidol glucoside were provided by Hopsteiner, S.S. Steiner, Inc. (Mainberg, Germany).

2.2.5 Extraction and UHPLC analysis of terpenophenolics

Hop cones were lyophilized for 24 h (2-4% moisture) and extracted in 100% MeOH by stir plate agitation for 20 min on a DPC Micromix 5 using program 40 (15 Hz at 900 rpm). The resulting extract was then subjected to UHPLC-PDA analysis. Seven terpenophenolics, xanthohumol desmethylxanthohumol, adhumulone, cohumulone, humulone, colupulone, and lupulone, were quantitated by UHPLC-PDA using a previously developed method (*68*).

2.2.6 Extraction and sample preparation of polyphenols

After removing 0.25 g of sample for terpenophenolic analysis, the remaining lyophilized tissue was extracted by supercritical carbon dioxide. Samples were extracted by CO_2 in a custom-built industrial extractor at the following parameters: 2,400 psi and 30 ºC. The resulting tissue was used to produce a polyphenol-rich extract. Aliquots of

CO_2 extracted tissue (0.25 g) were weighed out and extracted by sonication for 1 h in 10 mL of 80% MeOH. The resulting extract was centrifuged at 3,500 rpm for 15 min, and the supernatant was decanted. Liquid extract was dried under nitrogen gas, lyophilized, and weighed. Dried extracts were reconstituted in 80% MeOH at 10 mg/mL, filtered using a 0.45 µm nylon filter disk and syringe, and then subjected to HPLC-TOF-MS analysis. Serial dilutions (1:20) were also subjected to HPLC-TOF-MS analysis in order to quantitate major constituents within the linear range.

2.2.7 LC-TOF-MS analysis

Samples were analyzed by LC-MS using a Waters LCT Premiere XE Time-of-Flight (TOF) mass spectrometer (Waters Corp., Milford, MA). Ionization was achieved using a multimode source in electrospray (ESI) mode at the following conditions: +ESI capillary 3000 V, −ESI capillary 2800 V, 20 V, aperture 1:0 V, ion guide 1:0 V, and multichannel plate (MCP) 2500 V. Nitrogen was used for both cone and desolvation gases, with a cone gas flow of 20 L/h, and desolvation gas flow of 600 L/h at 400 °C. The source temperature was 120 °C. Leucine-enkephaline (*m/z* 556.2771) was used as a reference mass and infused by a secondary reference probe. The reference mass was scanned once every five scans for each positive and negative data collection. Both positive and negative ESI data were collected using a scan time of 0.2 s, with an interscan time of 0.01 s, and a polarity switch time of 0.3 s. MS data were collected in centroid mode using MassLynx V4.1 Scn 727 software (Waters Corp., Milford, MA).

LC separation was conducted using a Waters Alliance 2695 HPLC coupled to a Waters 2998 PDA detector. Separation was achieved on a 150 × 2.0 mm, 2.6 µm, Kinetex C-18 column (Phenomenex, Torrance, CA), held at a constant temperature of 45

°C and using a gradient system composed of A, 0.1% formic acid in water, and B, 0.1% formic acid in MeCN, at a flow of 0.2 mL/min (except where noted): 0-2.7 min B: 7-9%; 2.7-13.8 min B: 9%; 13.8-14.1 min B: 9-13%; 14.1-54.0 min B: 13-30%; 54.0-67.0 min B: 30-80.5%; 67.0-67.5 min B: 80.5-100%; 67.5-67.6 min B: 100%; flow increased to 0.3 mL/min, 67.6-78.0 min B: 100%. The LC eluent was diverted to waste at two points in the run (0-0.9 min and 67.0-78.0 min) to avoid salt and lipophilic compounds from entering the ESI source.

2.2.8 Quantitation

All MS data were processed using QuanLynx software (Waters). Quantitated components were identified by comparison of retention time and exact mass of both $[M+H]^+$ and $[M-H]^-$ ions with those of authentic standards. Phenolic acids were quantitated using –ESI mode as they produced a greater signal in negative mode; all other compounds reported here were quantitated using +ESI mode. QuantLynx mass tolerance was set to ±5 mDa with a retention time window of ±0.3 min.

UHPLC-PDA data were processed using Empower2 software (Waters). Prenylflavonoids were quantitated at 370 nm and desmethylxanthohumol quantitation was based on the calibration curve of xanthohumol; α- and β- acids were quantitated at a wavelength of 325 nm. This method was developed and validated in our previous work (*68*).

2.2.9 Validation

HPLC-TOF-MS analytical methods were validated for linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision. For each standard, LOD and LOQ were determined as 3:1 and 10:1 S/N, respectively. Calibration curves were

obtained using six concentrations of each analyte above the LOQ and within the linear range.

2.2.10 Statistical analysis

Statistical tests were conducted using JMP 8.01 (SAS, Cary, NC) software. Statistical significance was determined using Tukey-Kramer HSD analysis within a one-way ANOVA where significance was ascribed to differences among means where $p < 0.05$, and are described here as being significant. Values present in all tables include letter notations ascribed to values using Tukey-Kramer HSD analysis.

2.3 Results and Discussion

2.3.1 Morphological characterization of hop cones

The hop inflorescence contains multiple flowers, each with a reduced cup-shaped vestigial perianth, subtended by a bracteole. Pairs of flowers are subtended by a single bract (*10*). The inflorescence, or strobilus contains between 36 and 48 flowers (*11*). A mature cone is a leafy green structure, and bracts and bracteoles are photosynthetic. Photographs and phenological description of glands (**Figure 2.3 A1-E1**) and detached bracteoles (**Figure 2.3 A-E**) are used to assign stages to cones as follows:

Stage I: In basal cone flowers, stigma tips begin to senesce, which is evident as brown colored tissue. Young bracteoles on the abaxial side of the ovary are slightly longer than the ovaries and are flat, covering only the abaxial side of the flower (**Figure 2.3A**). The ovary is enclosed in a vestigial perianth upon which no peltate glandular trichomes are apparent at 50× (**Figure 2.3 A1**). Few bulbous trichomes are sparsely distributed on the abaxial and adaxial surfaces of the bracts and bracteoles. Observations

of the middle and apical cone flowers showed elongated stigmas, and inconspicuous bracts and bracteoles.

Stage II: Bracteoles of the basal cone flowers are cup-shaped and enclose the sides of the ovary. The stigmas have senesced and abscised from the ovaries of the basal flowers, but remain present on ovaries of middle and apical cone flowers (**Figure 2.3 B**). The perianth and abaxial surface of the base of the bracteoles are covered in peltate glandular trichomes, which are cup-shaped and do not appear to contain secretions (**Figure 2.3 B1**). Bulbous trichomes are densely distributed on the abaxial and adaxial surface of bracts and bracteoles. The number of bulbous trichomes does not appear to increase after this stage.

Stage III: Bracteoles have elongated to approximately half the length of the bract and almost completely enclose the ovary (**Figure 2.3 C**). The ovary begins to senesce; however the perianth surrounding the ovary remains intact (**Figure 2.3 C1**). Peltate trichomes present on the abaxial surface of the bracteoles begin filling with terpenophenolic secretions, whereas those on the perianth have not yet begun to secrete. Apical flowers have lost stigmas.

Stage IV: Bracteoles have increased in width causing them to open slightly so that they no longer closely enclose the ovary; rather the base of the bracteoles forms a small cup within which the ovary sits (**Figure 2.3 D**). The ovary has senesced, apparent from shrinkage in size and browning in color. The ovary is kept in place by the perianth, the outer surface of which is covered with peltate glandular trichomes that do not appear to contain secretions. In contrast, peltate glandular trichomes on the bracteoles continue to

fill with terpenophenolic secretions; only few trichomes remain cup-shaped (**Figure 2.3 D1**).

Stage V: Bracteoles have fully elongated to almost the same length as the bracts (**Figure 2.3 E**). The ovary is a small inconspicuous structure with necrotic tissue surrounded by the persistent perianth. The perianth and abaxial surface of the bracteoles contain fully mature peltate glandular trichomes, which are biconal in shape, filled with terpenophenolic secretions (**Figure 2.3 E1**).

2.3.2 Cone volume, mass, and density

In the Zeus cultivar, cones increased in average volume and mass over the five developmental cone stages (**Figure 2.4 A-C**). Zeus cone volume and mass increased between each stage from 1.5 (stage I) to 12.1 cm^3 (stage V), and 119.6 (stage I) to 302.6 mg (stage V), respectively. Average cone volume significantly increased between each sequential stage. Density followed an opposite trend, decreasing from stages I through V, with no significant changes occurring between stages III and V.

Willamette cones, as in Zeus, showed significant increases in volume and mass over the five developmental stages, while cone density significantly decreased overall (**Figure 2.4 D-F**). Willamette cones increased in volume from 0.6 (stage I) to 6.9 cm^3 (stage V) and this increment was statistically significant between all stages, except for between stages I and II. Cone mass increased over the five developmental stages, from 45.2 (stage I) to 145.2 mg (stage V), but the only significant increases occurred between stages II and III. Willamette cone density decreased from 79.5 (stage I) to 21.2 mg/cm^3 (stage V), with the only significant decreases occurring between stages II and III, and between stages III and IV.

In both cultivars a least-squared regression of average cone mass increases (Willamette, R^2 = 0.99; Zeus, R^2 = 0.98) and volume increases (Willamette, R^2 = 0.94; Zeus, R^2 = 0.97) were essentially linear over the five stages; and, thus, mass and volume are not useful in stage demarcation, but do allow tracking of relative growth progression amongst cones. Cone density decreases can be used to demarcate the early stages of bract and bracteole formation from the latter stages of elongation and expansion.

2.3.3 Validation of analytical methods

A sensitive and suitable HPLC-TOF-MS method to quantitate a large panel of hop constituents was achieved (**Figure 2.5**). The calibration curves indicated good linearity for all standards analyzed between the peak area and concentration ($R^2 \geq 0.99$), which occurred between 0.016-5 mg/mL for phenolic acids and flavonol aglycones, 0.26-100 mg/mL for flavonol glycosides, 0.26-100 mg/mL for flavan-3-ols and procyanidins, and 0.26-25 mg/mL for amino acids. The limit of detection (LOD) values for phenolic acids, flavonols and flavonol glycosides, flavan-3-ols and procyanidins and amino acids ranged from 3.9 to 4.9, 3.3 to 7.6, 22.9 to 33.2, and 21.6 to 24.2 ng/mL, respectively, and the limit of quantitation (LOQ) values, for the same metabolites, from 11.9 to 17.4, 9.9 to 22.6, 69.1 to 100.5, and 56.7 to 73.5 ng/mL, respectively.

Accuracy was determined using recovery experiments in which each analyte was spiked into the plant material prior to extraction. Recovery ranged from 97.2 to 101.3% for phenolic acids, 96.0 to 103.2% for flavonols and flavonol glycosides, 97.7 to 99.3% for flavan-3-ols and procyanidins, and 95.8 to 102.4% for amino acids.

The repeatability of the analytical method was evaluated in terms of the intermediate precision by analyzing the inter- and intraday variation, which were

calculated as the relative standard deviation (RSD) of each analyte quantitated from a mixture of the 21 polyphenols. Polyphenolic standards were quantitated at three concentrations that spanned the concentrations of analytes found in all samples. Intraday analysis occurred three times within the same day, and interday analysis occurred over three separate days. In general, all intraday and interday variations were below 4.3% and 7.1%, respectively. More specifically, intraday variation for phenolic acids, flavonols and flavonol glycosides, flavan-3-ols and procyanidins, and amino acids ranged from 2.2 to 3.4%, 1.6 to 4.3%, 0.8 to 3.6%, and 2.0 to 4.1%, respectively. Interday variation of the same compounds ranged from 2.0 to 5.0%, 2.2 to 7.1%, 1.5 to 3.4%, and 2.2 to 4.3%, respectively.

2.3.4 Quantitation of terpenophenolics and polyphenols

We quantitated seven terpenophenolics and 21 polyphenols by UHPLC-PDA and LC-TOF-MS, respectively, for Zeus and Willamette cones over five developmental stages.

Terpenophenolics: The accumulation of terpenophenolics over cone maturation directly corresponded to the morphological development of both bulbous and peltate glandular trichomes as observed over the five developmental stages of cone growth (**Figures 2.2 and 2.3**). All seven terpenophenolic constituents significantly increased in Zeus and Willamette cones between developmental stages I and V, and were coincident with morphological changes in trichomatous glands. The development of peltate glandular trichomes most closely corresponded with terpenophenolic accumulation. Prenylflavonoids desmethylxanthohumol and xanthohumol from Zeus significantly increased from 0.75 and 7.25 mg/g (stage I) to 2.73 and 14.86 mg/g (stage V),

respectively **(Figure 2.6 A)**. In Willamette cones, a significant increase in xanthohumol occurred from 3.53 (stage I) to 7.72 mg/g (stage V) **(Figure 2.7 A)**. In addition, levels of xanthohumol metabolic precursor desmethylxanthohumol followed the same trend in Willamette cones, significantly increasing from 1.34 (stage I) to 1.85 mg/g (stage V). In both cultivars significant increases also occurred in desmethylxanthohumol and xanthohumol between most sequential stages of development.

Like the prenylflavonoids, α- and β- acids also significantly increased over the five developmental stages in both Zeus and Willamette cones, which also corresponded with glandular trichome development **(Figures 2.6 B and 2.7 B)**. In general, the Zeus cultivar showed significant increases in α-acids adhumulone, cohumulone, and humulone, and β-acids colupulone, and lupulone over the five stages of cone development; levels of α-acids adhumulone, cohumulone, and humulone significantly increased from 8.53, 18.31, and 47.41 mg/g (stage I) to 71.23, 94.96, and 213.65 mg/g (stage V), respectively; levels of β-acids, colupulone and lupulone, followed the same trend, as colupulone significantly increased from 26.85 (stage I) to 58.04 mg/g (stage V), and lupulone from 29.56 (stage I) to 55.28 mg/g (stage V). In Willamette cones, α- and β-acids measured also significantly increased over the five developmental stages; levels of α-acids adhumulone, humulone, and cohumulone significantly increased from 0.83, 2.06, and 1.11 mg/g (stage I) to 39.93, 99.12, and 30.80 mg/g (stage V), respectively. In the case of β-acid accumulation, the majority occurred between stages I and II when colupulone increased from 4.80 to 30.68 mg/g, and lupulone increased from 7.34 to 33.10 mg/g, respectively. Colupulone and lupulone levels continued to increase over

developmental stages II-V, but increases were not as large as those between stages I and II.

Accumulation of terpenophenolics in both Zeus and Willamette hop cones directly correlated with the development and maturation of peltate glandular trichomes at most developmental stages. Numerous fully developed bulbous glandular trichomes, filled with biosecretions, were present on the abaxial and adaxial surface of bracts and bracteoles at stage II. Some terpenophenolic accumulation at stage II may be associated with developed bulbous glands. Further work is required to isolate the contribution of bulbous glands to metabolite accumulation. A coincidence between gland development and terpenophenolic accumulation of hop cones was incremental over developmental time, and these occurrences are key identifiers of developmental stage. In addition to terpenophenolic accumulations, trends in some classes of polyphenolics were similar over the developmental stages of Zeus and Willamette cones.

Amino acids: Phenylalanine is the precursor to several phenolic acids, and leucine and valine degradation products are the precursors to α- and β-acid synthesis; therefore the levels of these substrates were of interest to this study. In addition, they are also used in multiple and diverse cellular processes from signaling to protein production. Nevertheless, the decrease in levels of these compounds with the increased production of flavonoids and terpenophenolics might be related.

In Zeus cones, leucine and phenylalanine content showed a decrease over the five developmental stages while valine levels did not change (**Figure 2.6 C**). Leucine content decreased, but was not significantly different at stage V compared to stage I. Phenylalanine levels significantly decreased from 0.26 (stage I) to 0.10 mg/g (stage V),

and decreases were observed between each sequential stage. In contrast to Zeus, leucine and valine contents of Willamette cones decreased between almost all sequential developmental stages (**Figure 2.7 C**). Leucine and valine content significantly decreased from 86.4 and 27.9 mg/g (stage I) to 38.1 and 14.1 mg /g (stage V), respectively.

Overall, decreases, many of which were statistically significant between stages, were observed among select amino acids over the five stages in both Zeus and Willamette cones. However, due to the chloroplastic origins and multiple cellular fates of amino acids leucine, valine, and phenylalanine, changes in contents are a result of more than just utilization for terpenophenolic synthesis. Nonetheless, it is important to note that terpenophenolic production is a large metabolic sink late in development for the degradation products, namely isovaleryl-, isobutyl-, and coumaryl-coenzyme As, and may therefore coincide with decreased phenylalanine and reduction in branched amino acid production in late seasonal cone development. While only steady-state amino acid levels are reported here, we expect different pools of branch-chain amino acids to be synthesized in glandular secretory cell elioplasts and in bract and bracteole chloroplasts and for them to have different fates specific to organ types. It is not known whether amino acids produced in hop leaf chloroplasts are transported to secretory gland cells for the production of gland secondary metabolites.

Flavan-3-ols and procyanidins: Flavan-3-ols and their dimers, procyanidins, are highly antioxidant and of interest to several agricultural products such as tea and wine, but also for beer. These compounds are also well-known for their biomedical implications. Polyphenol levels are also of concern to brewers as antioxidants and haze-reducing agents in beer production and storage. Catechin, epicatechin, and procyanidins

B-1 and B-2 levels were highly variable in Zeus cones; the only significant decreases occurred at stage V, but a decreasing trend was apparent in all four compounds between stages III and V (**Figure 2.6 D**). In Willamette cones, flavan-3-ols, catechin and epicatechin, significantly decreased from 0.69 and 0.06 mg/g (stage I) to 0.30 and 0.03 mg/g (stage V), respectively (**Figure 2.7 D**). Procyanidin B-2 levels fluctuated in Willamette cones over the season, significantly increasing between stages I and II, and significantly decreasing between stages IV and V, but no overall trend was observed. Procyanidin B-1 was present below the LOQ in Willamette MeOH cone extracts, and was therefore not quantitated.

Flavonols and flavonol glycosides: Flavonol glycosides make up a large percentage of hop polyphenols. These compounds, especially the glycosides, are stable and soluble throughout the brewing process and control the haze and stability of beer. In Zeus cones, the content of the flavonols, quercetin and kaempferol, did not significantly change over the five developmental stages. Flavonol glycoside levels had mixed trends, where the levels of glucosides slightly increased, and contents of the galactosides and rutinosides slightly decreased over developmental time; only one of the five flavonol glycosides measured significantly changed from stage I to stage V (**Figure 2.6 E**). In Zeus hops, kaempferol-3-*O*-rutinoside levels significantly decreased from 0.90 (stage I) to 0.36 mg/g (stage V). In Willamette hops, quercetin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-rutinoside all significantly increased in content over the five developmental stages (**Figure 2.7 E**); all flavonol glycosides, with the exception of quercetin-3-*O*-galactoside, significantly

increased over the five developmental stages, although changes between each stage were variable.

Overall, with the exception of quercetin-3-*O*-galactoside, flavonol and flavonol glycoside levels significantly increased over Willamette cone development. These trends were generally not observed in Zeus cone flavonols and flavonol glycosides over cone development. Varietal and environmental variation may be large in hop flavonol accumulation, which may also specifically respond to abiotic and biotic stress, and additional developmental experiments may be necessary in order to elucidate repeatable trends among cultivars.

Phenolic acids: Phenolic acids are the precursors to flavonoids and were of interest in this study as they are precursors to prenylflavonoid production. Some phenolic acids changed over the five developmental stages, but no correlation among phenolic acids or among cultivars was apparent. In Zeus cones, chlorogenic acid levels significantly increased between stages I and II, and ferulic acid significantly decreased between stages I and II; no other major changes were noted in phenolic acids in Zeus cones (**Figure 2.6 F**). In Willamette cones, 4-hydroxybenzoic acid was the only phenolic acid which significantly changed over developmental time; 4-hydroxybenzoic acid decreased from 2.3 (stage I) to 1.0 mg/g (stage V), and decreases occurred between most developmental stages (**Figure 2.7 F**). Overall, phenolic acids were highly variable in both hops cultivars over the five developmental stages, and no overall trend was apparent.

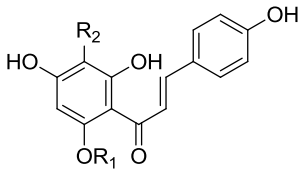
The entire hop industry, from growers to end users, holds great interest in phytochemical accumulation during hop cone development, mainly because of the impact of time-of-harvest on product quality. Therefore we have provided a detailed staging

index for hop cones spanning five stages of terpenophenol and polypenol accumulation. At stage V, terpenophenolics have accumulated to their maximum levels in each variety, which correlate to spectrophotometric measurements of commercially harvested cones over several seasons for these varieties (results not shown). Additionally, flavonoid and phenolic acid contents are sensitive to biotic and abiotic factors (*84*), which can also influence the time-of-harvest. However, phytochemical accumulation alone does not indicate complete maturation of hop cones for use in the brewing industry; additional characteristics, such as cone moisture content, cone size, cone density, and the onset of decline in ⟨-acid contents, are also used to determine commercial harvest time.

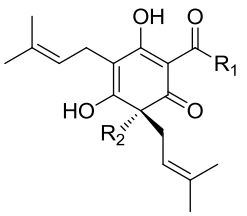
Our cone staging index can be used to inform future investigations into developmentally sensitive hop research. For example, transcriptomics has delivered a plethora of putative gene functions (*16, 85, 86*), often with multiple gene family members indicated, whose functional characterization depends on spatial and temporal placement within the compartmentalized biochemistry of cone development. Similarly, application of growth regulators during cone maturation, such as prohexidione-calcium, has produced agronomically positive yet developmentally sensitive effects on terpenophenolic and cone biomass yields (*87*). Successful time-of-application of growth regulators to increase yield and subsequent assessment of the chemical substantial equivalence of the agricultural food product can be based on developmental chemical profiling and analytical advances reported here.

In conclusion, here we reported the application of a new LC-TOF-MS method and a previously reported UHPLC-PDA method to quantitate 28 industrially pertinent compounds in hop cones. These methods provide a rapid and most detailed analysis to

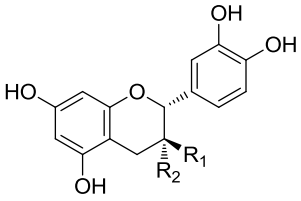
date of the polyphenolic and terpenophenolic makeup of hop cones with sensitive, accurate, and repeatable results. The analytical methods also have wider applicability beyond hops and can be used for targeted metabolite quantitation in other crops. Application of these methods to hops has generated a novel, useful picture of morphological and phytochemical development of hop cone maturation.



