JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Increase in Cone Biomass and Terpenophenolics in Hops (*Humulus lupulus* L.) by Treatment with Prohexadione-Calcium

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ABSTRACT: *Humulus lupulus* L. (hop), a specialty crop bred for flavor characteristics of the inflorescence, is an essential ingredient in beer. Hop inflorescences, commonly known as hop cones, contain terpenophenolic compounds, which are important for beer flavoring and of interest in biomedical research. Hop breeders focus their efforts on increasing cone biomass and terpenophenolic content. As an alternative to traditional breeding, hops were treated with prohexadione-calcium (Pro-Ca), a growth inhibitor previously shown to have positive agronomic effects in several crops. Application of Pro-Ca to hop plants during cone maturation 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. Induced changes in cone biomass production and terpenophenolic accumulation were most dependent on cultivar and the developmental stage at which plants were treated.

KEYWORDS: cone biomass, developmental stage, hops, *Humulus lupulus*, prohexadione-calcium, terpenophenolics

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 metabolically active structures and are the site of terpenophenolic and essential oil biosynthesis.[1,2] 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 1). Hops contain two types of prenylated acylphloroglucinols; humulones, or α-acids, the isomers of which have bitter flavor and lupulones, or β-acids, which have antimicrobial activity.[3]

Two main types of hop cultivars are grown for beer production; aroma hops are bred for essential oil content and flavor profiles, containing between 0.5 and 2% terpene-rich essential oils by total dry cone weight, and alpha hops, which are used for their high α-acid content, which may exceed 22% of the total kiln-dried cone weight.[4,5] Aroma hops tend to produce lower cone yields (total cone biomass per acre), whereas alpha hops often yield larger cone biomass per acre.[6] Aroma hops in general also reach flowering maturity earlier in the growing season than do alpha hops. To alter α- and β-acid accumulation and hop cone yield, we treated aroma and alpha hop cultivars with an enzyme inhibitor known as prohexadione-calcium (Figure 1).

Prohexadione-calcium (Pro-Ca) is a 2-oxoglutaric acid dependent dioxygenase (2-ODD) antimetabolite that has shown inhibitory effects on enzymes critical for the production of gibberellins, ethylene, and flavonoids.[7−9] 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.[10−12] 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.[8] 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.[13]

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.[14−17] In response to Pro-Ca treatment, contents of phenolic acids and flavanones found upstream from F3H increased, whereas compounds found downstream from F3H, including flavonols, flavan-3-ols, and procyanidins, significantly decreased in several species.[16] In addition to the alteration of known flavonoids, the presence of novel, antimicrobial 3-deoxyflavans were reported in some crops in which they were previously unknown.[14−17]

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 2 months prior to harvest or plant senescence. Pilot experiments showed increases in cone yield and terpenophenolic accumulation when plants were treated at early and middle stages of cone development with 50 ppm Pro-Ca.[18] To provide greater statistical confidence and investigate seasonal variation in

Received: February 17, 2011
Revised: May 5, 2011
Accepted: May 6, 2011
Published: May 20, 2011

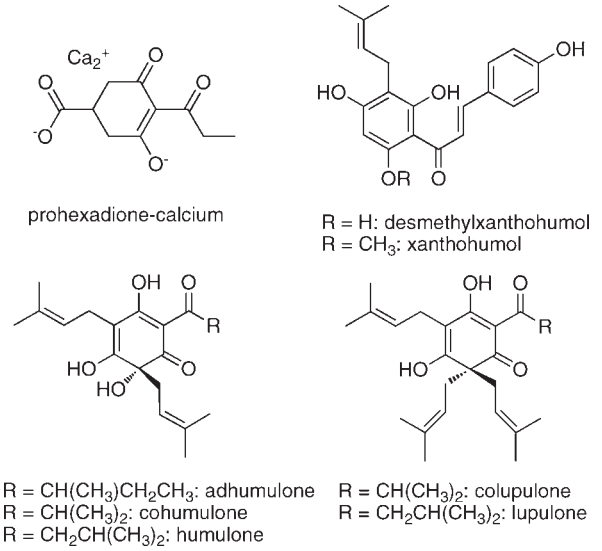

Figure 1. Structures of prohexadione-calcium and the seven terpenophenolics quantitated in this study.

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.

MATERIALS AND METHODS

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 deionized water with addition of 1% (v/v) Regulaid (Kalo Inc., Overland Park, KS), a nonionic surfactant. Control groups were treated with the surfactant in deionized 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 or 100 ppm, applied at five developmental stages.[19]

Mature cones were collected from the upper third section of plants and kiln-dried using standard commercial handling protocols (65 °C for about 12 h in a commercial kiln until moisture content reached 8−10%). Dried subsamples 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.