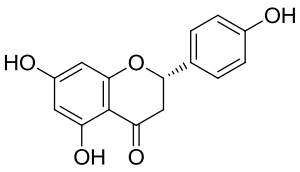
Desmethylxanthohumol	R_1 = H	R_2 = $CH_2CH(CH_3)_2$
Xanthohumol	R_1 = CH_3	R_2 = $CH_2CH(CH_3)_2$
Naringenin chalcone	R_1 = H	R_2 = H



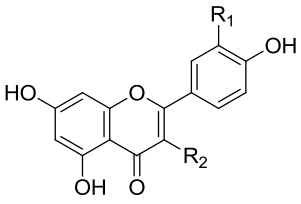
Adhumulone	R_1 = $CH(CH_3)CH_2CH_3$	R_2 = OH
Cohumulone	R_1 = $CH(CH_3)_2$	R_2 = OH
Humulone	R_1 = $CH_2CH(CH_3)_2$	R_2 = OH
Colupulone	R_1 = $CH(CH_3)_2$	R_2 = $CH_2CH(CH_3)_2$
Lupulone	R_1 = $CH_2CH(CH_3)_2$	R_2 = $CH_2CH(CH_3)_2$



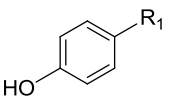
Catechin	R_1 = OH	R_2 = H
Epicatechin	R_1 = H	R_2 = OH



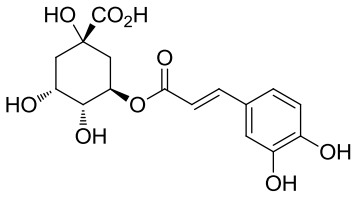
Naringenin



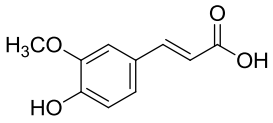
Kaempferol	R_1 = H	R_2 = OH
Quercetin	R_1 = OH	R_2 = OH
Kaempferol-3-*O*-glucoside	R_1 = H	R_2 = Glu
Kaempferol-3-*O*-rutinoside	R_1 = H	R_2 = Rut
Quercetin-3-*O*-glucoside	R_1 = OH	R_2 = Glu
Quercetin-3-*O*-galactoside	R_1 = OH	R_2 = Gal
Quercetin-3-*O*-rutinoside	R_1 = OH	R_2 = Rut



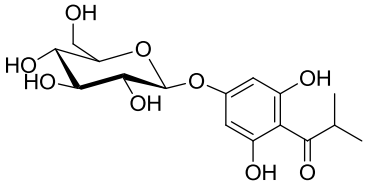
4-Coumaric acid	R_1 = $CHCHCO_2H$
4-Hydroxybenzoic acid	R_1 = CO_2H



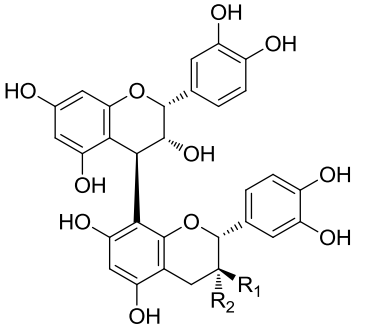
Chlorogenic acid



Ferulic acid



Co-multifidol-glucoside



Procyanidin B1	R_1 = OH	R_2 = H
Procyanidin B2	R_1 = H	R_2 = OH

Figure 2.1 Chemical structures of terpenophenolics, flavonoids, and phenolic acids quantitated in this study

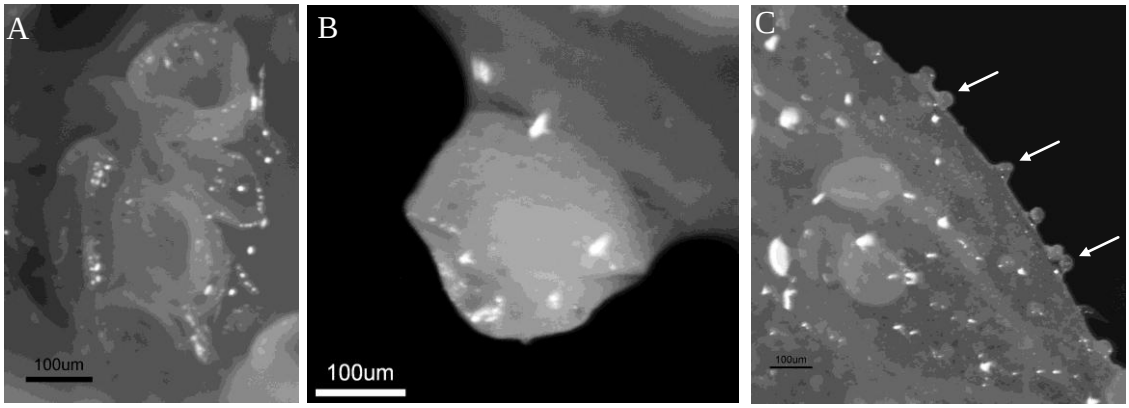


Figure 2.2 Hop cone trichomes

(A) Fully grown peltate trichomes prior to secretion

(B) Fully developed peltatetrichomes containing bio-secretions

(C) Fully developed bulbous trichomes (shown by arrows), much smaller than peltate trichomes, but also contain bio-secretions

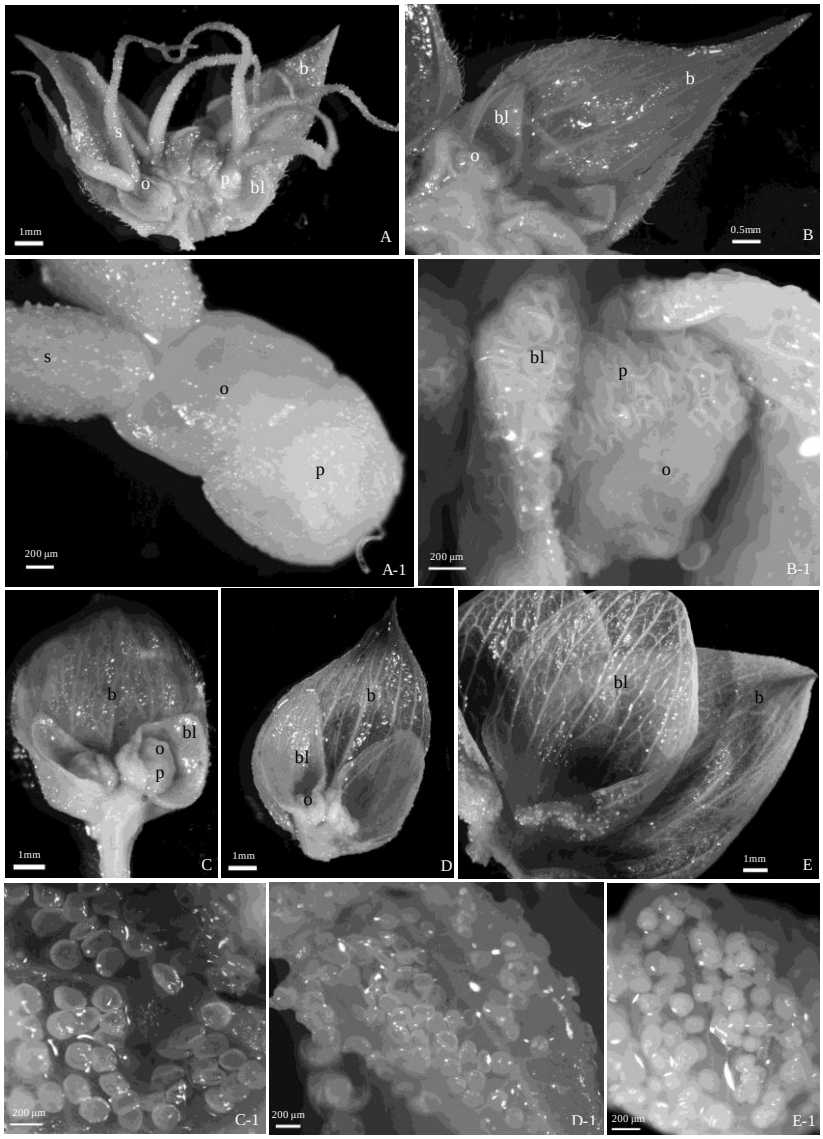


Figure 2.3 Basal flowers were photographed from (A) stage I, (B) stage II, (C) stage III, (D) stage IV, and (E) stage V. Letters represent: (b) bract, (bl) bracteole, (p) perianth, (o) ovary, and (s) stigma.

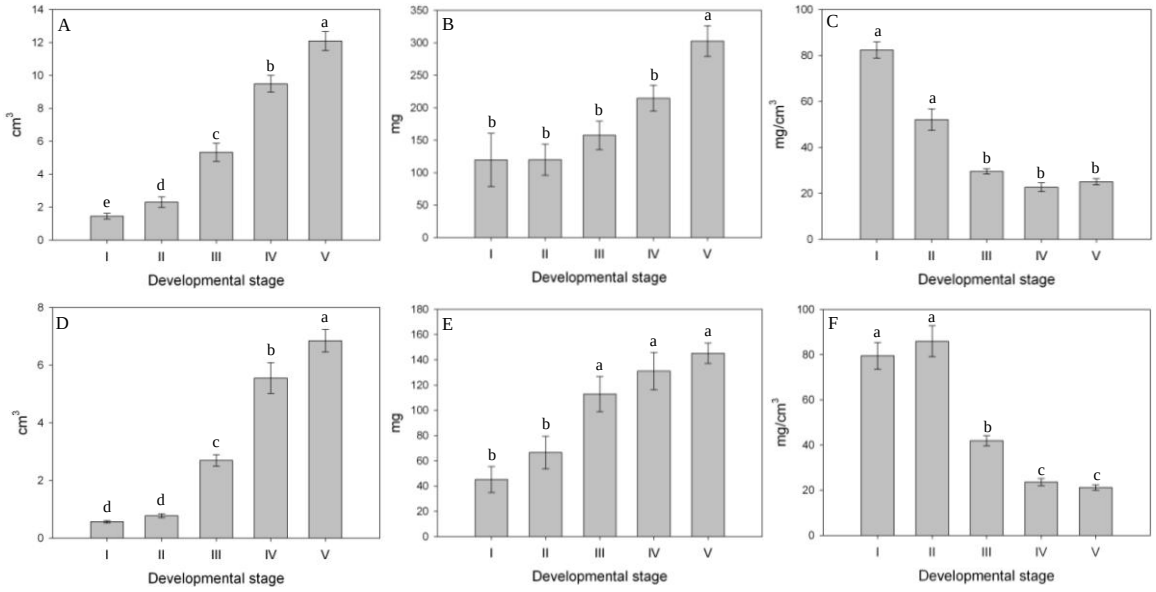


Figure 2.4 Zeus (A) cone volume, (B) cone mass, and (C) cone density over the five developmental stages. Willamette (D) cone volume, (E) cone mass, and (F) cone density over the five developmental stages. Values that share the same letters are not significantly different where p > 0.05 as calculated by Tukey-Kramer HSD analysis within a one-way ANOVA, and error bars Represent standard error.

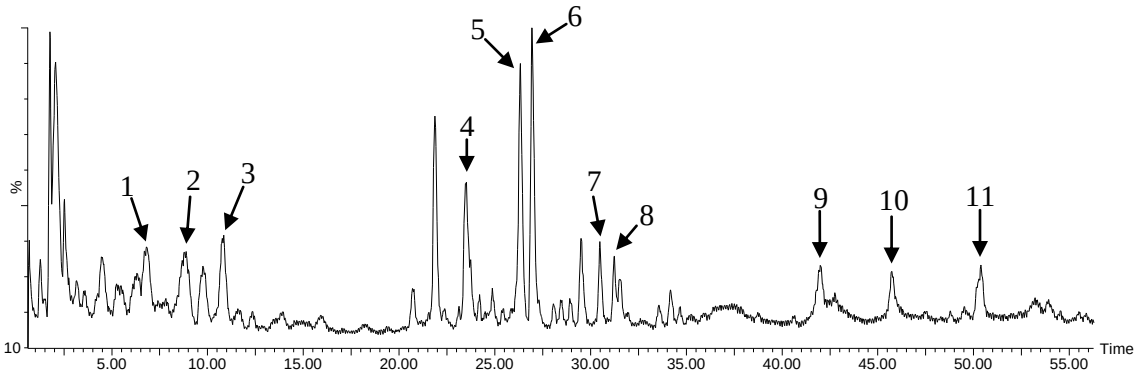


Figure 2.5 Total ion chromatogram (TIC) from +ESI-LC-TOF-MS analysis. Compounds showing peaks in TIC are labeled as: 1. catechin 2. procyanidin b1 3. epicatechin 4.co-multifidol glycoside 5. quercetin-3-*O*-rutinoside 6. quercetin-3-*O*-glucoside 7. kaempferol-3-*O*-rutinoside 8. kaempferol-3-*O*-glucoside 9. quercetin 10. chalcone naringenin 11. Kaempferol

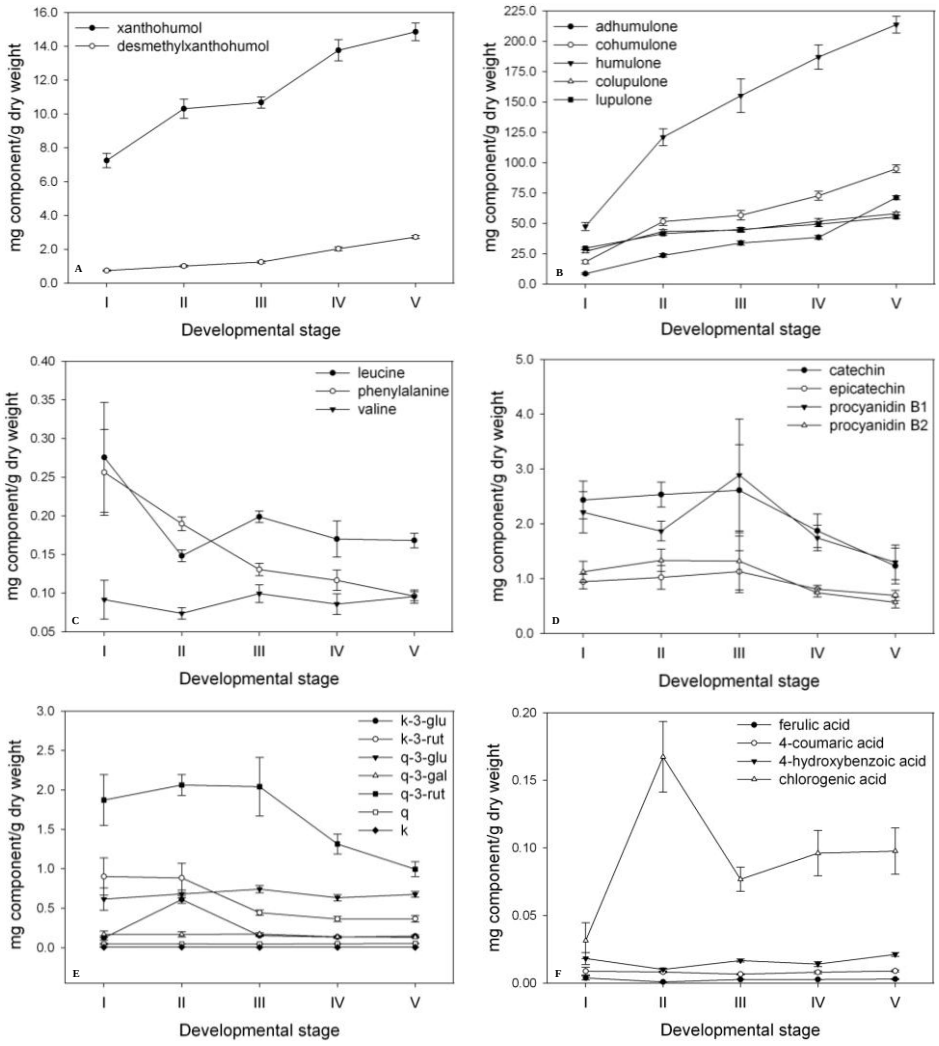


Figure 2.6 Contents of (A) prenylflavonoids, (B) α- and β-acids, (C) amino acids, (D) Flavan-3-ols and procyanidins, (E) flavonols and flavonol glycosides, and (F) phenolic acids of Zeus hop cones over five developmental stages, labeled I-V. For each measurement n = 15, and error bars represent standard error.

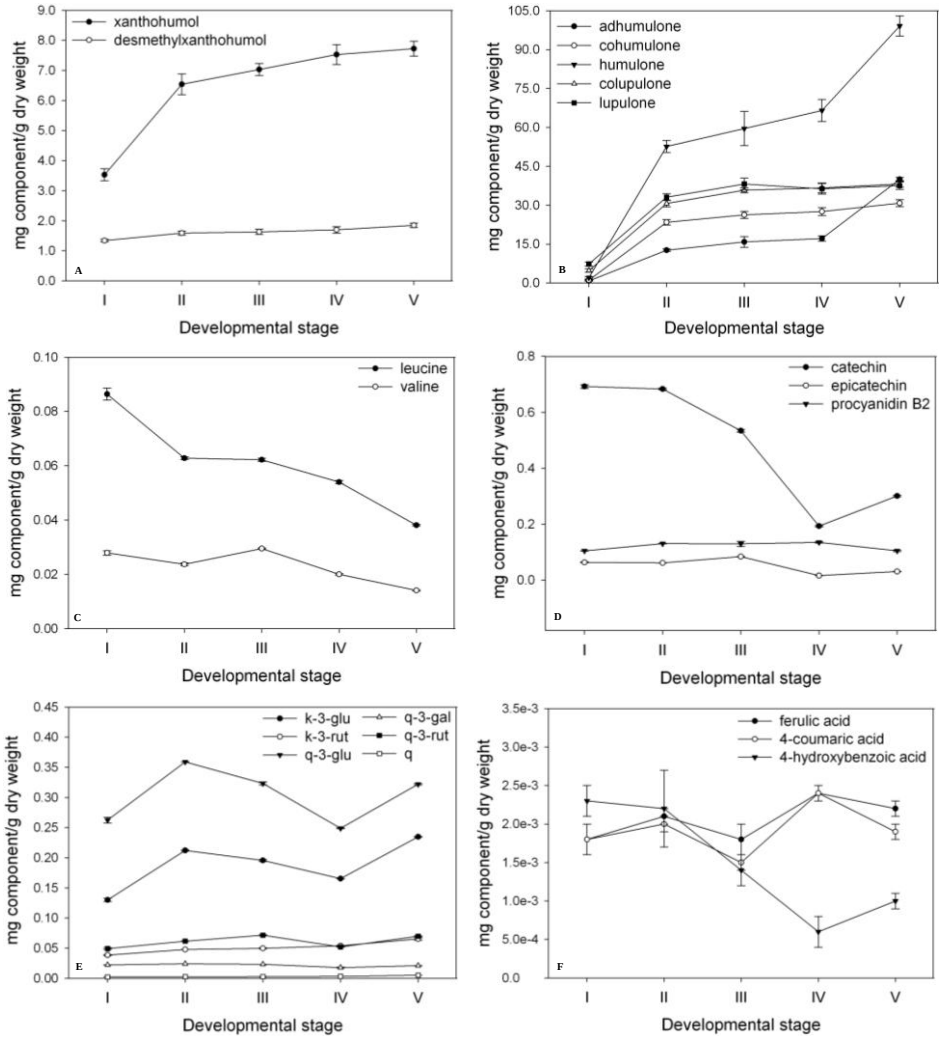


Figure 2.7 Contents of (A) prenylflavonoids, (B) α- and β-acids, (C) amino acids, (D) Flavan-3-ols and procyanidins, (E) flavonols and flavonol glycosides, and (F) phenolic acids of Willamette hops over five developmental stages, labeled I-V. For each measurement n = 15, and error bars represent standard error.

Table 2.1 Summary of morphological and terpenophenolic data for each developmental stage, I-V, for Zeus and Willamette hop cultivars. DMX: desmethylxanthohumol, XN: xanthohumol, CoHum: cohumulone, Hum: humulone, AdHum: adhumulone, CoLup: colupulone, Lup: lupulone.

Stage	Morphology	Cultivar	Mass (mg)	Volume (cm^3)	Density (mg/cm^3)	Prenylflavonoids (mg/g)	α-acids (mg/g)	β-acids (mg/g)
I	Stigma- tips browning Bracteoles- slightly longer than ovary and flat Peltate glands- not apparent	Willa-mette	45.2 ± 10.3	0.57 ± 0.04	79.5 ± 5.94	DMX 1.34 ± 0.04 XN 3.53 ± 0.20	CoHum 1.11 ± 0.19 Hum 2.06 ± 0.46 AdHum 0.83 ± 0.13	CoLup 4.80 ± 0.61 Lup 7.34 ± 0.77
		Zeus	119.6 ± 41.2	1.45 ± 0.18	82.36 ± 3.49	DMX 0.75 ± 0.04 XN 7.25 ± 0.43	CoHum 18.31 ± 1.46 Hum 47.41 ± 3.30 AdHum 8.53 ± 0.67	CoLup 26.85 ± 1.10 Lup 29.56 ± 0.85
II	Stigma- not present on basal flowers, present at mid and apical flowers Bracteoles- enclose ovary Peltate glands- cup shaped, no secretions	Willa-mette	66.6 ± 13.0	0.77 ± 0.07	85.97 ± 8.86	DMX 1.59 ± 0.07 XN 6.54 ± 0.34	CoHum 23.42 ± 1.03 Hum 52.65 ± 2.33 AdHum 12.66 ± 0.52	CoLup 30.68 ± 1.31 Lup 33.10 ± 1.30
		Zeus	120.1 ± 24.0	2.31 ± 0.32	52.07 ± 4.68	DMX 1.01 ± 0.06 XN 10.31 ± 0.56	CoHum 51.48 ± 3.19 Hum 121.02 ± 6.90 AdHum 23.67 ± 1.34	CoLup 43.28 ± 1.71 Lup 41.47 ± 1.46
III	Stigma- not present on any flowers Bracteoles- half length of bract Ovary- senescing Peltate glands- filling with secretions	Willa-mette	112.9 ± 14.0	2.69 ± 0.20	41.91 ± 2.26	DMX 1.63 ± 0.09 XN 7.03 ± 0.20	CoHum 26.29 ± 1.34 Hum 59.57± 6.59 AdHum 15.84 ± 2.02	CoLup 35.81 ± 0.96 Lup 38.18 ± 2.22
		Zeus	157.4 ± 21.8	5.33 ± 0.55	29.53 ± 1.14	DMX 1.25 ± 0.07 XN 10.68 ± 0.33	CoHum 56.67 ± 3.82 Hum 155.12 ± 13.88 AdHum 33.85 ± 1.74	CoLup 44.45 ± 1.79 Lup 45.02 ± 1.67
IV	Bracteoles- elongated, over half length of bract Ovary- senesced and brown Peltate glands- filling with secretions	Willa-mette	131.1 ± 14.7	5.55 ± 0.53	23.61 ± 1.61	DMX 1.70 ± 0.11 XN 7.53 ± 0.33	CoHum 27.57 ± 1.52 Hum 66.51 ± 4.26 AdHum 17.15 ± 1.04	CoLup 36.68 ± 1.84 Lup 36.33 ± 2.01
		Zeus	214.7 ± 19.8	9.49 ± 0.50	22.62 ± 1.92	DMX 2.03 ± 0.11 XN 13.77 ± 0.63	CoHum 72.79 ± 3.67 Hum 186.98 ± 9.97 AdHum 38.50 ± 1.55	CoLup 51.83 ± 2.20 Lup 49.25 ± 2.06
V	Bracteoles- fully elongated, almost bract length Ovary- inconspicuous Peltate glands- biconal shape, filled with secretions	Willa-mette	145.2 ± 8.1	6.85 ± 0.38	21.2 ± 1.20	DMX 1.85 ± 0.07 XN 7.72 ± 0.24	CoHum 30.80 ± 1.30 Hum 99.12 ± 3.95 AdHum 39.93 ± 0.79	CoLup 38.21 ± 1.23 Lup 37.55 ± 1.47
		Zeus	302.6 ± 23.8	12.09 ± 0.57	25.02 ± 1.31	DMX 2.73 ± 0.10 XN 14.86 ± 0.52	CoHum 94.96 ± 3.31 Hum 213.65 ± 6.81 AdHum 71.23 ± 1.75	CoLup 58.04 ± 1.31 Lup 55.28 ± 1.31

Chapter 3: Prohexadione-calcium increases cone biomass and terpenophenolics in hops (*Humulus lupulus* L.)

3.1 Introduction

Hops (*Humulus lupulus* L.) are large, dioecious, perennial vines valued for their female inflorescence, commonly known as "hop cones." Hop cones contain sessile glandular trichomes which are highly metabolically active structures and are the site of terpenophenolic and essential oil biosynthesis (*29, 77*). Two types of terpenophenolics are present in hops, prenylflavonoids, of interest for their biomedicinal use, and prenylated acylphloroglucinols, which make hops a key ingredient in beer (**Figure 3.1**). Hops contain two types of prenylated acylphloroglucinols; humulones, or α-acids, which have bitter flavor and lupulones, or β-acids, which have antimicrobial activity (*2*).

Two main types of hop cultivars are grown for beer production; aroma hops are bred for essential oil content and delicate flavor profiles, containing between 0.5-2% terpene-rich essential oils by total dry cone weight and super-alpha hops which are used for their high α-acid content, which may exceed 22% of the total kiln dried cone weight (*17, 88*). Aroma hops tend to produce lower cone yields (total cone biomass per acre), while super-alpha hops often yield larger cone biomass per acre (*89*). Aroma hops in general also reach flowering maturity earlier in the growing season than do super-alpha hops. In attempt to alter α- and β-acid accumulation and hop cone yield, we treated aroma and super-alpha hop cultivars with an enzyme inhibitor known as prohexadione-calcium.

Prohexadione-calcium (Pro-Ca) is a 2-oxoglutaric acid dependent dioxygenase (2-ODD) anti-metabolite that has shown inhibitory effects on enzymes critical for the

production of gibberellins, ethylene, and flavonoids (*49, 90-92*). Pro-Ca has become widely used for its favorable effects on canopy architecture, shoot elongation, rhizome growth, and fruit yield in pome trees, strawberries, grapes, wheat, and sorghum, among other agronomically valuable species (*93-97*). In addition to effective growth inhibition, Pro-Ca treatment increased fruit yield in specific genotypes of apple; the Golden Delicious apple cultivar exhibited a 15% increase in yield when treated with a single application of 175 ppm Pro-Ca (*49*). In addition to effects on growth and fruit production, Pro-Ca has also been shown to alter the timing and extent of flowering in some plants (*98*).

Treatments of several crops with Pro-Ca have altered the production of flavonoids by inhibiting flavanone-3-hydroxylase (F3H), a 2-ODD essential in the biosynthesis of flavonols and procyanidins (*52, 56, 99-101*). In response to Pro-Ca treatment, contents of phenolic acids and flavanones found upstream from F3H increased, while compounds found downstream from F3H, including flavonols, flavan-3-ols, and procyanidins, significantly decreased in several species (*52*). In addition to the alteration of known flavonoids, the presence of novel, antimicrobial 3-deoxyflavans were reported in some crops where they were previously unknown (*52, 56, 99-101*).

The effects of Pro-Ca on shoot elongation, fruit and flower production, and on flavonoid accumulation made an investigation of treatment of hops with the growth regulator compelling. We treated flowering hop plants with Pro-Ca and studied the effects on terpenophenolic content and total cone yield at harvest. The development of cones and terpenophenolic accumulation are rapid and complex processes, which occur during the two months prior to harvest or plant senescence. Pilot experiments showed

increases in cone yield and terpenophenolic accumulation when plants were treated at an early and middle stage of cone development with 50 ppm Pro-Ca (*68*). To provide greater statistical confidence and investigate seasonal variation in treatment outcomes, we increased experimental group sizes based on power analyses and conducted Pro-Ca treatments at five developmental stages of hop cones over two seasons.

3.2 Materials and methods

3.2.1 Plant material and treatments

Two hop (*H. lupulus)* cultivars, Willamette and Zeus, were grown under standard agronomic conditions at Golden Gate Ranches, Hopsteiner-S.S. Steiner, Inc, near Prosser, WA. Plants were treated with various concentrations of Pro-Ca dissolved in de-ionized water with addition of 1% (v/v) Regulaid™ (Kalo Inc., USA), a non-ionic surfactant,. Control groups were treated with the surfactant in de-ionized water. Plants were drenched "until run-off" at dusk to favor foliar absorption. Pro-Ca treatments were conducted over two seasons using treatment concentrations of 50-100 ppm, applied at five developmental stages (*102*).

Mature cones were collected from the upper third section of plants and kiln-dried using standard commercial handling protocols (65 ^{o}C for about 12 h in a commercial kiln until moisture content reached 8-10%). Dried sub-samples were stored at 4 °C until chemical analysis. Alternately, freshly cut bines were transported to a cone-picking machine for measurement of the mass of cone yield per each bine.

3.2.2 Analysis of yield

To measure the total cone mass (yield) per plant bine, each plant was separately cut at a stem height of ~ 0.5 m and removed from the overhead trellis. Total above-

ground biomass was recorded, then cones from each plant were separated from stems and leaves, using a single-bine picking machine (WOLF Anlagen-Technik GmbH & Co., Germany), and total cone mass was determined using digital scales coupled to a computer database.

3.2.3 Analysis of cone terpenophenolics by UHPLC-PDA

Kiln-dried hop cones were ground with liquid nitrogen, extracted using 100% MeOH and then analyzed by UHPLC-PDA. Seven terpenophenolics, desmethylxanthohumol, xanthohumol, adhumulone, cohumulone, humulone, colupulone, and lupulone were quantitated by UHPLC-PDA using a previously described method (*68*).

3.2.4 Developmental timing of applications

Experiments were conducted over two seasons, in 2007 (season 07) and 2009 (season 09). The same methods were employed in both seasons for treatment, collection, and sample analysis. Several protocols were followed to ensure consistency in maturity among cone samples. Plant stages were assigned based on phytochemical and morphological traits of cones present on the apex of the plant; cone stages I-V are described in detail in **Chapter 1** (*102*). Furthermore, we chose the near-apex of the bine for sampling to favor consistency of cone maturity as cones develop acropetally over several weeks. Willamette and Zeus plants were treated at three developmental stages (denoted as stages I, II, and III) in season 07. In season 09 Zeus plants were treated at stages III, IV, and V, and Willamette plants were treated at stages III and IV.

3.2.5 Treatments

In season 07 experiments, Willamette and Zeus plants were treated at either stage I, II, or III with either 50 or 100 ppm Pro-Ca. There were 50 plants per group and six groups per variety; one control and one treatment for each of the six treatment groups, and two treatment levels for each of the three developmental stages. Each plant was treated a single time with a single concentration of Pro-Ca.

In season 09 experiments Willamette and Zeus plants were treated at either stage III, IV, or V with 100 ppm Pro-Ca. There were 80 plants per group and six groups per variety, one control and one treatment for each of the three treatment groups, with one treatment level for each of the three treatment stages. Each plant was treated a single time with a single concentration of Pro-Ca. Plants were harvested following maximal terpenophenolic accumulation and after cone moisture content reached 80%, as is common practice in hop agriculture.

3.2.6 Statistical analysis

Statistical analysis was conducted using JMP 8.01 (SAS, Cary, NC) software. Group means were compared using least means square contrast within a two-way ANOVA crossing treatment level with developmental stage of treatment; statistical significance was ascribed to analysis that resulted in a difference among means with a p-value of 0.05 or less.

3.3 Results and discussion

Pro-Ca treatment induced significant changes in yield and terpenophenolic contents of hop cones in Zeus and Willamette plants. Application of Pro-Ca to hop plants

induced increases in cone biomass production by 1.5-19.6%, and increased terpenophenolic content by 9.1-87.3%; however some treatments also induced significant decreases in terpenophenolic content. Changes in both cone yield and terpenophenolics were highly dependent on the cultivar, developmental stage, and dosage of Pro-Ca treatment.

3.3.1 Cone yield and biomass

Dramatic dwarfing and flowering occurred in hops treated with Pro-Ca (500 ppm) during early vine development, likely the result of induced hormonal alterations (**Figure 3.2**). Since Pro-Ca has known effects in other plants on hormone-mediated processes through the inhibition of gibberellic acid metabolism and ethylene biosynthesis, we expected treatments with Pro-Ca late in the late maturity of hops to induce morphological changes in plant stature, inflorescence development, and yield. Cone yield was measured as the mass of all cones from each plant (kg cones/plant), which were removed from the above ground stem using a single-bine picking machine. In addition to cone yield, total above-ground biomass was measured as the mass of all leaves, stem, and cones.

Previous experiments showed significant increases in total cone yield from Willamette plants treated with 50 ppm Pro-Ca at developmental stage I (*68*). We conducted experiments over two additional seasons to measure the effects of 50 and 100 ppm Pro-Ca treatment over the five developmental stages for which we measured terpenophenolic contents.

Cone yield and total above ground biomass of Zeus plants changed slightly in response to season 07 treatments, but changes were not significant (**Figure 3.3A and 3.3B**). Cone yield decreased from 4.08 kg/plant to 3.81 kg/plant in plants treated with 50

ppm Pro-Ca at stage I, and increased in plants treated at stage III, from 3.57 kg/plant to 3.79 kg/plant. Total above ground biomass decreased in plants treated at stages I and II; stage I treatments decreased in biomass from 9.60 kg/plant to 8.84 kg/plant (50 ppm), and plants treated at stage II decreased from 9.85 kg/plant to 9.30 (50 ppm) and 9.24 kg/plant (100 ppm), but neither decrease was significantly different than controls.

We used a ratio of yield/biomass to normalize cone yield values, since hop plants can be highly variable in size, even among clones within the same field. While Zeus plants did not significantly change in either cone yield or above ground biomass in response to any treatments conducted in season 07, increased yield/biomass ratios were measured in plants treated at stages II and III; yield/biomass ratios increased in Zeus plants treated with 100 ppm Pro-Ca at stage II, and when treated with either 50 and 100 ppm Pro-Ca at stage III (**Figure 3.3C**). Plants treated with 100 ppm at stage II increase in yield/biomass ratios from 0.44 to 0.46 ($p = 0.024$). Yield/biomass ratios also increased in plants treated at stage III from 0.44 to 0.47 in both 50 ppm ($p=0.004$) and 100 ppm ($p = 0.007$) treatments.

In season 07, total cone yield increased from Willamette plants treated at stages I and III (**Figure 3.4A**). In response to stage I treatments, cone yield increased from 1.06 kg/plant to 1.22 kg/plant (50 ppm) and 1.24 kg/plant (100 ppm) ($p = 0.017$ and $p = 0.008$, respectively). Willamette cone yield also increased in plants treated with 100 ppm at stage III from 1.10 kg/plant to 1.25 kg/plant ($p = 0.038$). Cone yield from plants treated with 50 ppm Pro-Ca at stage II slightly increased from 1.08 kg/plant to 1.15 kg/plant, but this increase was not significant.

Total above ground biomass of Willamette plants significantly decreased in season 07 experiments when plants were treated with 50 and 100 ppm Pro-Ca at stage II and in plants treated with 50 ppm at stage III (**Figure 3.4B**). Willamette plants treated at stage II decreased in above-ground biomass from 5.64 kg/plant to 4.90 kg/plant (50 ppm) and 5.08 kg/plant (100 ppm) ($p = 0.004$ and $p = 0.034$, respectively). When Willamette plants were treated with 50 ppm Pro-Ca at stage III, decreases in biomass occurred from 5.86 kg/plant to 5.20 kg/plant ($p = 0.036$). Plants treated with 100 ppm Pro-Ca at stage III decreased in above-ground biomass from 5.86 kg/plant to 5.36 kg/plant, but this decrease was not significant. Above-ground biomass of Willamette plants increased slightly treated with either 50 or 100 ppm Pro-Ca at stage I, but this change was also not significant.

Yield/biomass ratios significantly increased in Willamette plants treated with either 50 or 100 ppm Pro-Ca at stages II and III (**Figure 3.4C**). Increased in yield/biomass ratios occurred in plants treated at stage II from 0.19 to 0.25 (50 ppm) and 0.22 (100 ppm) ($p = 0.004$ and $p = 0.034$, respectively). Yield/biomass ratios increased in Willamette plants treated at stage III, from 0.19 to 0.22 (50 ppm) and 0.24 (100 ppm) ($p = 0.031$ and $p < 0.001$, respectively). Plants treated with either 50 or 100 ppm Pro-Ca at stage I slightly increased in yield/biomass ratios, but the increases were not significant.

Results from season 07 experiments indicated increases in cone yield and cone yield/total biomass ratios, which occurred in Willamette plants treated with 100 ppm Pro-Ca at stages II and III. In season 09 experiments cone yield and total above ground biomass were measured for Willamette and Zeus hops treated at stages III, IV, and V.

In season 09, cone yield and yield/biomass ratios increased in Zeus and Willamette plants treated with 100 ppm Pro-Ca, but above ground biomass was not affected (**Figures 3.3D and 3.3E**). Zeus plants treated at stage III increased in both cone yield production and yield/biomass ratios, but neither increase was statistically significant. Cone yield increased in plants treated at stage IV from 2.78 kg/plant to 3.26 kg/plant ($p = 0.010$), and in plants treated at stage V from 2.86 kg/plant to 3.41 kg/plant ($p = 0.024$). Yield/biomass ratios from Zeus plants treated at stages IV and V also increased from 0.40 in both controls to 0.42 (stage IV) and 0.44 (stage V) ($p = 0.004$ and $p = 0.010$, respectively) (**Figure 3.3F**).

In season 09, treatment of Willamette plants also induced increases in cone yield and yield/biomass ratios; similar to Zeus, total above ground biomass was not affected (**Figures 3.4D-3.4F**). Cone yield from plants treated at stages III and IV increased from 1.40 kg/plant in both controls to 1.60 (stage III) and 1.68 kg/plant (stage IV) ($p = 0.026$ and $p = 0.002$, respectively). Yield/biomass ratios also increased in Willamette plants treated at both stages III and IV, from 0.29 to 0.34 in stage III treatments, and from 0.26 to 0.31 in stage IV treatments ($p \leq 0.001$ for both increases).

Results from both season 07 and season 09 experiments showed significant increases in the cone yield and cone yield/total plant biomass ratios from Zeus and Willamette plants treated with Pro-Ca at late developmental stages. These late stages are described here as stages III, IV, and V according to the developmental index we previously described (*102*).

3.3.2 Terpenophenolics

Levels of all seven terpenophenolics measured significantly changed following Pro-Ca treatment; effects varied according to the timing and dosages of Pro-Ca. treatment. Data from season 07 suggests that the impact of Pro-Ca treatment on terpenophenolic levels is stage-specific. Terpenophenolic levels in plants treated at stage I decreased, stage II changed slightly, and stage III increased. Zeus plants, when treated with either 50 or 100 ppm Pro-Ca at stage I, produced significantly lower concentrations of all seven terpenophenolics measured with respect to controls (**Figures 3.5A, 3.5B, 3.6A, and 3.6B**). Both prenylflavonoids decreased, desmethylxanthohumol from 0.70 mg/g to 0.13 mg/g (50 ppm) and 0.18 mg/g (100 ppm), and xanthohumol from 6.33 mg/g to 1.15 mg/g (50 ppm) and 1.59 mg/g (100 ppm). All three α-acids decreased, adhumulone from 25.93 mg/g to 5.35 mg/g (50 ppm) and 7.87 mg/g (100 ppm), cohumulone from 64.01 mg/g to 10.52 mg/g (50 ppm) and 15.64 mg/g (100 ppm), and humulone from 95.58 mg/g to 18.90 mg/g (50 ppm) and 26.35 mg/g (100 ppm). Both β-acids also decreased, colupulone from 37.09 mg/g to 8.90 mg/g (50 ppm) and 10.50 mg/g (100 ppm), and lupulone from 28.55 mg/g to 7.45 mg/g (50 ppm) and 8.55 mg/g (100 ppm). All terpenophenolics contents decreased in response to treatment of Zeus with either 50 or 100 ppm Pro-Ca at stage I, and all decreases were statistically significant where $p < 0.001$.

In contrast to the large decreases in terpenophenolics following season 07-stage I treatments of Zeus plants, stage II treatments induced only minor changes. adhumulone and lupulone increased in Zeus plants treated with 50 ppm Pro-Ca at stage II; no other terpenophenolics significantly changed in Zeus plants treated at stage II.

Treatments conducted at stage III in season 07 induced favorable agronomic effects in Zeus cones where all seven terpenophenolics increased in response to 50 and 100 ppm treatments. Both prenylflavonoids increased, desmethylxanthohumol from 0.70 mg/g to 1.12 (50 ppm) and 1.18 mg/g (100 ppm), and xanthohumol from 6.33 mg/g to 10.15 mg/g (50 ppm) and 11.03 mg/g (100 ppm). Levels of α-acids also increased, adhumulone from 25.93 mg/g to 48.56 mg/g (50 ppm) and 41.45 mg/g (100 ppm), cohumulone from 64.01 mg/g to 106.35 mg/g (50 ppm) and 112.12 mg/g (100 ppm), and humulone from 95.58 mg/g to 161.49 mg/g (50 ppm) and 161.28 mg/g (100 ppm). Additionally, both β-acids increased, colupulone from 37.09 mg/g to 63.12 mg/g (50 ppm) and 62.18 mg/g (100 ppm), and lupulone from 28.55 mg/g to 48.10 mg/g (50 ppm) and 47.49 mg/g (100 ppm). Levels of all terpenophenolics measured increased in response to treatment of Zeus with either 50 or 100 ppm Pro-Ca at stage III during season 07 experiments, and all increases were highly significant where $p < 0.001$.

Similar changes in terpenophenolic contents of Zeus were also observed in Willamette plants treated with 50 ppm Pro-Ca during season 07 (**Figures 3.5D and 3.6D**). Willamette plants treated at stage I with 50 ppm Pro-Ca produced cones with decreased levels of all seven terpenophenolics measured. Both prenylflavonoids decreased, desmethylxanthohumol from 0.43 mg/g to 0.31 mg/g ($p = 0.001$), and xanthohumol from 3.82 mg/g to 3.21 mg/g ($p = 0.021$). All three α-acids decreased, adhumulone from 9.25 mg/g to 7.45 mg/g ($p = 0.012$), cohumulone from 16.37 mg/g to 13.16 mg/g ($p = 0.013$), and humulone from 34.82 mg/g to 29.14 mg/g ($p = 0.033$). β-Acid contents also decreased, colupulone from 23.51 mg/g to 16.99 mg/g, and lupulone from 21.40 mg/g to 15.00 mg/g ($p < 0.001$ for both decreases). In summary, all

terpenophenolics measured decreased in response to treatment of Willamette with 50 ppm Pro-Ca at stage I, and all decreases were statistically significant.

Few changes occurred in Willamette cones in season 07 when plants were treated with 50 ppm Pro-Ca at stage II. Significant increases did occur in levels of xanthohumol and colupulone, but no other terpenophenolics changed in response to stage II treatments.

Willamette plants treated in season 07 with 50 ppm Pro-Ca at stage III produced cones with increased levels of prenylflavonoids desmethylxanthohumol and xanthohumol, and β-acids colupulone and lupulone. Levels of α-acids adhumulone, cohumulone, and humulone slightly increased in response to Pro-Ca treatment at stage III, but no increase was significantly different from controls. Both prenylflavonoids increased, desmethylxanthohumol from 0.43 mg/g to 0.54 mg/g ($p < 0.001$), and xanthohumol from 3.82 mg/g to 4.55 mg/g ($p = 0.003$). β-Acid contents also increased, colupulone from 23.51 mg/g to 29.88 mg/g, and lupulone from 21.40 mg/g to 27.78 mg/g ($p < 0.001$ for both increases).

In season 07, levels of terpenophenolics in cones from Willamette plants treated with 50 ppm Pro-Ca showed similar responses as cones from Zeus plants treated with both 50 and 100 ppm Pro-Ca; however, when Willamette plants were treated with 100 ppm Pro-Ca the cones did not have the same altered terpenophenolic profiles as the other three treatment groups (**Figures 3.5E and 3.6E**). Willamette plants treated with 100 ppm Pro-Ca at stage I produced cones with slightly decreased levels of prenylflavonoids and α-acids; the only significant decrease occurred in adhumulone from 9.25 mg/g to 8.16 mg/g ($p = 0.043$). Levels of both β-acids also decreased, colupulone from 23.51 mg/g to 19.68 mg/g ($p = 0.001$), and lupulone from 21.40 mg/g to 17.99 mg/g ($p = 0.002$). No

significant changes or trends in terpenophenolic contents were observed in cones from Willamette plants treated with 100 ppm Pro-Ca at stages II or III.

The results of season 07 experiments indicate that levels of all three groups of terpenophenolics, the prenylflavonoids, α-acids, and β-acids, significantly change in response to Pro-Ca treatment. Treatment of Zeus plants with either 50 or 100 ppm Pro-Ca, and treatment of Willamette plants with 50 ppm Pro-Ca induced the same responses; terpenophenolic levels in plants treated at stage I decreased, stage II changed slightly, and stage III increased. Our results indicate that increases in terpenophenolics occur in response to mid-late season cone treatments, characterized as stage III (*102*). We therefore conducted a second season (season 09) of experiments in order to test the repeatability of treatments conducted at stage III, and assess the effects of Pro-Ca treatment on plants at later developmental stages.

In season 09 experiments Zeus and Willamette plants were treated with 100 ppm Pro-Ca at three and two stages during late cone development, respectively. Zeus plants were treated at developmental stages III, IV, and V, and Willamette plants were treated at stages III and IV; Willamette plants mature several weeks earlier than Zeus, so a stage V treatment was omitted. In season 09 experiments, the first treatment, which occurred at stage III, corresponded to the final treatment in season 07 experiments, which was also noted as stage III; stage III for both seasons were characterized as similar using a previously established index of developmental stages (*102*).

Treatment of Zeus plants in season 09 induced significant increases in all terpenophenolics measured, but prenylflavonoids and α- and β- acids accumulated at variable rates, depending on the developmental stage at which treatments occurred

(**Figures 3.5C and 3.6C**). Prenylflavonoids increased in cones from Zeus plants treated at stage IV. Both prenylflavonoids increased in response to stage IV treatments, desmethylxanthohumol from 1.12 mg/g to 1.29 mg/g, and xanthohumol from 4.88 mg/g to 5.42 mg/g ($p < 0.001$ for both increases). Prenylflavonoid content did not change in response to treatments conducted at stages III or V. Bitter acids, however, increased in cones from plants treated at stage III. All three α-acids increased, adhumulone from 20.89 mg/g to 24.14 mg/g ($p < 0.001$), cohumulone from 58.58 mg/g to 65.83 mg/g ($p < 0.001$), and humulone from 107.59 mg/g to 116.02 mg/g ($p = 0.006$). Levels of β-acids also increased, colupulone from 36.13 mg/g to 40.42 mg/g ($p = 0.003$), and lupulone from and 39.02 mg/g to 43.47 mg/g ($p = 0.008$). No changes in α- or β- acid contents occurred in response to treatment at stages IV and V, with the exception of a significant decrease in humulone content from 107.59 mg/g to 97.37 mg/g ($p = 0.027$) in cones from plants treated at stage V. While α- and β-acids and prenylflavonoids significantly increased in Zeus plants treated at stages III and IV in season 09 experiments, the magnitude of change was much less in comparison to the increases measured from treatments conducted at stage III in season 07 experiments.

In season 09, the treatment of Willamette plants induced significant increases in levels of cone terpenophenolics; as in Zeus, changes in Willamette were also dependent upon the developmental stage at which plants are treated. The prenylflavonoid xanthohumol and β-acids colupulone and lupulone significantly increased in response to season 07 treatments of Willamette plants at stage III; xanthohumol from 3.35 mg/g to 3.55 mg/g ($p < 0.01$), colupulone from 33.79 mg/g to 37.45 mg/g ($p < 0.001$), and lupulone from 26.73 mg/g to 30.99 mg/g ($p < 0.001$). Willamette plants treated at stage

IV produced cones with decreased levels of xanthohumol, two α-acids and the β-acid lupulone; prenylflavonoid xanthohumol from 3.35 mg/g to 3.17 mg/g ($p = 0.002$), α-acids cohumulone from 15.25 mg/g to 13.26 mg/g ($p = 0.008$), and humulone from 27.19 mg/g to 23.99 mg/g ($p = 0.045$), and the β-acid lupulone from 26.73 mg/g to 25.23 mg/g ($p = 0.017$). adhumulone and colupulone also decreased in response to stage IV treatments, but the decreases were not statistically significant.

In both seasons I and II, effects of Pro-Ca on hop terpenophenolics was dependent on the developmental stage at which Pro-Ca was applied. Smaller changes in terpenophenolics occurred in season 09 treatments, in comparison with changes observed in season 07. Overall, treatments which occurred during mid-inflorescence development, noted here as stages III and IV, had the most agronomically positive outcomes, with significant increases in hop cone terpenophenolics. Of the two hop cultivars treated, Zeus and Willamette, Zeus cones showed more consistent results between season 07 and season 09. Willamette hops may be more sensitive to Pro-Ca treatment, as a dosage of 50 ppm Pro-Ca induced more agronomically positive results than treatments with 100 ppm Pro-Ca. Willamette is a less vigorous cultivar that produces lower levels of terpenophenolics and may be more sensitive to low concentrations of Pro-Ca.

Prenylflavonoid and α- and β-acid contents responded similarly to Pro-Ca treatment; in several cases both groups of compounds either increased or decreased in the same direction and to the same extent. While these two groups of compounds are prenylated terpenophenolics and present in glandular trichomes, their biosynthetic origins are quite different. Bitter acids are produced via the prenylation of polyketides derived from branched-chain amino acid degradation products, whereas prenylflavonoids are

produced by the prenylation of chalcone naringenin, a polyketide derived from phenolic acid biosynthesis (*16*). The changes in these compounds of different biosynthetic origin may indicate multi-mechanistic effects of Pro-Ca on hop metabolism and terpenophenolic accumulation. Pro-Ca has been shown to inhibit biosynthesis of gibberellic acid, ethylene, and flavonoids; treatment may therefore affect a number of enzymes involved in the synthesis of the metabolic precursors of terpenophenolic biosynthesis as well as hormone levels that have gross effects on cone morphology. Pro-Ca has been shown to inhibit F3H, which caused increases in phenolic acids in several species (*49, 52*). Chalcone naringenin, a polyketide derived from the phenolic acid 4-coumaric acid, has been shown to be the precursor compound to prenylflavonoid biosynthesis in hops (*16, 29*). Therefore, an inhibition of F3H in hop cones may influence the contents of phenolic acids and chalcone naringenin; however, changes in these substrates would not likely have a direct effect on terpenophenolic biosynthesis, since the compartmentalization of F3H (in mesophyll or epidermal cells) and the terpenophenolic biosynthetic pathway enzymes (in trichome gland secretory cells) are different.

The branched-chain amino acid degradation products and prenyl groups required to supply the large demand for α- and β-acid biosynthesis are products of multiple, distinct biosynthetic pathways (*16*). More specifically, degradation products of valine and leucine provide precursor compounds for production of α- and β-acids. While no known 2-ODDs are responsible for the degradation of valine or leucine, or the biosynthesis of α- and β-acids, 2-oxoglutaric acid (or 2-oxoglutarate) is a substrate of aminotransferases, which catalyze the transfer of amino groups from branched amino acids in the first step of amino acid degradation. It is suggested that Pro-Ca inhibits 2-ODDs by competitive

inhibition, possibly due to its structural similarity to 2-oxoglutaric acid (*59*, *103*). If Pro-Ca mimics 2-oxoglutarate, there could be some effects on aminotransferases, which may effect amino acid degradation and α- and β-acid metabolism. In addition to amino acid degradation, 2-oxoglutarate is an essential intermediate in the tricarboxylic acid cycle. Inhibition of the tricarboxylic acid cycle could have major effects on primary metabolism and the primary flux of carbon in plant metabolism. While it is possible that Pro-Ca has effects on any process involving 2-oxoglutarate, the effects that have been observed are non toxic, and therefore Pro-Ca inhibition is not likely causing major changes in central metabolism, such as the tricarboxylic acid cycle. Our results suggest that rather than the product of a single enzyme inhibition, Pro-Ca effects on hop secondary metabolism may be due to multiple mechanisms, possibly involving amino acid, phenolic acid, and hormone biosynthesis; the involvement of multiple pathways may be the source of the developmental, genotypic, and seasonal variation we have observed. As part of our ongoing investigation of the effects of Pro-Ca treatment on hop cone terpenophenolic biosynthesis, we are conducting comprehensive polyphenol quantitation including the analysis of branched amino acids, chalcone naringenin, and other precursor metabolites related to terpenophenolic biosynthesis.

The effects of Pro-Ca treatment on hop cone production, and possibly terpenophenolic content, may be related to inhibition of enzymes within the gibberellic acid (GA) and ethylene biosynthetic pathways. Pro-Ca treatment inhibits the production of active gibberellic acids, specifically GA_1, and has been shown to decrease internode elongation; primarily Pro-Ca is used to induce favorable canopy architecture in fruit trees, reducing the need for pruning (*59*, *91*). We have also observed significant decreases

in internode elongation in hops treated with 500 ppm Pro-Ca (**Figure 3.2**); although no changes in internode lengths were observed when hop plants were treated with 50 or 100 ppm Pro-Ca as was conducted in this study (data not shown). Early season Pro-Ca treatment has been shown to inhibit the 3β-hydroxylation of GA_{20}, an inactive form of gibberellic acid, which normally produces the active GA_1; this inhibition resulted in decreases in internode elongation in several agricultural species (*59*). However, it has also been shown that late season treatment with Pro-Ca may increase active gibberellic acid contents as Pro-Ca also inhibits the 2β-hydroxylation of GA_1, which normally produces GA_8, an inactive gibberelic acid; this is a catabolic process which occurs as vegetative growth declines, and is known as gibberellic acid inactivation (*104, 105*). In summary, early season application of Pro-Ca inhibits the production of GA_1 from GA_{20}, and thereby decreases the content of active gibberellic acids, whereas late season application, following GA_1 accumulation, inhibits the inactivation of GA_1 to GA_8, which results in an increased level of active gibberellic acids. Active GAs can induce or suppress flowering, depending on the plant species treated, and the dosage and time point of treatment (*106-108*). The treatment of hops with active gibberellic acids has previously been shown to delay flower production and extend the period of flowering and cone development; this resulted in an increase in cone number and total cone yield by weight (*109-111*). Measurements of endogenous GAs in hops showed a gradual increase in GA_1 during vegetative growth, followed by decreased levels during flowering; GA_3 and GA_4 were present at increased levels during the early production of hop flower buds (*112*). Gibberellic acid application has also been shown to increase α- and β-acid content in hop

cones, which may help explain the significant increases in terpenophenolics we observed in response to treatment of plants with Pro-Ca at stage III (*111*).

Effects of GA on glandular trichome formation may be related to the increase in α- and β-acid levels we have observed. It is well known that treatment of *Arabidopsis thaliana* with active gibberellic acids stimulate early trichome formation (*113-115*). Conversely, when paclobutrazol, a GA biosynthesis inhibitor, which inhibits biosynthesis at early stages, i.e., between *ent*-kaurene and *ent*-kaurenoic acid, was applied to plants, trichome initiation was suppressed (*113*). The GA-deficient *Arabidopsis* mutant ga1-3 does not produce trichomes; however, when GA_3 is added exogenously trichome development is restored (*113*). Stimulatory effects of GA on trichome formation has also been reported in *Zea mays* L., where GA-deficient mutants have also showed delayed trichome formation (*116*). However, conflicting results on the effects of active gibberellic acid treatment of plants on trichome formation have been reported; *Origanum* x *intercedens*, an aromatic plant which produces glandular trichomes containing essential oils, exhibited decreases in the density of glandular trichomes present on the surface of leaves in response to exogenous treatment with GA_3 (*117*). In hops, the development of glandular trichomes occur on the perianth and abaxial surface of bracteoles, they are difficult to observe because they are concealed by the bracts and bracteoles, and the tissue is concave along the axis of the strig, making for quantitation of glandular trichomes a challenging task. In summary, late season application of Pro-Ca to hop plants may increase the content of active gibberellic acids by decreasing the catabolic breakdown of GA_1, and thereby alter flower and cone development, trichome formation, and terpenophenolic contents.

Pro-Ca treatment has also been shown to inhibit ACC oxidase, which catalyzes the final step in ethylene biosynthesis; ethylene is a plant hormone, which stimulates flower senescence (*49, 91*). We have previously reported the production of hop cones with increased density in response to Pro-Ca treatment (*68*). An increase in density may be associated with younger cones as we have also found decreases in cone density corresponding to later developmental stages of hop cones (*102*). In these experiments we also observed the production of new young shoots with young hop cones in response to stage III treatments, although these observations were not quantitated. Changes in inflorescence and cone development, including the induction of new flowers and cones during mid to late inflorescence development, would also likely effect terpenophenolic accumulation.

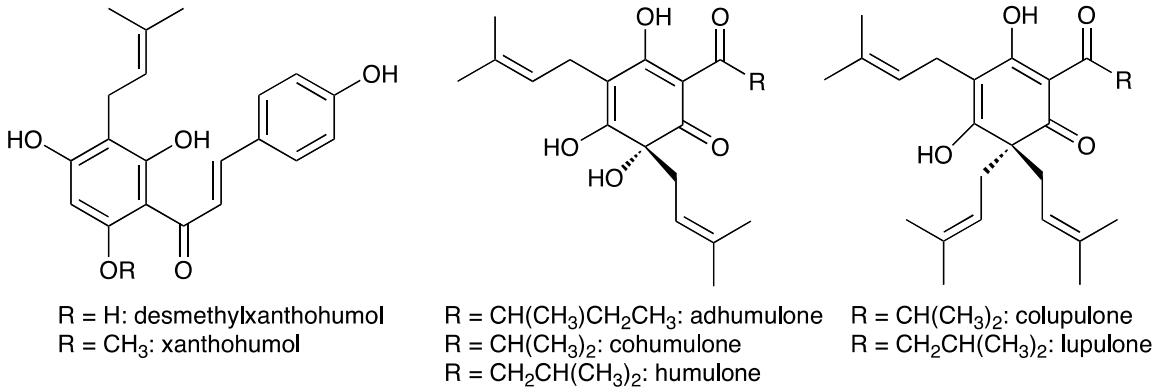
Ethylene also effects trichome initiation and development; in tomato plants, ethylene-overproducer mutant *epi* develops much lower densities of both glandular and non-glandular trichomes on both the abaxial and adaxial surface of leaves (*118*). Morphological changes in trichomes have been also induced by exogenous ethylene treatment including increased number of cells in cucumber (*Cucumis sativus*) seedlings and increased branching observed in *Arabidopsis* trichomes (*119, 120*). While no studies of ethylene in hops have been published, ethylene is well known to be a hormone that initiates flowering and senescence (*121-123*). The promotion of flower senescence has been demonstrated by exogenous treatment of ethylene in several species, and treatment of several plant species with inhibitors of ethylene production have shown to delay the onset of flower senescence (*121, 123*). The increased production of cone yield and

cone/biomass ratios, as we observed in Pro-Ca treated hop plants, may be attributed to decreased senescence of cones, induced by an inhibition of ethylene production.

In summary, the effects of Pro-Ca treatment on hop cone yield, cone/biomass ratios, and terpenophenolics were specific to the developmental stage at which plants were treated. Depending upon the stage at which Pro-Ca was applied, results were either agronomically favorable or, in some cases, agronomically unfavorable. Hop cone development is a highly complex process involving hormonal changes and secondary metabolite accumulations**;** these processes are inter-related and Pro-Ca has large effects on enzymes involved in hormone production and on substrates in secondary metabolism (*59*). Hop flower and cone development can be variable between seasons, cultivars, and individual plants (*80*). In addition, the potentially multiple pleotropic effects of Pro-Ca treatment on hop cone yield and terpenophenolic biosynthesis could induce the changes we observed through multiple mechanisms.

In these experiments we have produced favorable agronomic responses in hop plants using Pro-Ca. Two seasons of large-scale field experiments have helped identify the most effective dosage and developmental time-points for treatment. As prohexadione is a transient enzyme inhibitor, with a half-life of only a few weeks within the plant, the timing of application is vital to agronomically desirable outcomes. In order for a short-lived enzyme inhibitor to have significant effects on these events, application must be conducted at a precise time point. As has occurred in other specialty crops under treatment with Pro-Ca, more experimental work at an agronomically relevant scale is needed to ensure efficient use of Pro-Ca treatment for hop crop improvement, including treatments of various varieties over multiple seasons. Further work on the mechanisms

and inter-actions of Pro-Ca effects on metabolism and growth of hops, especially changes in flavor-intense polyphenolics and terpenophenolics in the hop cone and trichomatous gland systems are in progress in our laboratories.



R = H: desmethylxanthohumol
R = CH_3: xanthohumol

R = $CH(CH_3)CH_2CH_3$: adhumulone
R = $CH(CH_3)_2$: cohumulone
R = $CH_2CH(CH_3)_2$: humulone

R = $CH(CH_3)_2$: colupulone
R = $CH_2CH(CH_3)_2$: lupulone

Figure 3.1 Structures of the seven terpenophenolics quantitated in this study including two prenylflavonoids, three humulones (α-acids), and two lupulones (β-acids).

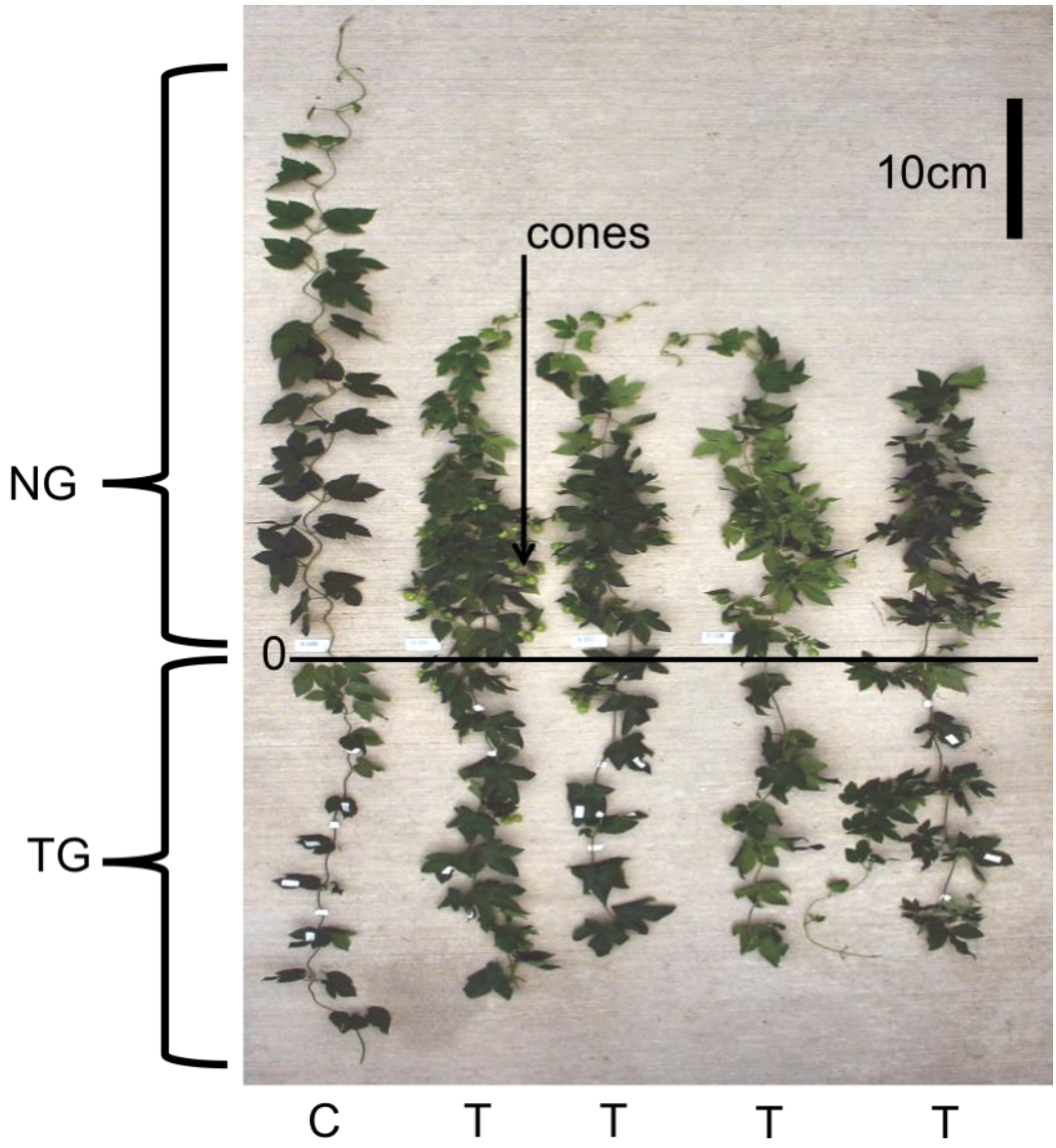


Figure 3.2 Immature hop plants treated with high doses (500 ppm) of Pro-Ca show effects of inhibition of hormone biosynthesis. Treated plants (T) and a control plants (C), which were treated at the extent of growth (TG) shown below the line (time 0), and 6 weeks of new growth (NG), above the time 0 line, are shown for detached bines. Growth effects include: shortened internodes and petioles, small dark-green leaves, epinastic leaf curl, and induction of premature flowering.

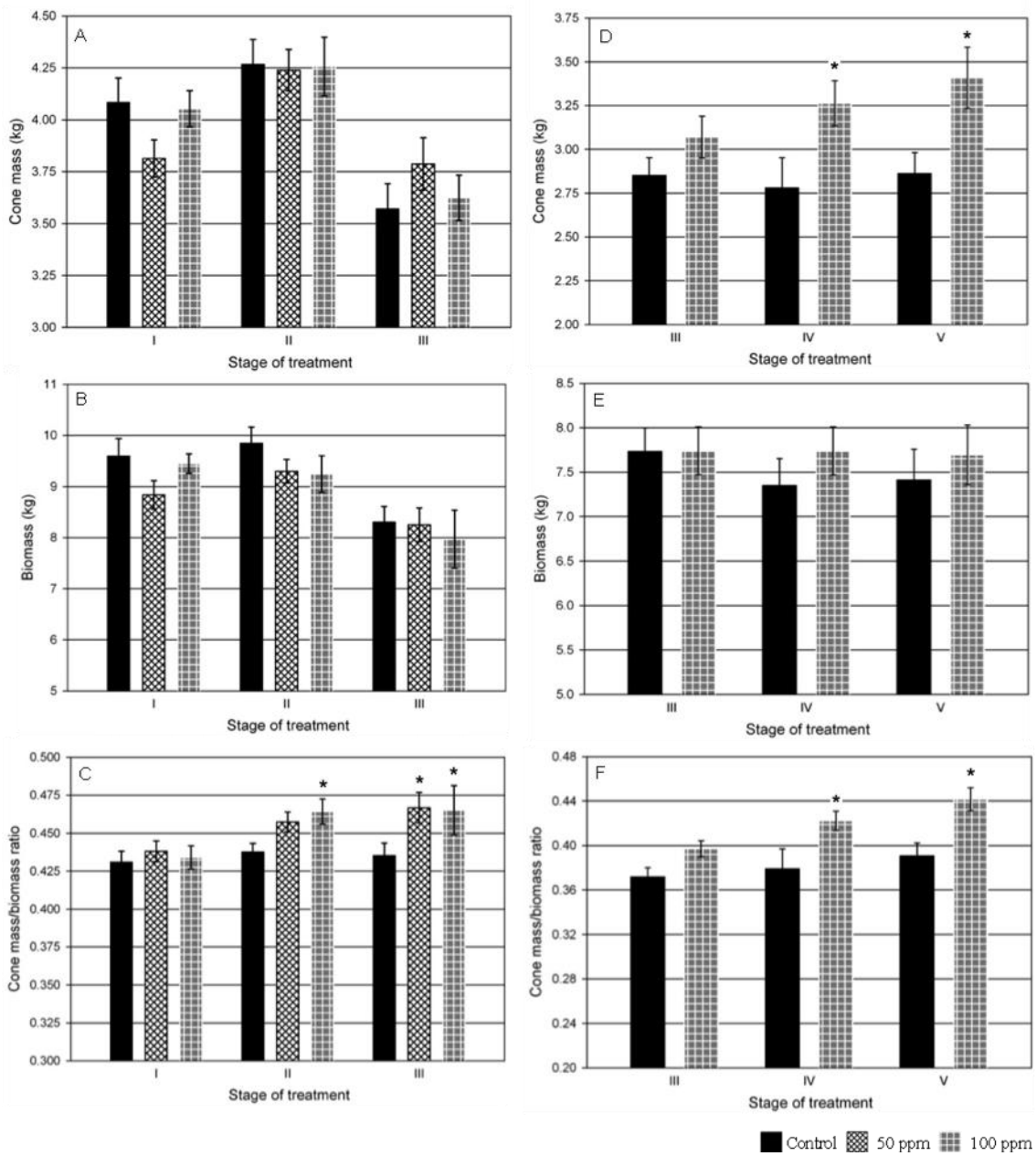


Figure 3.3 Changes in (A) yield, (B) biomass, and (C) yield/biomass ratios of Zeus plants after treatments with 50 and 100 ppm Pro-Ca at stages I, II, and III in season 07. Changes in (D) yield, (E) biomass, and (F) yield/biomass ratios in Zeus plants treated with 100 ppm Pro-Ca at stages III, IV, and V in season 09.

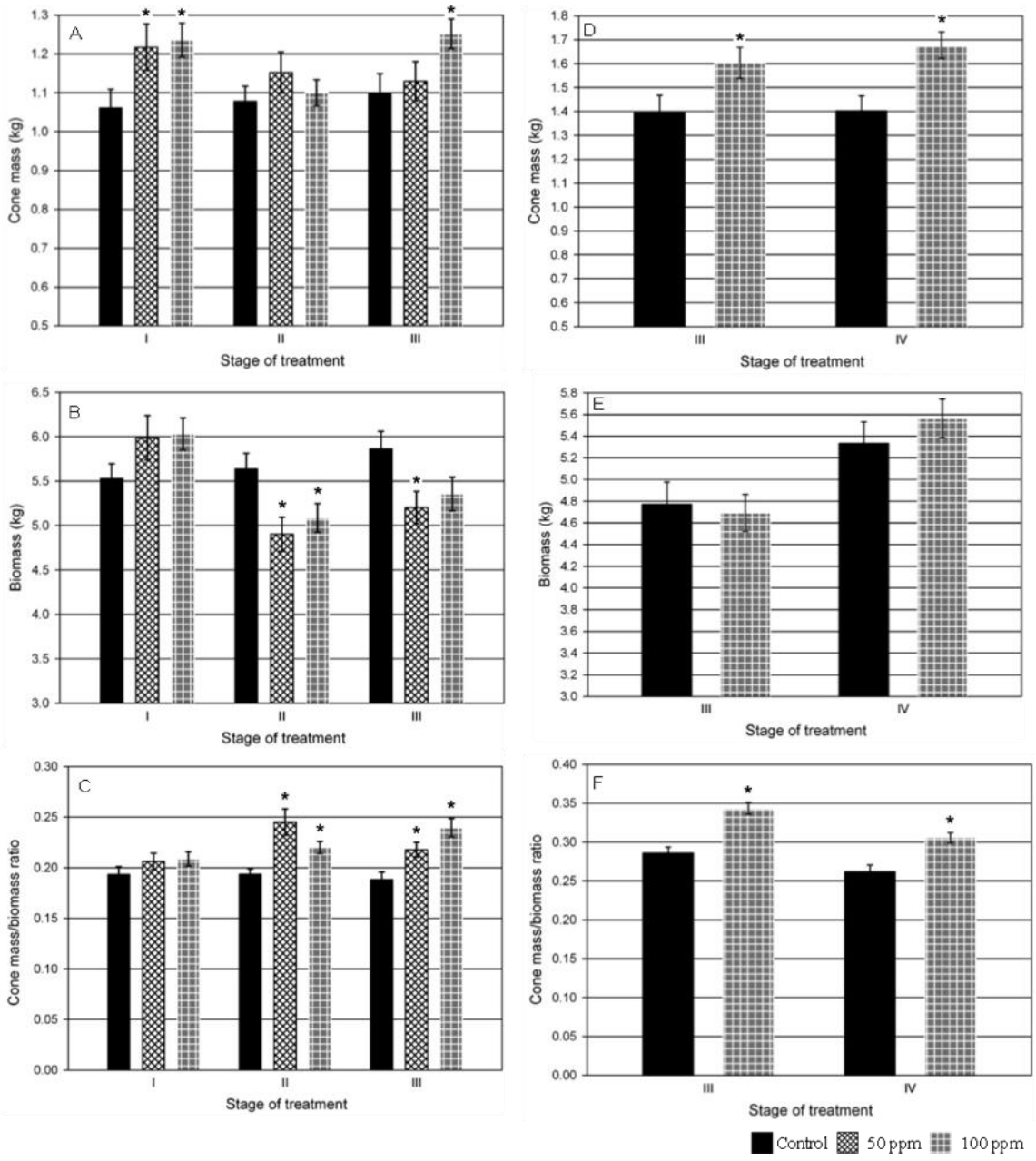


Figure 3.4 Changes in (A) yield, (B) biomass, and (C) yield/biomass ratios of Willamette plants after treatments with 50 and 100 ppm Pro-Ca at stages I, II, and III in season 07. Changes in (D) yield, (E) biomass, and (F) yield/biomass ratios in Willamette plants treated with 100 ppm Pro-Ca at stages III and IV in season 09.

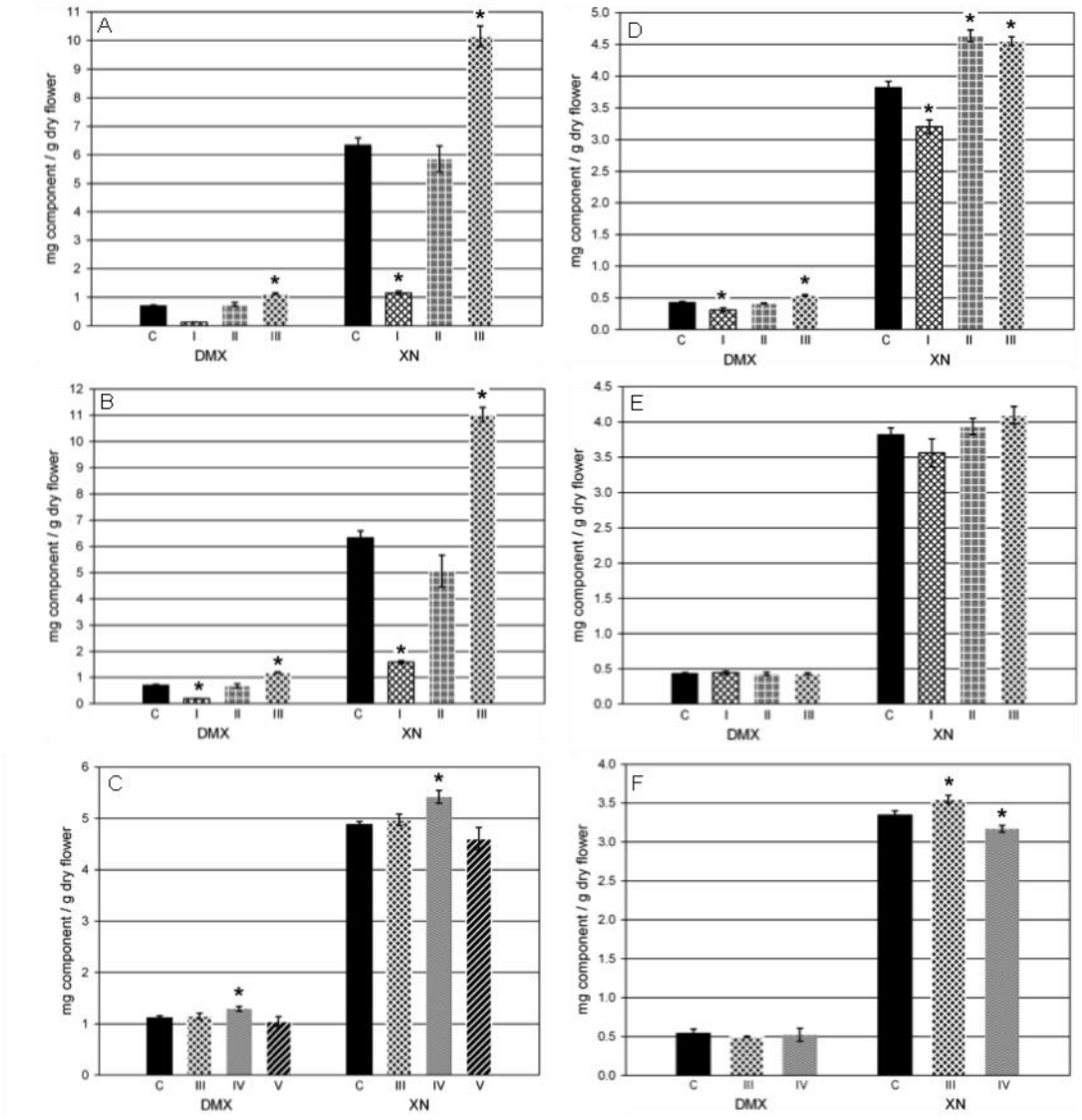


Figure 3.5 Changes in prenylflavonoids desmethylxanthohumol (desmethylxanthohumol) and xanthohumol (xanthohumol) in response Pro-Ca treatment over two seasons. In season 07 Zeus hops were treated with 50 ppm Pro-Ca (A), and 100 ppm Pro-Ca (B) at stages I, II, and III, and season 09; Zeus hops were treated with 100 ppm Pro-Ca (C) at stages III, IV, and V. In season 07 Willamette hops were treated with 50 ppm Pro-Ca (D), and 100 ppm Pro-Ca (E) at stages I, II, and III; and in season 09 Willamette hops were treated with 100 ppm Pro-Ca (F) at stages III and IV.

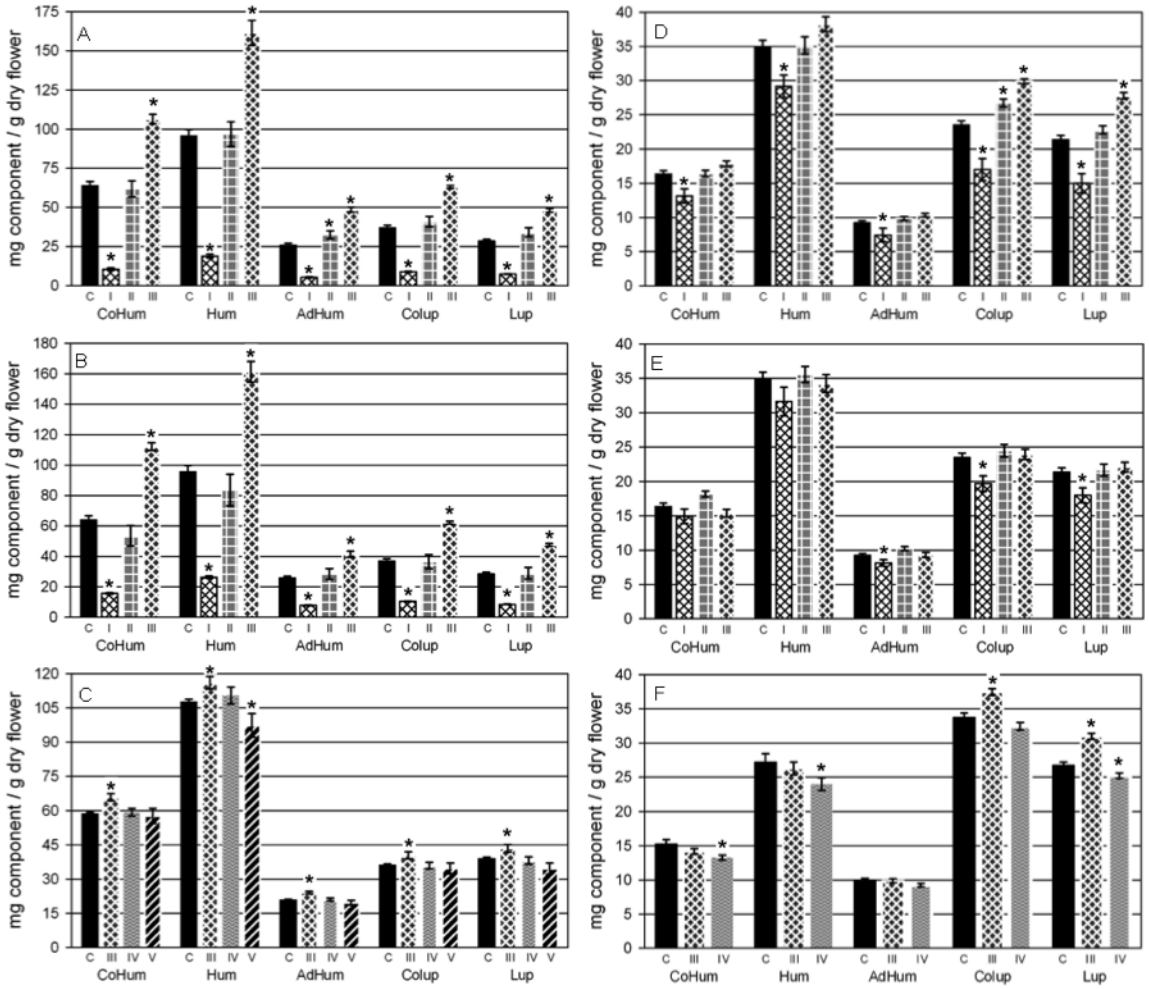


Figure 3.6 Changes in alpha acids cohumulone (cohumulone), humulone (humulone), and adhumulone (adhumulone), and beta acids colupulone (colupulone) and lupulone (lupulone) in response to treatment with Pro-Ca over two seasons. In season 07 Zeus hops were treated with 50 ppm Pro-Ca (A), and 100 ppm Pro-Ca (B) at stages I, II, and III; and in season 09 Zeus hops were treated with 100 ppm Pro-Ca (C) at stages III, IV, and V. In season 07 Willamette hops were treated with 50 ppm Pro-Ca (D), and 100 ppm Pro-Ca (E) at stages I, II, and III; and in season 09 Willamette hops were treated with 100 ppm Pro-Ca (F) at stages III and IV.

Chapter 4: Prohexadione-calcium impacts phenolic acids, flavonoids, and terpenophenolics in hops (*Humulus lupulus* L.) during cone development

4.1 Introduction

Humulus lupulus L., hops, is a perennial vine that produces inflorescences, commonly known as "hop cones," which contain economically and medicinally important compounds. Traditionally, hop cones have been valued for their high content of prenylated acylphloroglucinols, known as α- and β-acids, or humulones and lupulones, which have bittering flavors and antimicrobial properties that make hops a key ingredient in beer (*2*). More recently, a group of biologically active prenylflavonoids, unique to hops, has been identified (*6, 71*).

Prenylated acylphloroglucinols and prenylflavonoids, collectively known as terpenophenolics, are biosynthetically produced via the phenolic acid, polyketide, and methylerythritol 4-phosphate (MEP) pathways (**Figure 4.1**) (*17*). These compounds are produced in hop glandular trichomes, whereas flavonoids and phenolic acids, non-prenylated polyphenols, are found in the photosynthetic bract tissue of the hop inflorescence (**Figure 4.2**) (*18, 79*). In flavonoid biosynthesis malonyl-coenzyme A condenses with a single 4-coumaroyl-coenzyme A to produce chalcone naringenin, the precursor to naringenin. Naringenin can be hydroxylated by flavanone-3-hydroxylase (F3H) to produce polyphenols such as catechins, procyanidins, and flavonols, including kaempferol, quercetin, and their respective glycosides; these compounds are produced in the photosynthetic flower bract tissue of hop cones (**Figure 4.2)** (*18, 79*). Alternatively, in glandular trichomes, chalcone naringenin can be prenylated by hop isoprenyl transferase 1 with the substrate dimethylallyl pyrophosphate (DMAPP) to produce

prenylflavonoids (**Figure 4.1**) (*86*). Production of α- and β- acids occurs by the condensation of malonyl-coenzyme A with isobutryl-coenzyme A or isovaleryl-coenzyme A, degradation products of leucine and valine, respectively. This condensation reaction produces acylphloroglucinols, which are prenylated by aromatic prenyltransferases to produce humulones and lupulones.

Prohexadione-calcium (Pro-Ca) is a 2-oxoglutaric acid-dependent dioxygenase (2-ODD) inhibitor that has determined effects on critical enzymes involved in the production of gibberellins, ethylene, and flavonoids in apples (*49, 91, 92*). Pro-Ca has also been shown to have dramatic growth effects on hop plants when applied to immature stages at high doses (*87*), consistent with its favorable effects among several other agronomically valuable species (*92-96, 124*). In addition to changes in growth and development, Pro-Ca has been shown to alter the production of flavonoids by inhibiting flavanone-3-hydroxylase (F3H), a 2-ODD essential to the biosynthesis of flavonols and procyanidins (**Figure 4.2**) (*52, 56, 99, 100*). In response to Pro-Ca treatment, levels of phenolic acids and flavanones found upstream from F3H increased, while concentrations of compounds found downstream from F3H, including flavonols, flavan-3-ols, and procyanidins, significantly decreased in several species (*52*). In addition to the alteration of known flavonoids, the presence of antimicrobial flavan-4-ols and 3-deoxyflavans were reported in several species, where they were previously unknown (**Figure 4.2**) (*52, 56, 99, 100*).

Hop cones grow and mature over the final two months of the agricultural season, the time when glandular trichomes develop and terpenophenolics and essential oils accumulate in secretory cavities of these structures. Previously, we found significant

changes in accumulation of terpenophenolic contents of hop cones induced by Pro-Ca treatment; the direction and extent of change in terpenophenolics was highly dependent on the developmental stage of cones when the hop plants were treated (*87*). In order to gain a more accurate assessment of the metabolic changes induced by Pro-Ca treatment in hops, 29 compounds including precursors and products of the phenolic acid, flavonoid, and terpenophenolic pathways were quantified from the "Zeus" cultivar at four previously characterized developmental stages (stages I, II, III, and V) (*102*) over 22 days following treatment with Pro-Ca. We also mined the raw MS data in search of additional changes and found two new marker compounds induced by treatment.

4.2 Materials and methods

4.2.1 Plant Material and Treatments and Analyte Standards

Hop cultivar, *H. lupulus* 'Zeus', was grown and treated at Golden Gate Ranches, Hopsteiner-S.S. Steiner, Inc, near Prosser, WA as previously described (*102*). Plants were treated with 100 ppm Pro-Ca in conjunction with 1% (v/v) Regulaid™ (Kalo Inc., USA), a non-ionionic surfactant, in deionized water. Control groups were treated with the surfactant in deionized water, Cones were collected at 2, 7, 15, and 22 days following treatment.

We have previously described five developmental stages of hop inflorescences, which were based on morphological and phytochemical properties of cones collected from near the plant apex (*102*). In the present experiment, we conducted Pro-Ca treatment on plants at what has been previously described as developmental stage I (*102*).

Ten biological replicates were collected for each group of five plants at each time-point; for each sample, ~10 g of fresh cones were collected near the apex of each bine. Samples were flash frozen in field using liquid nitrogen, then stored at -20 ºC until extraction.

4.2.2 Extraction and UHPLC-PDA analysis of terpenophenolics

Hop cones were lyophilized, ground with liquid nitrogen, then extracted in 100% methanol. Prenylflavonoids desmethylxanthohumol and xanthohumol, α-acids adhumulone, cohumulone, and humulone, and β-acids colupulone and lupulone were quantified using a previously developed UHPLC-PDA method (*68*).

4.2.3 Extraction and sample preparation of polyphenols

After removing tissue for terpenophenolic analysis, the remaining lyophilized tissue was extracted by supercritical carbon dioxide using a previously described method (*102*). Dilutions (1:20) were also subject to HPLC-TOF-MS analysis in order to quantify major constituents within the linear response-range of the detectors.

4.2.4 HPLC-TOF-MS analysis of polyphenols

Twenty-two polyphenolic constituents were quantified using a previously developed HPLC-TOF-MS method (*102*). Authentic phytochemical standards were used for all phytochemical quantifications as previously described (*102*). MS data was collected in centroid mode using MassLynx V4.1 Scn 727 and processed using QuanLynx.

4.2.5 MS data analysis

MS data was mined for the differential presence of marker ions in treated samples. Raw MS data was processed using MarkerLynx XS software (Waters Inc.) set at

the following parameters: retention time (t_R) 0.8-55.0 min, mass range 100-1,000 Da, mass tolerance 0.1 Da, noise elimination level of 6.0, ion peak intensity was set to 100 counts, and the t_R window was set to 0.5 min.

4.2.6 Statistical analysis

Statistical tests were conducted using JMP 8.01 (SAS, Cary, NC) software. Statistical significance was determined using the least mean squares within a two-way ANOVA crossing treatment level (0 or 100 ppm Pro-Ca) with post treatment collection time-point (day 2, 7, 14, or 22). Significance was ascribed to differences among means were $p < 0.05$, and are described here as being significant.

4.3 Results and discussion

Previous work has shown value-added changes in cone biomass production and terpenophenolic accumulation resulting from Pro-Ca treatment (*68, 87*). The substantial yield increases with low doses Pro-Ca suggest the crop treatment is industrially relevant, but further work on chemical changes and substantial equivalence of the hop food products was essential. We hypothesized that changes in terpenophenolics are likely paralleled by changes in flavonoids and phenolic acids, which have been altered in several crops following Pro-Ca treatment. Furthermore, due to the transient nature of Pro-Ca inhibition, we hypothesized that effects of treatment would change over time; therefore we assessed the effects of Pro-Ca treatment on terpenophenolics, phenolic acids, and flavonoids from hop cones collected at four developmental stages over a course of twenty-two days.

Hop polyphenols have roles in flavor, stability, foaming, and process of brewed beverages (*45, 46*). Hop polyphenols or "hard resins" are present in raw hops but absent from many extracted, commercial hop products. Careful developmental staging of cones allowed us to show many metabolite changes, which are partially concomitant with increased α- and β-acid accumulation (*102*). Using a previously developed UHPLC-PDA method, we quantitated seven major terpenophenolics including two prenylflavonoids (desmethylxanthohumol and xanthohumol), three α-acids (adhumulone, cohumulone, and humulone), and two β-acids (colupulone and lupulone) (*68*). In addition to terpenophenolics, we quantitated polyphenolic constituents present in photosynthetic bract tissue: three subclasses of flavonoid metabolites found biosynthetically downstream from F3H, (1) flavan-3-ols (catechin and epicatechin) (2) procyanidins (B-1 and B-2) and (3) flavonols (kaempferol and quercetin) and flavonol glycosides (kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-rutinoside); And five subclasses of metabolites found biosynthetically upstream from F3H were also measured, (1) a chalcone (naringenin chalcone) (2) a flavanone (naringenin) (3) phenolic acids (chlorogenic acid, 4-coumaric acid, ferulic acid, and 4-hydroxybenzoic acid) (4) a stilbene (resveratrol) and (5) three amino acids (phenylalanine, leucine, and valine), many of which are precursors in the biosynthesis of the flavonoids and polyketides of interest (**Figures 4.1 and 4.2**). Synthesis and storage of these different metabolite classes are compartmentalized in different mesophyll, epidermis, and glandular trichome secretory cells (*18, 79*).

Significant changes in the content of 24 of the 29 compounds were measured, where steady-state accumulations were quantified at four time-points over 22 days

following treatment of hop plants with Pro-Ca. Compounds within the same structural classes changed in quantity with similar trends in respect to the direction, extent, and timing of changes in treated samples as compared to controls. Increases in terpenophenolics, present in glandular trichomes, were accompanied by changes in the contents of flavonoids, chalcones, and phenolic acids found in the cone bract tissue.

4.3.1 Changes in accumulation of terpenophenolics

We have previously reported significant increases in the terpenophenolic contents of mature hop cones, from the Zeus cultivar, when plants were treated at middle and late stages of flowering (*87*). However, when Zeus plants were treated at an early stage of development, significant decreases in terpenophenolics were measured by the time of harvest, when hop cones had reached full maturity (*87*). In this study, we treated hop plants at an early stage of development and monitored accumulation of terpenophenolics in the days immediately following treatment in order to investigate a wider time-course of changes. The maturity of the cones at time of treatment was morphologically and phytochemically characterized as stage I in our prior work (*102*).

Prenylflavonoid contents increased on day 15 following treatment, then returned to levels found in controls by day 22 after treatment (**Figure 4.3a**). No changes in α- or β-acids were observed through day 15, on day 22 following treatment all five compounds increased (**Figure 4.3b**). Co-multifidol glycoside, a glycosylated prenylated acylphloroglucinol, also increased on day 22 after treatment, but this increase was not statistically significant. With the exception of co-multifidol glycoside, all prenylated compounds studied showed minor decreases 2 days after Pro-Ca treatment, but none of these decreases were statistically significant.

4.3.2 Changes in accumulation of metabolites downstream from F3H

Flavonoids are secondary metabolites resulting from a linear array of pathways, thus, blockage of the early pathway enzymes by inhibitors, leads to decreased contents of the secondary metabolites. A homeostatic network of metabolism (or complex catabolism) is not expected for flavonoid accumulation in terminal, reproductive organs. Reactivation of stored glycosides to aglycones in response to stress is well-documented (*84*), but is not likely to be a major contributor to content distribution in our agronomically-regulated field environment: no major water stress, herbivory, or insect damage was observed during our studies.

Since flavan-3-ols, procyanidins, flavonols, and flavonol glycosides are products derived from dihydroflavonols, and are the terminal products of the hydroxylation of flavanones catalyzed by F3H (**Figure 4.2**), we hypothesized decreased contents of these compounds in response to F3H inhibition. Dihydroflavonols can be oxidized by flavonol synthase (FLS) to produce flavonols; alternatively, dihydroflavonols can be reduced by dihydroflavonol reductase (DFR) to produce flavan-3,4-ols, which can be further reduced by leucoanthocyanidin reductase (LAR) to produce flavan-3-ols. Flavan-3-ols can also form polymeric compounds known as procyanidins. Procyanidans are important to flavor of hops, the processing of beer but also, potentially, to the ecology of the hop crop, as they are antioxidants and anti-herbivory compounds (*43, 73, 74*). The effects of Pro-Ca on accumulation of compounds found downstream from F3H were assessed by the quantitation of flavan-3-ols, procyanidins, flavonols, and flavonol glycosides. Dihydroflavonols and leucocyanidins were not present in crude extracts above the limit of quantitation and were, therefore, not analyzed in this study.

Flavan-3-ols, catechin and epicatechin, significantly decreased in cones collected 2, 7, and 15 days following treatment, with the exception of 2 days following treatment when the decreases in epicatechin were not statistically significant (**Figure 4.3c**). Similar to flavan-3-ols, procyanidins B1 and B2, dimers of catechin and epicatechin, decreased in cones from treated plants throughout the experiment but only significantly decreased on days 7 and 15 following treatment (**Figure 4.3c**). Significant changes in both flavan-3-ols and procyanidins reflect a large and rapid decrease in contents of metabolites present downstream from F3H in response to Pro-Ca treatment. Hop cones also contain several polymeric procyanidins; these compounds were not quantified in this study, but may also be affected by the inhibition of F3H. Current methods for the analysis of polymeric procyanidins are not are compatible with the exploratory nature (large number of samples) of our study design (*43, 73, 74*). High-throughput methods for measuring polymeric procyanidins are being developed in our laboratory.

Flavonols quercetin, kaempferol, and their respective glycosides also significantly decreased following Pro-Ca treatment (**Figure 4.3d**). Quercetin levels decreased on days 15 and 22 following treatment, and kaempferol contents decreased 2 days after treatment. All quercetin glycosides measured, quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside, and quercetin-3-*O*-glucoside, significantly decreased by day 7 after treatment, additionally quercetin-3-*O*-rutinoside levels were significantly lower in treatment plants on day 15 following Pro-Ca application. Kaempferol glycosides, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside, significantly decreased 2 days following treatment. Levels of flavonol glycosides had similar trends in their responses to Pro-Ca treatment as their respective aglycones. Kaempferol, kaempferol-3-*O*-glucoside, and

kaempferol-3-*O*-rutinoside levels all significantly decreased on day 2 following treatment. Similar to kaempferol and kaempferol glycosides, levels of quercetin and quercetin glycosides shared the same trend in content changes in response to Pro-Ca treatment; quercetin content did not significantly decrease until 15 days after treatment and quercetin glycoside levels significantly decreased by day 7 following treatment.

The timing of accumulation of quercetin and quercetin glycosides in comparison to kaempferol and kaempferol glycosides may be related to activity of F3'H on flavonol hydroxylation. Quercetin and kaempferol differ in the extent of hydroxylation on the flavonoid B ring (**Figure 4.1**). Kaempferol has a single OH group on ring B attached at the 4' carbon; whereas quercetin contains two OH groups attached at carbons 3' and 4'. Kaempferol may be hydroxylated on the 3' carbon by F3'H to produce quercetin. Naringenin and dihydrokaempferol, the 4'-hydroxylated precursors to kaempferol, were present in the 80% methanol extract at detectable levels by LC-TOF-MS, while eriodictyol and dihydroquercetin, the 3',4'-hydroxylated precursors to quercetin, were not. Quercetin and quercetin glycosides, however, are present at greater levels than kaempferol and kaempferol glycosides in hop cones. It is therefore likely that F3'H activity is responsible for the accumulation of quercetin in hop cones, and the additional time between Pro-Ca treatment and the responses of quercetin and quercetin glycosides levels, as compared to kaempferol and kaempferol glycosides, may be due to the F3'H catalyzed hydroxylation. Additionally, F3'H is a 2-ODD that may also be inhibited by Pro-Ca. Flavonol production may be further inhibited at flavonol synthase (FLS), which is also a 2-ODD that has been shown to be inhibited by Pro-Ca treatment (*52*). In summary, while varying in the timing and extent of changes, levels of flavan-3-ols,

procyanidins, flavonols, and flavonol glycosides, all biosynthetically produced downstream from F3H, significantly decreased following Pro-Ca treatment (**Figure 4.3c and 4.3d**). These changes paralleled those seen in several other crops and support our hypothesis that Pro-Ca inhibits F3H in hop cones (*52, 56, 99*).

4.3.3 Changes in accumulation of metabolites upstream from F3H

Although previous results from apple trees treated with Pro-Ca showed significant increases in eriodictyol, a 3',4'-hydroxylated flavanone (*52*), we were interested in levels of prenyflavonoid precursor naringenin chalcone, the precursor of naringenin, a 4' hydroxylated flavanone. While hops contain quantifiable levels of naringenin and naringenin chalcone, eriodictyol and eriodictyol chalcone have not been reported in the plant, and we were unable to detect these compounds by TOF-MS. Naringenin and chalcone naringenin contents doubled 2 days following Pro-Ca treatment and slowly returned to values found in controls over 22 days (**Figure 4.3e**). Naringenin contents were significantly greater in treated cones on days 2, 7, and 15 following treatment, and chalcone naringenin levels were significantly greater on days 2 and 7 following treatment. While chalcones have not previously been studied in Pro-Ca experiments, the increases in naringenin we measured in response to treatment parallel previous findings in which flavanones and flavanone glycosides accumulated following Pro-Ca treatment in several crops (*52, 56, 99*).

Ferulic, 4-hydroxybenzoic, 4-coumaric, and chlorogenic acids were measured to gauge the changes in steady-state accumulations of phenolic acids to Pro-Ca treatment. These compounds are significant as ferulic, chlorogenic, and 4-hydroxybenzoic acids are all produced from 4-coumaric acid, which is a precursor compound in naringenin

chalcones synthesis. Levels of all phenolic acids measured significantly increased following Pro-Ca treatment (**Figure 4.3f**). Coumaric acid and 4-hydroxybenzoic acid levels significantly increased on days 7 and 15 after treatment. Ferulic acid contents significantly increased 7 days after treatment, and chlorogenic acid levels significantly increased on day 15 following treatment. Contents of all four phenolic acids in treated cones returned to levels found in controls by day 22 days following treatment. Our results parallel those from other Pro-Ca studies where treatment of several crops induced accumulation of phenolic acids, which represents more widespread metabolic changes as phenolic acids are present several biosynthetic steps upstream from F3H (*52, 56, 99*).

Coumaric acid and naringenin chalcone are precursor compounds to prenylflavonoid biosynthesis (*16, 17, 29*). However, a direct correlation between the increases in these precursor compounds and the accumulation of terpenophenolics is not likely. The inhibition of F3H by Pro-Ca treatment has previously shown to increase flavanones and phenolic acids in vegetative tissues; based on previous studies we hypothesize that the effects on phenolic acids and chalcone naringenin is likely occurring in the vegetative bract tissue of hop cones. Terpenophenolics accumulate in the glandular trichomes present on the abaxial surface of bracteoles present in hop cones, whereas flavonoid and phenolic acid constituents are found in the leafy bract tissue (*18*). Partitioning is likely to occur at the level of primary metabolism, especially by influences on the carbohydrates and amino acids pools that are shared across different organs of the inflorescences (*19*).

Despite increases in terpenophenolic accumulation, we did not detect changes in the levels of phenylalanine, leucine, or valine after Pro-Ca treatment. Phenylalanine is an

aromatic precursor to phenolic acid, flavonoid, and prenylflavonoid production, leucine, from which the activated degradation product, butyryl-CoA, is used in adhumulone, cohumulone, and humulone synthesis, and valine, from which the activated degradation product, valeryl-CoA, is used in colupulone and lupulone **(Figure 4.1**) While terpenophenolic production does provide a significant sink for phenylalanine and the degradation products of leucine and valine, these substrates are used in multiple cellular processes and their relative contents are likely the result of multiple sink allocations, replenishment from branch-chain amino acid metabolism, and transport. We have previously discussed the potential inhibitory effects of Pro-Ca on amino transferases, but this is not suggested from our results (*87*). Overall, we measured significant increases in several metabolites found upstream from F3H in the flavonoid and phenolic acid pathways following treatment of hop plants with Pro-Ca (**Figure 4.3e and 4.3f**).

Production of novel constituents. MS data mining revealed an ion detected at Rt 24.5 min in +ESI mode with a *m/z* of 275.0941 amu; the elemental composition of this ion corresponded to $C_{15}H_{15}O_5$ (2.2 mDa). The marker ion was present at low levels in all treatment extracts and absent from controls (**Figure 4.4**). The elemental composition of this ion matches the $[M+H]^+$ ion of the 3-deoxyflavan luteoliflavan and flavan-4-ol apigenin. Given the presence of naringenin and the lack of eriodictyol in hops, it is possible that DFR catalyzed the production of apigenin from the increased pool of naringenin, which accumulated following Pro-Ca treatment. Additional experiments are necessary for unambiguous identification of this metabolite. Due to the low levels accumulation of this component in our treatment regime, large-scale extraction followed by extensive separation is likely required.

Our results match those from previous experiments where novel compounds were also produced in several agricultural species following treatment with Pro-Ca. Flavan-4-ols and 3-deoxyflavans including luteoforol, luteoliflavan, and luteoliflavan 5-glucoside, as well as eriodictyol glucosides accumulated in *Malus* x *domestica, Pyrus communis*, *Vitis vinifera*, *Rosa* x *hybrida*, *Fragaria* x *ananassa, Actinidia* sp., *Prunus persica,* and *Vaccinium macrocarpun*, where they had never previously been found, when treated with Pro-Ca (*52, 55, 56, 99, 125, 126*). These are phytoalexic compounds and induced resistance against pathogens including *Erwinia amylovora*, the source of a devastating disease in pome fruit trees known as fire blight (*52, 55, 91, 126*). Luteoforol and luteoliflavan occur naturally in *Sorghum* where they are produced by the reduction of the carbonyl group on carbon 4, which is catalyzed by flavanone 4-reductase (FNR) (*65*). Dihydroflavonol reductase (DFR) shares significant homology with FNR, and DFR has demonstrated FNR activity *in vitro* where it produced flavan-4-ols from flavanones; crude enzyme extracts from *Malus* x *domestica* have been shown to reduce eriodictyol to luteoforol, but did not accept naringenin as a substrate (*64, 127*). Although the substrate specificity of DFR varies among different species and has been shown to catalyze the reduction of naringenin to produce the flavan-4-ol apiferol in some plants (*128, 129*), under normal conditions DFR catalyzes the reduction of dihydroxyflavonols to produce leucocyanidins; however, with to Pro-Ca treatment, and with the accumulation of eriodictyol, DFR activity was responsible for the production of luteoforol (**Figure 4.1**). In both control and treated hop cones, no eriodictyol or eriodictyol derivatives were detected, while significant accumulation of naringenin occurred with Pro-Ca treatment.

Marker analysis also revealed an additional ion that produced 2.2-2.5 times less MS signal in treatment samples in comparison to controls, when collected on days 7, 15, and 22 following Pro-Ca treatment. The marker compound produced four ions at Rt 21.6 min, with masses detected in +ESI mode of *m/z* 649.1369, 627.1550, 465.1018, and 303.0480 amu; elemental composition analysis of each ion resulted in the molecular formulas of $C_{27}H_{30}O_{17}Na$ (1.2 mDa), $C_{27}H_{31}O_{17}$ (1.1 mDa), $C_{21}H_{21}O_{12}$ (1.5 mDa), and $C_{15}H_{11}O_7$ (2.5 mDa), respectively. We have putatively identified this compound as a quercetin dihexoside, as the detected ions matched the sodium adduct of the molecular ion ($C_{27}H_{30}O_{17}Na$), the molecular ion ($C_{27}H_{31}O_{17}$), loss of a single hexose (−172 amu, $C_{21}H_{21}O_{12}$), and a second hexose (−172 amu, $C_{15}H_{11}O_7$) units. The later marker ion matched with the aglycone ion of quercetin ($C_{15}H_{11}O_7$, −2.5 mDa). Significant decreases in this proposed flavonol glycoside correlates to the effects we measured in known flavonol glycosides.

An additional two ions were detected at Rt 23.9 min, with masses in +ESI mode of *m/z* 367.1615 and 389.1424 amu; elemental composition analysis matched well with what is likely to be a molecular ion with a formula of $C_{15}H_{27}O_{10}$ (1.1 mDa) and its sodium adduct $C_{15}H_{26}O_{10}Na$ (0.0 mDa). These ions were present in all treated samples and absent from controls (**Figure 4.4**). While the number of carbons matched those found in the basic flavonoid skeleton, the number of oxygen and hydrogen atoms is much greater than those normally found in this group of compounds. Both marker compounds were present at very low levels and therefore structure information based on TOF signals was limited. Isolation and structure elucidation of these components is a topic of ongoing investigation.

In summary, following Pro-Ca treatment of hop plants, cones accumulated higher levels of terpenophenolics than untreated hops; increases in phenolic acids, chalcone naringenin, and naringenin also occurred. Increased production of these compounds, located biosynthetically upstream from F3H, coincided with significant decreases in catechins, procyanidins, flavonols, and flavonol glycosides, which are found biosynthetically downstream from F3H in the flavonoid pathway. In most cases, steady-state contents of compounds found downstream from F3H rapidly decrease in content in response to Pro-Ca treatment, which occurred on days two and seven following treatment. As the season progressed several compounds in treated cones returned to levels found in controls. This is likely due to the metabolism of Pro-Ca within the plant; Pro-Ca has a short half-life of a few weeks and is rapidly broken down into endogenous plant metabolites (*130*).

Other studies of metabolic perturbation induced by Pro-Ca treatment including effects on flavonoid and phenolic acid biosynthesis have been reported, but no other reports of Pro-Ca effects on glandular trichome secondary metabolite accumulation over time have been reported. Our results parallel those found in leaves of apple trees following treatment with Pro-Ca and add additional insight into changes of phenolic acids, flavonoids, and hop-specific terpenophenolic metabolism induced by Pro-Ca. Previously, analysis of metabolic effects of Pro-Ca on the phenolic acid and flavonoid pathways in apple included quantification of 12 components found throughout these pathways (*52*). In this study we developed a more comprehensive analysis and obtained leads for the production of two marker ions in hops, using TOF-MS, and have built on previous studies which suggest the value of Pro-Ca for crop improvement in hops (*68*,

87). Hops treated with Pro-Ca, exhibit changes in phytochemical content, many of which seem not to extend throughout maturity. Changes in chemical content are consistent with other Pro-Ca modified agricultural products which have met with deregulatory approval and widespread industrial use. Larger-scale agronomics experiments are proposed, as has occurred during agrichemical deregulation with other crops, and further application knowledge for successful seasonal, varietal and climatic variants in treatment are imminent.

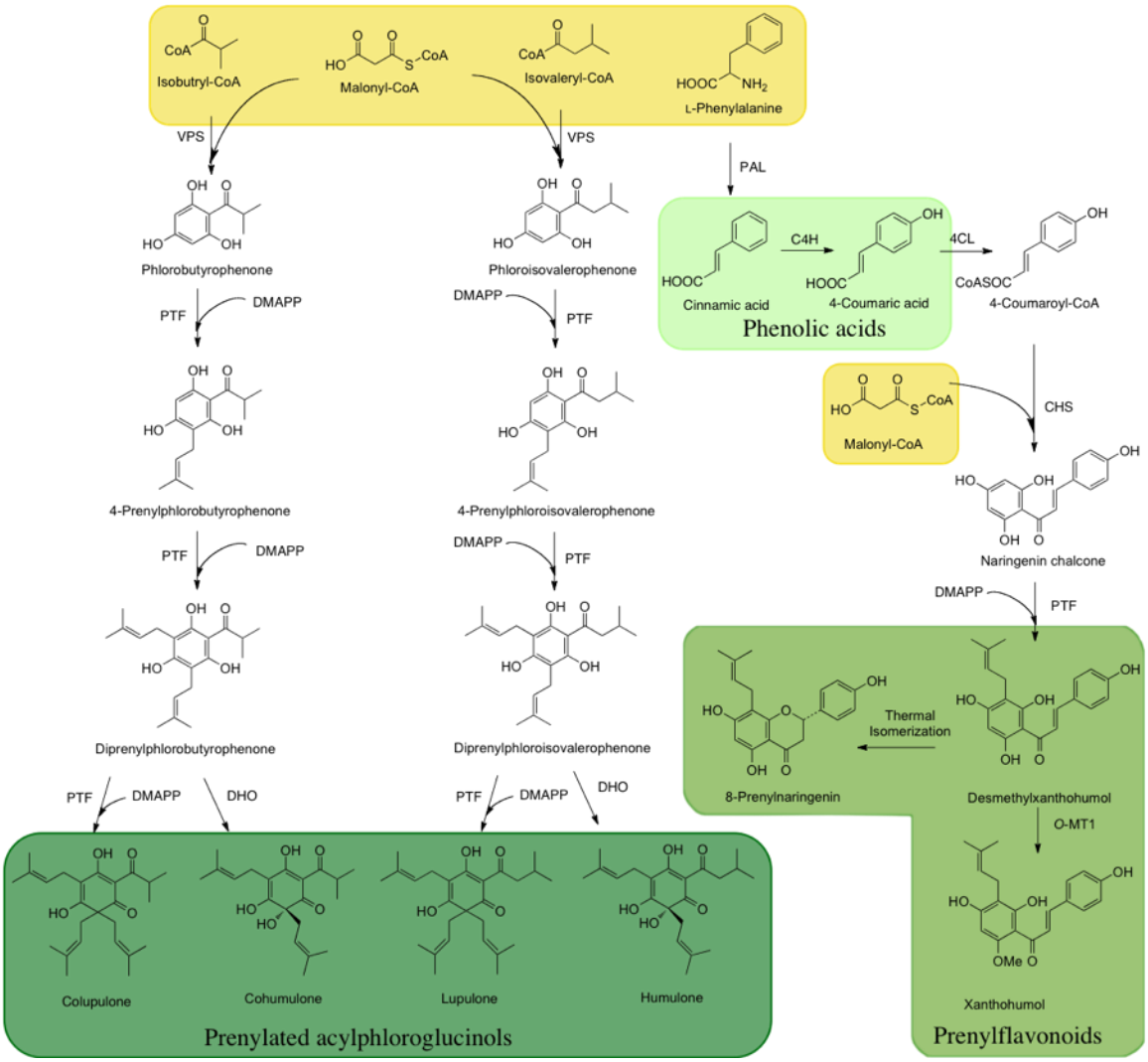


Figure 4.1 Terpenophenolic biosynthetic pathways from hop glandular trichomes. Primary metabolites are seen in yellow and groups containing phenolic acids and terpenophenolics quantitated in this study are various grades of green. Abbreviations are VPS: valerophenone synthase, PTF: aromatic prenyltransferase, DHO: deoxyhumulone oxidase, PAL: phenylalanine ammonia lygase, C4H: cinnamate 4-hydroxylase, 4-CL: coumarate coenzyme A ligase, CHS: chalcone synthase, *O*-MT1: *O*-methyl transferase 1, DMAPP: dimethylallyl diphosphate.

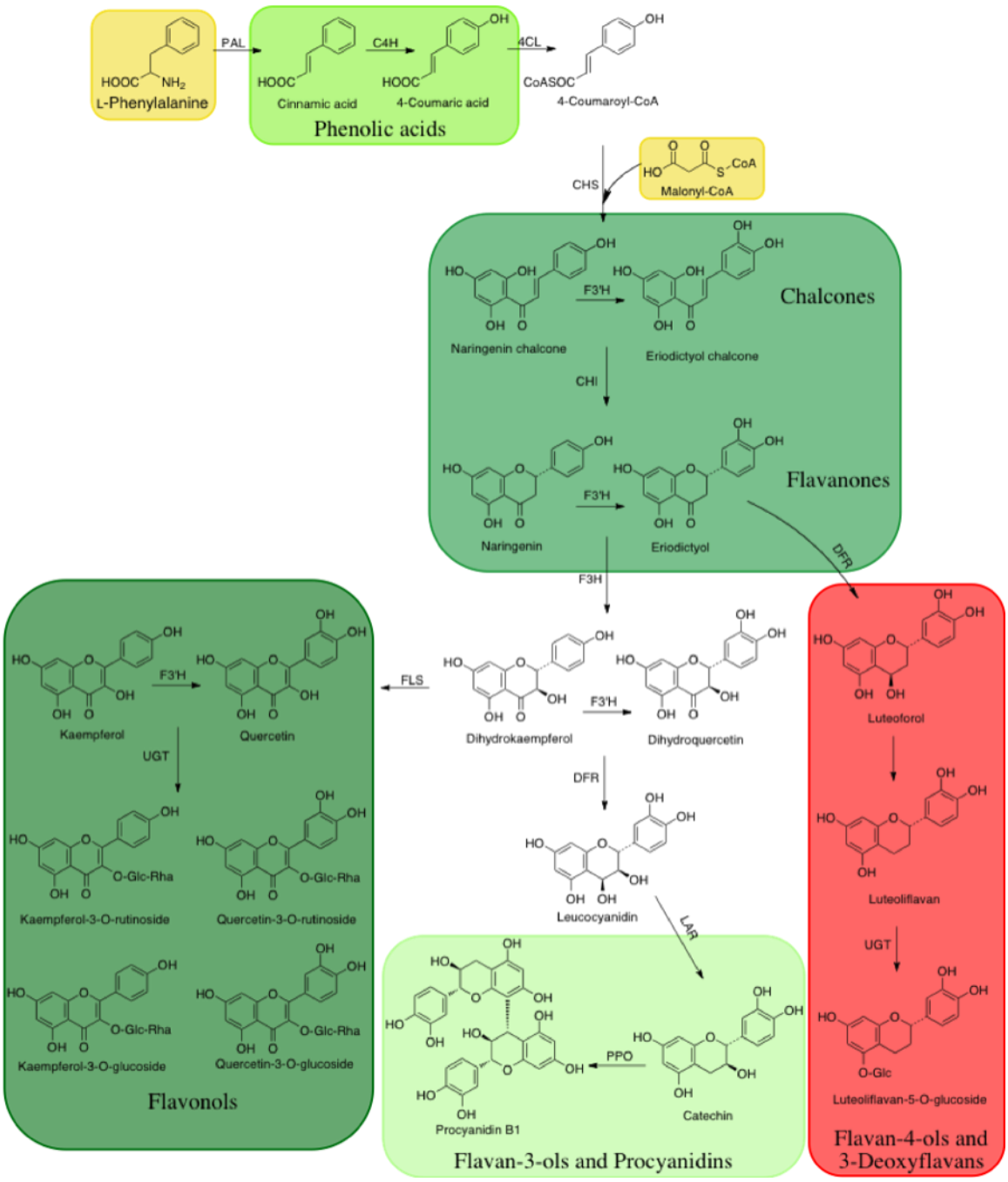


Figure 4.2 Flavonoid and phenolic acid biosynthetic pathways from bracts and bracteoles of hop cones, including biosynthesis of flavan-4-ols and 3-deoxyflavans (red) which occurred in several crops following treatment with Pro-Ca. Primary metabolites are seen in yellow and groups containing phenolic acids and flavonoids quantitated in this study are various grades of green. Abbreviations are PAL: phenylalanine ammonia lygase, C4H: cinnamate 4-hydroxylase, 4-CL: coumarate coenzyme A ligase, CHS: chalcone synthase, CHI: chalcones isomerase, F3H: flavanone-3-hydroxylase, F3'H: flavanone-3'-hydroxylase DFR: dihydroflavonol reductase, LAR: leucocyanidins reductase, PPO: polyphenol oxidase, UGT: glycosyl transferase.

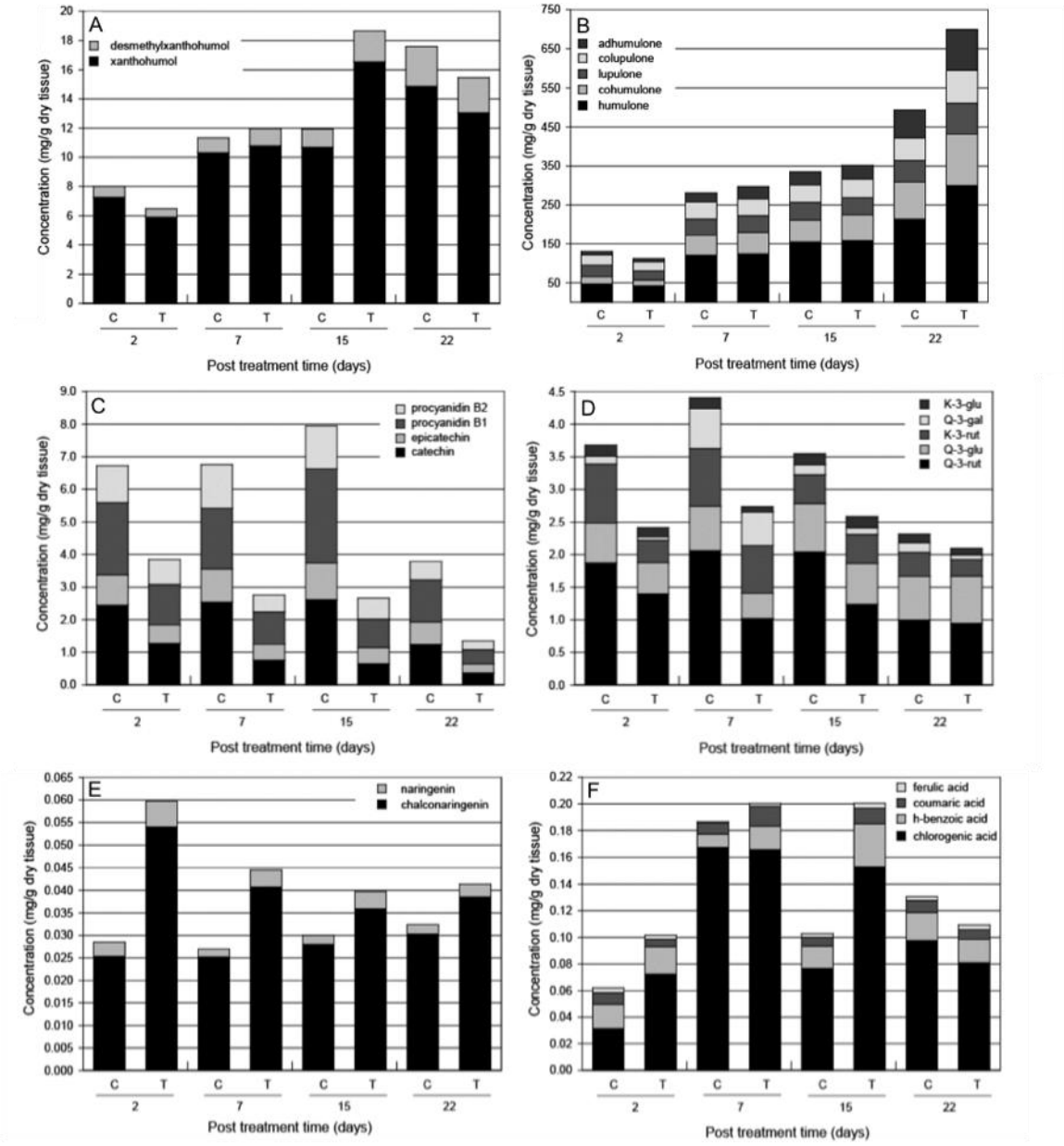


Figure 4.3 Effects of Pro-Ca on (A) prenylflavonoids xanthohumol and desmethylxanthohumol, (B) α- acids adhumulone, cohumulone, humulone and β-acids colupulone and lupulone, (C) flavan-3-ols and procyanidins, and (D) flavonol glycosides,(E) naringenin chalcones and naringenin and (F) phenolic acids chlorogenic acid, 4-hydroxybenzoic acid, 4-coumaric acid and ferulic acid. Control (C) and Pro-Ca treated (T) were quantitated on days 2, 7, 15, and 22 following Pro-Ca application. Compounds abbreviations are K-3-glu: kaempferol-3-*O*-glucoside, Q-3-gal: quercetin-3-*O*-galactoside, K-3-rut: kaempferol-3-*O*-rutinoside, Q-3-glu: quercetin-3-*O*-glucoside, Q-3-rut: quercetin-3-*O*-rutinoside, and h-benzoic acid: 4-hydroxybenzoic acid.

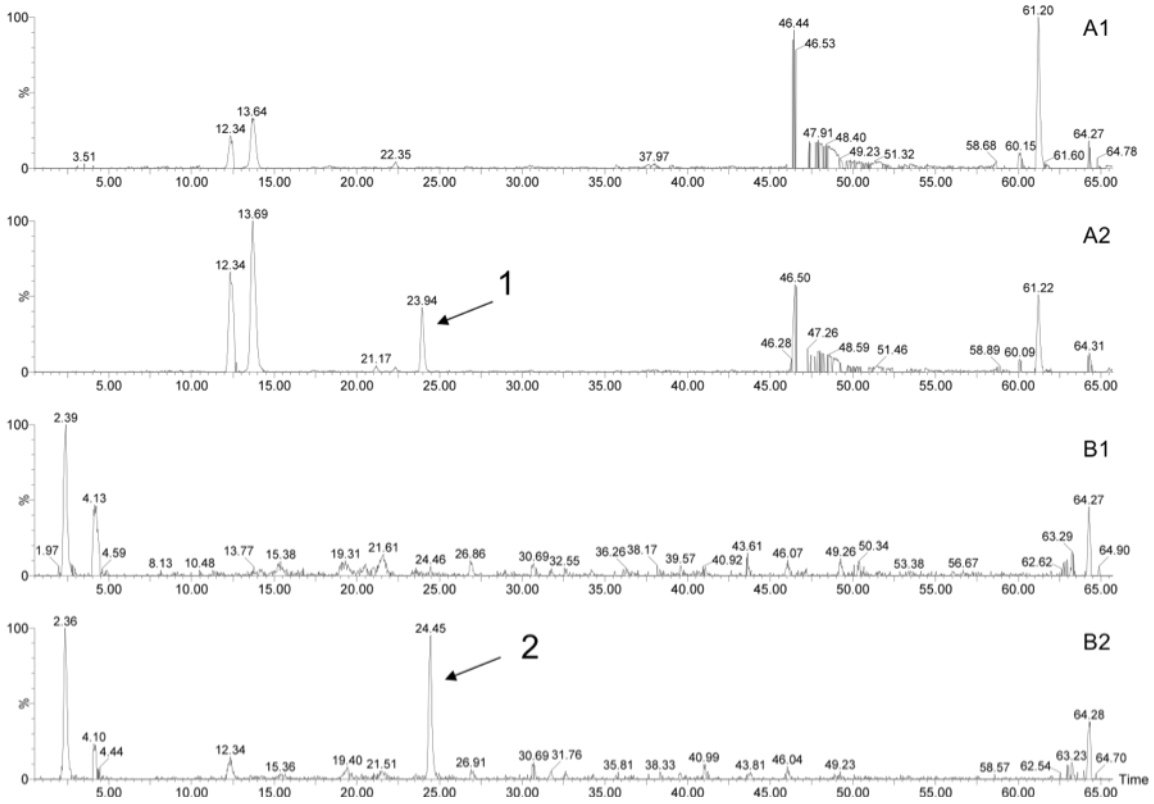


Figure 4.4 Extracted ion chromatograms from controls (A1 and B1) and treated (A2 and B2). Chromatograms were extracted at (A) *m/z* 367.1615 amu and (B) *m/z* 275.0941 amu. Marker compounds are labeled as compounds 1 and 2.

Chapter 5: Conclusions and discussion

5.1 Summary of results

Since hop cone chemical physiology is intimately associated with growth and morphological development of the agricultural product, I have presented gross and detailed morphological observations of hop cones during maturation for two hop cultivars. I measured morphological and phytochemical changes in hop cones over developmental time and then measured the effects of prohexadione-calcium (Pro-Ca) on these processes. In late stages of cone development, when terpenophenolic compounds, of interest to both the brewing industry and biomedical research, are produced, senescence of the stigmas and ovaries coincides with the elongation of bracts and bracteoles, which contain polyphenols, and development of sessile glandular trichomes, in which terpenophenolics accumulate. I focused my experiments on these stages, as they are most agriculturally relevant and developmentally complex.

Specifically, I quantitated terpenophenolic and polyphenolic compounds from hop cones collected at each of the five time points at which Pro-Ca was applied. I used gross cone morphology, including volume, mass, and density, and detailed inflorescence morphological observations, such as elongation of bracts and bracteoles, and development of glandular trichomes in order to describe each collection. In compiling this data I established an index of morphological and phytochemical characters to distinguish the five developmental stages (I-V) when we applied Pro-Ca (**Table 2.1**).

I conducted two groups of experiments to test the effects of Pro-Ca on hops. The first experimental design focused on industrially relevant measurements, such as terpenophenolic content of kiln dried cones, total plant biomass, and total cone biomass. I

measured significant increases in terpenophenolic content, cone biomass, and cone biomass/total above ground biomass ratios, which occurred following Pro-Ca treatments at middle and late stages of cone development (stages III-V). Treatment of hops during earlier stages of development (stage I) induced significant decreases in terpenophenolics; and only a few increases in cone biomass were observed from these treatments. While no major changes in either terpenophenolic content or cone biomass were observed following treatments conducted at stage II, when Zeus and Willamette hops were treated at stages III-V (III-IV in Willamette), favorable agronomically effects occurred. In conducting this research I have developed a general guideline for use of Pro-Ca on hops to produce value-added effects.

Willamette plants had greater variability in changes in terpenophenolic contents in their response to Pro-Ca; variability itself was Pro-Ca dosage-dependent. For example, Willamette plants treated with 50 ppm Pro-Ca produced cones with similar terpenophenolic profiles as Zeus hops; plants treated at stage I produced cones with significantly decreased levels of all terpenophenolics, those treated at stage II showed little to no changes, and those treated at stage III had increased contents of terpenophenolics. However, Willamette plants treated with 100 ppm Pro-Ca at the same stages produced cones with few changes in terpenophenolic contents. My results suggest that Pro-Ca effects on terpenophenolic contents vary dependent on cultivar and developmental stage and in some cases on dosage of treatment.

I conducted additional experiments to investigate the developmental effects of Pro-Ca on polyphenols and terpenophenolics over 22 days following treatment. Pro-Ca is a transient enzyme inhibitor, with a half-life of a few weeks *in planta*; therefore it is of

interest to understand the changes in plant metabolism induced over time after a single time-point of application. Therefore, I collected cones at four time-points following a single treatment; collections were made two, seven, 15, and 22 days following treatment. My results showed rapid changes in polyphenols and phenolic acids, which occurred within the first few days after treatment. Polyphenols, such as flavonols and flavan-3-ols found biosynthetically downstream from F3H significantly decreased, while compounds found biosynthetically upstream, including flavanones, chalcones, and phenolic acids, significantly increased following treatment. These changes were likely the result of the inhibition of flavanone-3-hydroxylase (F3H) as Pro-Ca has been shown to inhibit F3H in several species (*52, 56, 99, 100*). The changes in flavonoids are significant for the agricultural and food industry, as they coincide with significant increases in prenylflavonoids and terpenophenolics that were also measured on days 15 and 22 following Pro-Ca treatment.

5.2 Future directions

Chapter 2 relates the morphological and phytochemical changes over five stages of cone development (**Table 2.1**). This work provides a scaffold for future analysis of hops from additional seasons, cultivars, and locations. Seasonal variation has significant impacts on the hop industry and key markers used to accurately identify developmental stages can inform agronomists and researchers of when best to apply chemicals, conduct experiments, and time harvests to maximize product quality. Much importance lies in continuation of the scaffold, but also of interest is the incorporation of less-well studied hop phytochemicals than the compounds presented here. Polyphenol accumulation over

time has not been as well studied, and does not show as strong of a correlation to changes in morphological characters. Additional seasons, and possibly quantitation of additional polyphenols, may help address remaining questions.

Essential oil accumulation is a major interest in hop agriculture (*2*). Methods for the analysis of essential oils in hops have been established and quantitation of these compounds should be added to this developmental portrait (*131, 132*). Since essential oils are produced in glandular trichomes, glandular trichome development will likely correlate with terpene accumulation (*11, 77*). Polymeric procyanidins are additional flavor compounds to consider. In this study I quantitated dimeric procyanidins (procyanidins B1 and B2); additional oligomeric polyphenols have been identified from hops and may have significant impact on flavor (*43, 74*). Quantitation of these compounds remains a challenging task, as the availability of standards is limited and extraction procedures are time-consuming and do not lend themselves to high-throughput methodologies (*43, 74*). In this work I developed methods for wide-scale quantitation of polyphenols and terpenophenolics. By adding polymeric polyphenol and essential oil quantitation through further work, my studies may be extended towards a better understanding of flavor compounds in hops.

While Pro-Ca treatment of hops has potential for the hop industry, the outcomes are highly dependent upon the timing and methods of Pro-Ca application. Genotype, Pro-Ca dosage, and timing-of-application were all significant variables that determined the outcome of treatments on cone biomass and terpenophenolic contents. My results demonstrate that treatment of hops at developmental stages III and IV induces significant increases in cone biomass production and terpenophenolic content. Future studies should

focus on treatments conducted at or near stages III and IV, potentially including between-stage treatments, which may also be a useful addition to the five stage developmental index.

These experiments were conducted on small plots with limited numbers of plants. The methods I employed allowed for collection of maximum data with my available resources; over three seasons more than 1500 plants were analyzed for their cone terpenophenolic content, total above ground biomass, and total cone biomass. Large labor resources were required to collect this amount of biological sample and analytical data. In order to further assess the industrial potential for use of Pro-Ca on hops, future studies should use more agronomically relevant methods; for example large-scale treatments with industrial sprayers, mechanical harvest and terpenophenolic analysis of large bulked samples, protocols including the use of harvest combines, industrial hop picking machines, kilns and food packaging systems. While bulk methods may be more relevant to an agronomic assessment of the efficacy of Pro-Ca treatment of hops, variation data would be sacrificed as large industrial sized measurements do not include numerous data points. In order to most effectively evaluate the industrial potential of Pro-Ca application to hops, a combination of methods presented here and industrial methods should be conducted, as feasible. While industrial-scale methods can help assess the utility of Pro-Ca in hop agriculture, only small data sets are produced from these experiments. Smaller research-scale experiments, as were conducted in this work, apply methods that produce large data sets which provide greater power in the statistical analysis of the results. Therefore, a combination of large industrial-scale experiments and smaller research-scale experiments are necessary to assess the implications of Pro-Ca treatment on hop

agriculture and provide strong scientific evidence for results. Use of industrial sprayers is important for future studies, as these machines are streamlined for maximal coverage of plants, and may therefore increase the efficiency of application and use of Pro-Ca.

It is also imperative to assess the effects of Pro-Ca on the essential oil content of hop cones. Hop essential oils, which impart aroma to beer, are a main focus of breeding in aroma hop cultivars. If broad changes in carbon resource partioning are induced by Pro-Ca treatment, and general responses in primary and secondary metabolism are occurring, it is highly probable that essential oil biosynthesis would also be affected. Methods for quantitation of essential oils have been developed for hops, and application of these methods to Pro-Ca treated samples would help assess the effects of Pro-Ca on hop quality (*131, 132*).

In addition to future experiments addressing the agronomic and industrial potential of Pro-Ca treatment of hops, understanding the biochemical, genetic, and physiological mechanisms underlying the positive agronomic effects are compelling for future research.

Significant increases in cone biomass production and terpenophenolic content may be related to the effects of Pro-Ca on plant hormone biosynthesis. Pro-Ca has been shown to inhibit enzymes critical for gibberellic acid and ethylene biosynthesis in a number of agricultural crops (*49, 90-92*). As discussed in **Chapter 3**, Pro-Ca application may either decrease or increase levels of active gibberellic acids in plants, depending upon timing of treatment and species of active GA. Application of Pro-Ca early in cone development has been shown to inhibit GA3ox, which catalyzes the synthesis of the active gibberellic acid GA_1 from its inactive precursor GA_{20}. However, Pro-Ca has also

been shown to inhibit GA2ox, an enzyme that catalyzes the catabolic deactivation of GA_1 to the inactive gibberellic acid GA_8. Gibberellic acid profiles change as the growing season progresses; in hops active gibberellic acid content has been shown to increase over the vegetative growth period, which is followed by significant decreases just prior to the transition to reproductive phases (*112*). When Pro-Ca was applied in the early vegetative season, active gibberellic acid synthesis was inhibited and internode elongation significantly decreased in several crops (*59*). However, when plants were treated late in the season, after most vegetative growth had occurred, deactivation of the active gibberellic acid was inhibited and internode elongation and overall growth increased (*104, 105*). In my experiments I treated hop plants after the majority of vegetative growth had occurred. It is therefore possible that treatment of hop plants during late season development may increase levels of active gibberellic acids. When active gibberellic acids were applied to hops, levels of α- and β- acids and cone biomass production significantly increased (*109-111*). Methods for gibberellic acid quantitation have been developed for hops (*112*) and applying these methods to Pro-Ca treated hops may help elucidate potential underlying mechanisms by which Pro-Ca changes hop cone biomass production and terpenophenolic accumulation. Furthermore, comparisons of changes in cone biomass production and terpenophenolic contents of hop plants treated with Pro-Ca with plants treated with active gibberellic acids may provide insight into the relationship of Pro-Ca induced responses and effects induced by active gibberellic acids.

Gibberellic acid also plays an important role in glandular trichome formation in some species; treatment with active gibberellic acids has induced early initiation of trichome development in *Arabidopsis thaliana* (*113-115*). Glandular trichome density has

also been altered in response to exogenous treatment with active gibberellic acids as has been observed in *Origanum* x *intercedens* (see **Chapter 3**). In hops, glandular trichome density is likely correlated with terpenophenolic accumulation, and an increase in trichome density could account for the increases in terpenophenolic contents, which were observed following Pro-Ca treatment. Gland counting presents several challenges and a high-throughput method to collect this information has not yet been developed. In early stages of my method development I tried to establish a video-graphic method to count glands, however this remains a challenge.

Pro-Ca also inhibits 1-aminocyclopropane-1-carboxylate oxidase which catalyzes the final step in ethylene biosynthesis (*51*). Increases in fruit yield and changes in the timing and extent of flowering occurred in response to Pro-Ca treatment of *Malus* x *domestica*; these effects are likely due to inhibition of ethylene production (*49, 98*). The effects of ethylene on hops is unknown; there have been no published studies on this topic. Methods have been developed to quantitate ethylene content in other plants; however plants must be grown in contained environments or special collection devices must be used (*133, 134*). Method development for ethylene poses challenges due to hop plant stature. Correlation of gibberellic acid and ethylene quantities with terpenophenolic and polyphenol contents, and gross cone morphology, including total cone biomass measurements, could provide more comprehensive understanding of hormonal effects on these processes.

In **Chapter 3**, my developmental study of the effects of Pro-Ca on hops over time revealed several changes in secondary metabolism. Significant changes were measured in most polyphenols and terpenophenolics quantitated. Changes in polyphenols were likely

a direct result of the inhibition of F3H, while the increases in terpenophenolics I measured were likely due to indirect effects of F3H inhibition and/or effects of Pro-Ca on other biochemical pathways. Polyphenol biosynthesis is localized in the photosynthetic cone tissue of the bracts and bracteoles, while terpenophenolic biosynthesis occurs in glandular trichomes present on the perianth and abaxial surface of bracts in hop cones. Therefore, changes in polyphenol accumulation are most likely not directly related to increases in terpenophenolic accumulation. In order for these processes to be directly related, the biosynthetic precursors, such as chalcone naringenin and phenolic acids, which increased in response to treatment, would have to be translocated from bract and bracteole cells to glandular trichomes where essential enzymes are present for terpenophenolic biosynthesis. Rather, it is more likely that a decrease in polyphenols, namely flavonols and flavan-3-ols, would result in decreased sink loads on primary metabolites used in terpenophenolic biosynthesis, such as sugars and amino acids. These decreased sink loads may provide increased sources of primary metabolites for terpenophenolic biosynthesis, and the translocation of biosynthetic resources to gland secretory cells. Results from these studies of hop cone phytochemical development and the effects of Pro-Ca on these processes provide insight for future investigation of flux and partitioning relationships between plant secondary metabolites.

In order to test these hypotheses several experiments need to be conducted. The first, most basic experiments include quantitation of amino acid and sugar contents from treated and control hop cones. While I saw no changes in contents of phenylalanine, valine, or leucine in response to treatment, inter-conversion of amino acids is a well-known phenomenon (*135*). Established LC-MS methods are available for assessment of

the organ-specific compartmentalization of metabolites (*136*). For example, methods have been developed to efficiently separate glandular trichomes from hop cones (*16*). The resulting glandular fractions likely contains small amounts of bract tissue, the resulting gland tissue is therefore referred to as a "gland enriched fraction" (*16*). Gland enriched fractions can provide insight into the different metabolic events occurring in the bracts and bracteoles compared with those occurring in glandular trichomes. Additionally, single gland analysis is possible by UHPLC-TOF-MS and allows for the unadulterated analysis of glandular metabolites. The combination of radiolabeled precursor feeding experiments, and single gland analysis may help establish flux relationships in response to Pro-Ca treatment. Such analyses of flux and metabolite partitioning hold promise for identification of the causes of variation in terpenophenolic accumulation. MALDI-MS (matrix assisted laser desorption mass spectrometry) imaging allows for highly sensitive and accurate depiction of mass spectral data over two-dimensional surfaces and has been applied to plant tissue (*137-139*). Using this technology it may be possible to discern the location of metabolites. Therefore, with or without labeling experiments, it may soon be possible to use MALDI-MS to analyze tissue localization of hop cone metabolites.

More complex experiments may provide a deeper understanding of the primary and secondary metabolic responses to Pro-Ca and how these responses may be related. Flux experiments, in which stable isotopes of precursor compounds are fed to hops before and after Pro-Ca treatment, could help elucidate metabolic responses in more detail. By feeding plants isotopically labeled primary metabolites such as amino acids, sugars, and CO_2, changes in primary metabolic precursors, their translocation, and potential incorporation into polyphenols or terpenophenolics can be measured. Labeled precursor

feeding experiments have been developed in several plants (*140, 141*). These experiments have not yet been performed in hops, and would require significant effort to develop, but may provide the essential information to elucidate the relationships among secondary metabolic pathways and the localization of products.

In addition to the small molecule quantitation conducted in these experiments, measurement of transcript and protein levels in response to Pro-Ca treatment, may provide additional data indicating the biological mechanisms underlying the phenotypic changes of hop plants to Pro-Ca treatment; genomic responses to enzyme inhibition and hormone levels might be discovered with targeted proteome or transcriptome analytics. Interest in further elucidating the biochemical genetics of terpenophenolic biosynthesis has fuelled gene expression and enzyme prevalence studies (*16, 77*). Application of quantitative proteomic methods to Pro-Ca treated hops can also help elucidate effects on protein abundance, which may be related to the phenotypic responses. Multidimensional protein identification technology (MudPIT) is an efficient process by which the entire proteome of an organism can be sequenced using mass spectrometry and bioinformatics (*142*). Preliminary studies have suggested the utility of MudPIT methods for analysis of hop cone tissue (unpublished data). These tools are being applied to molecular breeding of hops and are applicable to further understanding of Pro-Ca treated hops.

Hop cone development is a complex process involving changes in morphology, hormone fluctuation, and accumulation of secondary metabolites. Here I have provided a framework to study several secondary metabolites as they change during this process using quantitative time-of-flight mass spectrometry. My results have implications for producers, researchers, and brewers alike as I describe the application of quantitative data

to agronomic and industrial processes. The results from this work also provide a significant scaffold for future developmental studies of hop cones, including layering of additional ‘omics datasets onto the scaffold towards a systems approach, and the pursuit of industrial applications of Pro-Ca to hop plants.

In summary, our results show that treatment of hop plants with Pro-Ca may induce increases in biomass and terpenophenolic contents of hop cones. These changes are agronomically positive, and if used properly, Pro-Ca treatment may have a significant impact on the hop agricultural industry. In order to further identify the most effective developmental time-points, dosage levels, and varieties for Pro-Ca treatment, additional experiments must be conducted. To assess the applicability of Pro-Ca treatment of hops larger-scale experiments should be conducted including the use of commercial sprayers and industrial harvest machines. Additional measurements such as quantitation of essential oil and polymeric procyanidin contents can provide a further assessment of Pro-Ca effects on hop quality. Finally, application of metabolomic, genomic, transcriptomic, and proteomic methods can provide more descriptive data to assess the mechanisms underlying the phenotypic effects of Pro-Ca treatment on hop cone metabolism. The development of key methods to assess hop cone development, and quantitation of the morphological and phytochemical effects of Pro-Ca treatment on hop cone biomass and chemistry, provide useful tools and generate informed hypothesis to expand future research into these areas.

Appendix

Methods for application of prohexadione-calcium in hop agricultural fields

Apogee contains 27.5% prohexadione-calcium by weight. Use **Table A1** as a guide to mix the correct amounts of water to Apogee in order to obtain accurate concentrations (ppm) prohexadione. Additionally, add 1% Regulade as a non-ionic surfactant to maximize foliar absorption.

Treat plants from the top down using a large volume (100 L) battery powered (12 V) hand sprayer. Use approximately 2 L of Pro-Ca solution per plant. Treatments should be conducted immediately following dusk on calm nights, when no precipitation is expected and winds are less than 5 mph. It is also suggested to maintain a barrier of either seven plants, or one field row of plants between those being treated with Pro-Ca and those being used for control samples in analytical experiments.

Table A.1 Masses of Apogee (g) and concentrations of Pro-Ca solutions

L of mixture/ Pro-Ca (ppm)	1	25	50	75	100
25	0.091	2.273	4.545	6.818	9.091
50	0.182	4.545	6.364	7.273	8.182
75	0.273	6.818	9.545	10.909	12.273
100	0.364	9.091	12.727	14.545	16.364
150	0.545	13.636	19.091	21.818	24.545
200	0.727	18.182	25.455	29.091	32.727
250	0.909	22.727	31.818	36.364	40.909
300	1.091	27.273	38.182	43.636	49.091
350	1.273	31.818	44.545	50.909	57.273
400	1.455	36.364	50.909	58.182	65.455
450	1.636	40.909	57.273	65.455	73.636
500	1.818	45.455	63.636	72.727	81.818
1000	3.636	90.909	127.273	145.455	163.636

Figure A. 1 Gang-plank truck. Golden Gate Ranches, near Prosser, WA. Use of this truck provided access to the hop plant apex where we conducted Pro-Ca treatments and obtained sample collections. Treatment of hop plants using this "top down" approach enables maximum foliar application. Collection of cones from the plant apex allows for homogenous sample collection.

Table A.2. Quantitation (mg/gdw) of terpenophenolics by UHPLC-PDA and polyphenols by LC-TOF-MS in Zeus hop cones over five developmental stages. Values that share the same letters are not significantly different where p > 0.05 as calculated by Tukey-Kramer HSD analysis within a one-way ANOVA.

Compound	I	II	III	IV	V
Xanthohumol	7.2481 ± 0.4280^{c}	10.3110 ± 0.5647^{b}	10.6806 ± 0.3283^{b}	13.7662 ± 0.6299^{a}	14.8577 ± 0.5189^{a}
Desmethylxanthohumol	0.7474 ± 0.0440^{d}	1.0068 ± 0.0611^{c}	1.2490 ± 0.0693^{c}	2.0322 ± 0.1121^{b}	2.7267 ± 0.1021^{a}
Adhumulone	8.5251 ± 0.6749^{d}	23.6706 ± 1.3363^{c}	33.8506 ± 1.7430^{b}	38.5033 ± 1.5463^{b}	71.2281 ± 1.7456^{a}
Cohumulone	18.3095 ± 1.4571^{c}	51.4821 ± 3.1928^{b}	56.6741 ± 3.8216^{b}	72.7897 ± 3.6709^{b}	94.9570 ± 3.3113^{a}
Humulone	47.4104 ± 3.3047^{c}	121.0204 ± 6.9016^{b}	155.118 ± 13.8762^{ab}	186.9767 ± 9.9734^{a}	213.6455 ± 6.8097^{a}
Colupulone	26.8466 ± 1.0966^{c}	43.2765 ± 1.7134^{b}	44.4497 ± 1.7939^{b}	51.8330 ± 2.1967^{b}	58.0405 ± 1.3077^{a}
Lupulone	29.5625 ± 0.8501^{c}	41.4670 ± 1.4584^{b}	45.0177 ± 1.6656^{b}	49.2458 ± 2.0592^{b}	55.2810 ± 1.3147^{a}
Chalcone naringenin	0.0254 ± 0.0085^{a}	0.0252 ± 0.0031^{a}	0.0280 ± 0.0014^{a}	0.0298 ± 0.0026^{a}	0.0303 ± 0.0018^{a}
Naringenin	0.0032 ± 0.0008^{ab}	0.0040 ± 0.0004^{a}	0.0018 ± 0.0001^{b}	0.0020 ± 0.0001^{b}	0.0021 ± 0.0002^{b}
Comultifidol glucoside	0.7655 ± 0.2191^{b}	0.6228 ± 0.0568^{b}	1.5598 ± 0.0690^{a}	1.6454 ± 0.1697^{a}	1.3365 ± 0.0448^{a}
Leucine	0.2756 ± 0.0711^{a}	0.1483 ± 0.0076^{b}	0.1987 ± 0.0075^{ab}	0.1700 ± 0.0232^{ab}	0.1682 ± 0.0094^{ab}
Phenylalanine	0.2562 ± 0.0555^{a}	0.1897 ± 0.0090^{ab}	0.1305 ± 0.0080^{b}	0.1166 ± 0.0132^{bc}	0.0961 ± 0.0057^{c}
Valine	0.0916 ± 0.0250^{a}	0.0738 ± 0.0074^{a}	0.0994 ± 0.0114^{a}	0.0858 ± 0.0133^{a}	0.0955 ± 0.0087^{a}
Catechin	2.4352 ± 0.3430^{ab}	2.5344 ± 0.2265^{ab}	2.6120 ± 0.8325^{a}	1.8731 ± 0.3050^{ab}	1.2322 ± 0.3268^{b}
Epicatechin	0.9458 ± 0.1333^{a}	1.0223 ± 0.2177^{a}	1.1278 ± 0.3844^{a}	0.8077 ± 0.0703^{a}	0.6929 ± 0.0942^{a}
Procyanidin B-1	2.2111 ± 0.3742^{a}	1.8662 ± 0.1793^{ab}	2.8892 ± 1.0212^{a}	1.7421 ± 0.2343^{a}	1.2971 ± 0.3158^{b}
Procyanidin B-2	1.1250 ± 0.1904^{a}	1.3327 ± 0.2023^{a}	1.3185 ± 0.5205^{a}	0.7431 ± 0.0770^{ab}	0.5671 ± 0.0987^{b}
k-3-glu	0.1232 ± 0.0258^{b}	0.6103 ± 0.0498^{a}	0.1522 ± 0.0103^{b}	0.1343 ± 0.0137^{b}	0.1464 ± 0.0140^{b}
k-3-rut	0.9022 ± 0.2369^{a}	0.8842 ± 0.1863^{a}	0.4432 ± 0.0345^{b}	0.3630 ± 0.0327^{b}	0.3649 ± 0.0403^{b}
q-3-glu	0.6153 ± 0.1423^{a}	0.6814 ± 0.0515^{a}	0.7416 ± 0.0454^{a}	0.6326 ± 0.0398^{a}	0.6751 ± 0.0365^{a}
q-3-gal	0.1691 ± 0.0401^{a}	0.1683 ± 0.0318^{a}	0.1715 ± 0.0214^{a}	0.1370 ± 0.0089^{a}	0.1328 ± 0.0064^{a}
q-3-rut	1.8711 ± 0.3226^{ab}	2.0622 ± 0.1334^{a}	2.0406 ± 0.3721^{a}	1.3141 ± 0.1255^{ab}	0.9945 ± 0.0946^{b}
Quercetin	0.0470 ± 0.0090^{a}	0.0466 ± 0.0022^{a}	0.0448 ± 0.0020^{a}	0.0481 ± 0.0020^{a}	0.0529 ± 0.0014^{a}
Kaempferol	0.0063 ± 0.0025^{a}	0.0078 ± 0.0019^{a}	0.0034 ± 0.0002^{a}	0.0034 ± 0.0003^{a}	0.0036 ± 0.0001^{a}
Ferulic acid	0.0038 ± 0.0014^{a}	0.0009 ± 0.0002^{b}	0.0027 ± 0.0002^{ab}	0.0027 ± 0.0001^{ab}	0.0030 ± 0.0003^{a}
4-coumaric acid	0.0088 ± 0.0029^{a}	0.0082 ± 0.0006^{a}	0.0066 ± 0.0004^{a}	0.0080 ± 0.0011^{a}	0.0090 ± 0.0007^{a}
4-Hydroxybenzoic acid	0.0182 ± 0.0045^{ab}	0.0101 ± 0.0011^{b}	0.0167 ± 0.0011^{ab}	0.0141 ± 0.0019^{ab}	0.0212 ± 0.0014^{a}
Chlorogenic Acid	0.0314 ± 0.0133^{b}	0.1673 ± 0.0262^{a}	0.0768 ± 0.0089^{b}	0.0961 ± 0.0167^{ab}	0.0976 ± 0.0171^{ab}

Table A.3. Quantitation (mg/gdw) of terpenophenolics by UHPLC-PDA and polyphenols by LC-TOF-MS in Willamette hop cones over five developmental stages. Values that share the same letters are not significantly different where p > 0.05 as calculated by Tukey-Kramer HSD analysis within a one-way ANOVA.

Compound	I	II	III	IV	V
Xanthohumol	3.5295 ± 0.1985^{c}	6.5401 ± 0.3422^{b}	7.0311 ± 0.1978^{ab}	7.5270 ± 0.3342^{a}	7.7244 ± 0.2440^{a}
Desmethylxanthohumol	1.3400 ± 0.0416^{c}	1.5894 ± 0.0698^{b}	1.6277 ± 0.0850^{ab}	1.6981 ± 0.1058^{ab}	1.8464 ± 0.0728^{a}
Adhumulone	0.8284 ± 0.1304^{d}	12.6552 ± 0.5155^{c}	15.8385 ± 2.0198^{bc}	17.1495 ± 1.0403^{b}	39.927 ± 0.7949^{a}
Cohumulone	1.1127 ± 0.1861^{c}	23.4224 ± 1.0349^{b}	26.2886 ± 1.3411^{ab}	27.5738 ± 1.5203^{a}	30.8014 ± 1.3009^{a}
Humulone	2.0593 ± 0.4550^{d}	52.6515 ± 2.3263^{c}	59.5663 ± 6.5896^{bc}	66.5125 ± 4.2621^{b}	99.1188 ± 3.9516^{a}
Colupulone	4.8000 ± 0.6131^{c}	30.6817 ± 1.3125^{b}	35.8076 ± 0.9636^{a}	36.6846 ± 1.8416^{a}	38.2125 ± 1.2279^{a}
Lupulone	7.3425 ± 0.7715^{b}	33.0950 ± 1.2971^{a}	38.1831 ± 2.2183^{a}	36.3300 ± 2.0140^{a}	37.5496 ± 1.4674^{a}
Comultifidol glucoside	0.1155 ± 0.0036^{b}	0.1365 ± 0.0006^{a}	0.1361 ± 0.0018^{a}	0.1184 ± 0.0015^{b}	0.0907 ± 0.0005^{c}
Leucine	0.0864 ± 0.0022^{a}	0.0628 ± 0.0005^{b}	0.0622 ± 0.0006^{b}	0.0540 ± 0.0006^{c}	0.0381 ± 0.0003^{d}
Valine	0.0279 ± 0.0008^{a}	0.0237 ± 0.0005^{b}	0.0295 ± 0.0002^{a}	0.0200 ± 0.0003^{c}	0.0141 ± 0.0002^{d}
Catechin	0.6924 ± 0.0068^{a}	0.6834 ± 0.0029^{a}	0.5343 ± 0.0048^{b}	0.1932 ± 0.0023^{d}	0.3009 ± 0.0028^{c}
Epicatechin	0.0636 ± 0.0015^{b}	0.0616 ± 0.0005^{b}	0.0842 ± 0.0005^{a}	0.0162 ± 0.0004^{d}	0.0308 ± 0.0008^{c}
Procyanidin B-2	0.1045 ± 0.0005^{b}	0.1304 ± 0.0011^{a}	0.1299 ± 0.0093^{a}	0.1348 ± 0.0020^{a}	0.1049 ± 0.0030^{b}
k-3-glu	0.1300 ± 0.0028^{e}	0.2121 ± 0.0009^{b}	0.1955 ± 0.0017^{c}	0.1653 ± 0.0003^{d}	0.2347 ± 0.0007^{a}
k-3-rut	0.0385 ± 0.0008^{c}	0.0479 ± 0.0001^{b}	0.0498 ± 0.0007^{b}	0.0540 ± 0.0008^{b}	0.0654 ± 0.0021^{a}
q-3-glu	0.2625 ± 0.0047^{c}	0.3589 ± 0.0001^{a}	0.3233 ± 0.0025^{b}	0.2488 ± 0.0009^{d}	0.3220 ± 0.0014^{b}
q-3-gal	0.0222 ± 0.0004^{bc}	0.0242 ± 0.0001^{a}	0.0232 ± 0.0001^{ab}	0.0178 ± 0.0006^{d}	0.0211 ± 0.0003^{c}
q-3-rut	0.0493 ± 0.0016^{c}	0.0616 ± 0.0002^{b}	0.0715 ± 0.0007^{a}	0.0502 ± 0.0001^{c}	0.0699 ± 0.0008^{a}
Quercetin	0.0024 ± 0.0001^{c}	0.0025 ± 0.0001^{c}	0.0027 ± 0.0001^{c}	0.0033 ± 0.0001^{b}	0.0051 ± 0.0001^{a}
Ferulic Acid	0.0018 ± 0.0002^{a}	0.0021 ± 0.0001^{a}	0.0018 ± 0.0002^{a}	0.0024 ± 0.0001^{a}	0.0022 ± 0.0001^{a}
4-coumaric acid	0.0018 ± 0.0002^{bc}	0.0020 ± 0.0001^{ab}	0.0015 ± 0.0001^{c}	0.0024 ± 0.0001^{a}	0.0019 ± 0.0001^{bc}
4-Hydroxybenzoic acid	0.0023 ± 0.0002^{a}	0.0022 ± 0.0005^{ab}	0.0014 ± 0.0002^{bc}	0.0006 ± 0.0002^{c}	0.0010 ± 0.0001^{c}

Table A.4. Standard curve equation, linear range, r-squared, limit of detection (LOD), limit of quantification (LOQ), interday RSD, and intraday RSD values for polyphenolics quantified by LC-MS-TOF.

Compound	Regression equation	Linear range (ug/ml)	r-sq	LOD (ng/ml)	LOQ (ng/ml)	Inter RSD (%)	Intra RSD (%)
Chalcone Naringenin	y=104066x-6.6.23389	0.26-5	0.996977	12.1	36.7	4.2	3.6
Naringenin	y=356657x+16.0612	0.26-5	0.993326	3.5	10.7	3.8	3.9
CoMultifidol glucoside	y=43947.1x+6.52753	0.26-75	0.992554	28.7	86.9	0.4	1.0
Leucine	y=79499.1x+498.688	0.26-25	0.997149	24.2	73.5	4.1	3.2
Phenylalanine	y=196350x+247.766	0.26-25	0.996697	21.6	65.3	2.6	4.3
Valine	y=39290.5x+8.17279	0.26-25	0.998114	22.1	56.7	2.0	2.2
Catechin	y=75411x-1.09155	0.26-125	0.997136	28.2	85.3	0.8	3.4
Epicatechin	y=89486.7x+0.235437	0.26-75	0.996516	29.6	89.8	2.9	1.9
Procyanidin B-1	y=36906.5x-11.932	0.26-100	0.996503	22.9	69.1	1.1	1.5
Procyanidin B-2	y=14362x-6.95688	0.26-100	0.998886	33.2	100.5	3.6	2.2
k-3-glu	y=252641x+497.157	0.26-25	0.998006	3.3	9.9	2.9	2.7
k-3-rut	y=112856x+8.34289	0.26-75	0.995885	7.3	22.2	2.7	5.4
q-3-glu	y=354285x+18.6251	0.26-50	0.997623	6.2	18.7	1.8	3.0
q-3-gal	y=142003x+6.19358	0.26-25	0.995954	3.4	10.2	4.1	2.2
q-3-rut	y=169123x+13.9	0.26-100	0.992223	7.5	22.6	1.6	2.3
Quercetin	y=124999x-48.8684	0.026-5	0.994879	7.6	23.1	4.3	6.5
Kaempferol	y=444137x-26.7229	0.026-5	0.997407	4.7	14.2	3.3	7.1
Ferulic acid	y=281072x+3.72899	0.026-5	0.993179	4.5	13.6	3.4	5.0
4-coumaric acid	y=243152x+12.8367	0.026-5	0.995724	3.9	11.9	2.8	2.5
4-Hydroxybenzoic acid	y=43328.7x+101.117	0.026-5	0.996298	4.6	13.3	3.1	3.4
Chlorogenic acid	y=73917x+2.9075	0.026-5	0.997421	4.9	17.4	2.2	2.0

Table A.5. Terpenophenolic contents of Zeus (Z) and Willamette (W) cultivars treated at stages I, III, or III, with either 0, 50, or 100 ppm Pro-Ca in 07 experiments. Values are reported in mg/g kiln dried tissue (10% moisture), and treatments that significantly differ from controls are noted with the superscript a.

Group	AdHum	CoHum	CoLup	DMX	Lup	Hum	XN
Z 0	25.93 ± 1.14	64.01 ± 2.56	37.09 ± 1.39	0.70 ± 0.03	28.55 ± 1.08	95.58 ± 4.12	6.33 ± 0.26
Z 100 I	7.87 ± 0.29[a]	15.64 ± 0.54[a]	10.50 ± 0.27[a]	0.18 ± 0.01[a]	8.55 ± 0.22[a]	26.35 ± 0.82[a]	1.59 ± 0.05[a]
Z 100 II	28.50 ± 3.50	53.56 ± 6.73	36.47 ± 4.59	0.67 ± 0.08	29.03 ± 3.77	83.62 ± 10.40	5.06 ± 0.60
Z 100 III	41.45 ± 2.02[a]	112.12 ± 2.50[a]	62.18 ± 0.94[a]	1.18 ± 0.02[a]	47.49 ± 0.87[a]	161.28 ± 6.82[a]	11.03 ± 0.27[a]
Z 50 I	5.35 ± 0.36[a]	10.52 ± 0.76[a]	8.90 ± 0.29[a]	0.13 ± 0.01[a]	7.45 ± 0.22[a]	18.90 ± 1.13[a]	1.15 ± 0.06[a]
Z 50 II	32.41 ± 2.57[a]	61.87 ± 5.02	40.96 ± 3.27	0.76 ± 0.06	34.13 ± 2.89[a]	96.83 ± 7.88	5.86 ± 0.45
Z 50 III	48.56 ± 1.61[a]	106.35 ± 3.05[a]	63.12 ± 0.90[a]	1.12 ± 0.03[a]	48.10 ± 1.15[a]	161.49 ± 7.88[a]	10.15 ± 0.36[a]
W 0	9.25 ± 0.30	16.37 ± 0.47	23.51 ± 0.63	0.43 ± 0.02	21.40 ± 0.57	34.82 ± 1.09	3.82 ± 0.1
W 100 I	8.16 ± 0.47[a]	14.94 ± 1.05	19.68 ± 1.15[a]	0.44 ± 0.03	17.99 ± 1.08[a]	31.64 ± 2.08	3.56 ± 0.2
W 100 II	10.24 ± 0.31	18.17 ± 0.40	24.48 ± 0.92	0.43 ± 0.02	21.66 ± 0.87	35.57 ± 1.16	3.94 ± 0.12
W 100 III	9.32 ± 0.36	15.39 ± 0.58	23.96 ± 0.75	0.42 ± 0.01	22.10 ± 0.70	34.25 ± 1.28	4.09 ± 0.12
W 50 I	7.45 ± 1.00[a]	13.16 ± 1.02[a]	16.99 ± 1.61[a]	0.31 ± 0.03[a]	15.00 ± 1.42[a]	29.14 ± 1.64[a]	3.21 ± 0.11[a]
W 50 II	9.88 ±0.27	16.44 ± 0.47	26.71 ± 0.57[a]	0.41 ± 0.01	22.79 ± 0.58	35.18 ± 1.24	4.63 ± 0.09[a]
W 50 III	10.33 ± 0.26	17.86 ± 0.43	29.88 ± 0.37[a]	0.54 ± 0.01[a]	27.78 ± 0.47[a]	38.27 ± 1.07	4.55 ± 0.06[a]

Table A.6. Terpenophenolic contents of Zeus (Z) and Willamette (W) cultivars treated at stages III, IV, or V, with either 0, or 100 ppm Pro-Ca in 09 experiments. Values are reported in mg/g kiln dried tissue (10% moisture), and treatments that significantly differ from controls are noted with the superscript a.

Group	DMX	XN	AdHum	CoHum	Hum	CoLup	Lup
W 0	0.54 ± 0.11	3.35 ± 0.11	9.97 ± 0.27	15.25 ± 0.63	27.19 ± 1.27	33.79 ± 0.60	26.73 ± 0.46
W 100 III	0.5 ± 0.02	3.55 ± 0.14[a]	9.88 ± 0.28	14.16 ± 0.41	26.26 ± 0.94	37.45 ± 0.51[a]	30.99 ± 0.43[a]
W 100 IV	0.52 ± 0.15	3.17 ± 0.11[a]	9.24 ± 0.25	13.26 ± 0.38[a]	23.99 ± 0.92[a]	32.47 ± 0.55	25.23 ± 0.40[a]
Z 0	1.12 ± 0.04	4.88 ± 0.10	20.89 ± 0.23	58.58 ± 0.63	107.59 ± 1.18	36.13 ± 0.57	39.02 ± 0.72
Z 100 III	1.15 ± 0.11	4.97 ± 0.21	24.14 ± 0.52[a]	65.83 ± 1.53[a]	116.02 ± 2.71[a]	40.42 ± 1.51[a]	43.47 ± 1.64[a]
Z 100 IV	1.29 ± 0.13[a]	5.42 ± 0.29[a]	21.05 ± 0.60	59.26 ± 1.62	110.45 ± 3.70	35.92 ± 1.41	38.13 ± 1.65
Z 100 V	1.05 ± 0.20	4.61 ± 0.51	19.7 ± 1.02	57.68 ± 3.26	97.37 ± 5.13[a]	34.7 ± 2.35	34.58 ± 2.43

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