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

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.[18]

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. Cones were characterized by using a previously described index of development.[19]

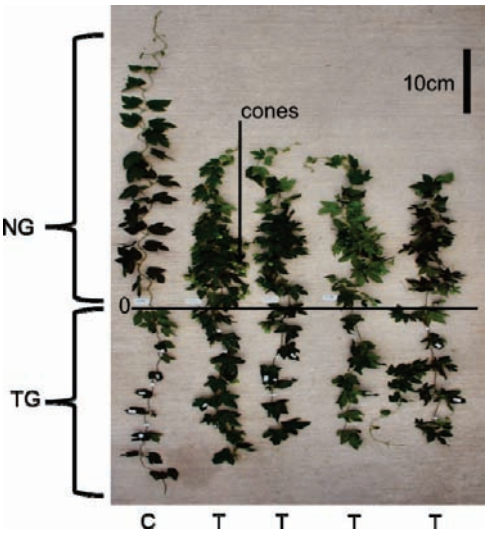

Figure 2. Immature hop plants treated with high doses (500 ppm) of Pro-Ca. Treated plants (T) and a control plants (C), treated at the extent of growth (TG) shown below the line (time 0) and 6 weeks of new growth (NG).

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 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.

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.

Statistical Analysis. Statistical analysis was conducted using JMP 8.01 (SAS, Cary, NC) software. Group means were compared using least mean 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.

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.

Cone Yield and Biomass. Dramatic dwarfing and flowering occurred in hops treated with Pro-Ca (500 pm) during early vine development, likely the result of induced hormonal alterations (Figure 2). Because Pro-Ca has known effects in other plants on hormone-mediated processes through the inhibition of

Table 1. Cone Biomass, Total Biomass, and Cone/Total Biomass Ratios of Zeus and Willamette Hop Cultivars following Pro-Ca Treatments Conducted over Two Seasons[a]

cultivar	season	treatment	stage	cone biomass (kg)	total biomass (kg)	cone/total biomass
Zeus	2007	0	I	4.08 ± 0.71	9.60 ± 2.04	0.43 ± 0.04
			II	4.27 ± 0.81	9.85 ± 2.15	0.44 ± 0.04
			III	3.57 ± 0.84	8.30 ± 2.12	0.44 ± 0.06
		50	I	3.81 ± 0.58	8.84 ± 1.81	0.44 ± 0.04
			II	4.24 ± 0.53	9.30 ± 1.22	0.46 ± 0.03
			III	3.79 ± 0.71	8.25 ± 1.86	0.47 ± 0.06 a
		100	I	4.05 ± 0.54	9.45 ± 1.24	0.43 ± 0.05
			II	4.26 ± 0.71	9.24 ± 1.80	0.46 ± 0.04 a
			III	3.62 ± 0.59	7.97 ± 3.26	0.47 ± 0.09 a
	2009	0	III	2.85 ± 0.65	7.73 ± 1.73	0.37 ± 0.05
			IV	2.78 ± 1.14	7.35 ± 1.99	0.38 ± 0.12
			V	2.86 ± 0.57	7.41 ± 1.66	0.39 ± 0.06
		100	III	3.07 ± 0.82	7.74 ± 1.84	0.40 ± 0.05
			IV	3.26 ± 0.87 a	7.74 ± 1.81	0.42 ± 0.06 a
			V	3.41 ± 0.96 a	7.7 ± 1.83	0.44 ± 0.06 a
Willamette	2007	0	I	1.06 ± 0.29	5.53 ± 0.99	0.19 ± 0.05
			II	1.08 ± 0.25	5.64 ± 1.15	0.19 ± 0.03
			III	1.10 ± 0.27	5.86 ± 1.06	0.19 ± 0.04
		50	I	1.22 ± 0.38 a	5.99 ± 1.59	0.21 ± 0.05
			II	1.15 ± 0.34	4.90 ± 1.24 a	0.25 ± 0.08 a
			III	1.13 ± 0.26	5.20 ± 0.95 a	0.22 ± 0.04 a
		100	I	1.24 ± 0.28 a	6.03 ± 1.17	0.21 ± 0.05
			II	1.10 ± 0.21	5.08 ± 1.03 a	0.22 ± 0.04 a
			III	1.25 ± 0.22 a	5.36 ± 1.09	0.24 ± 0.05 a
	2009	0	III	1.40 ± 0.59	4.77 ± 1.66	0.29 ± 0.06
			IV	1.40 ± 0.51	5.33 ± 1.63	0.26 ± 0.07
		100	III	1.60 ± 0.49 a	4.69 ± 1.28	0.34 ± 0.06 a
			IV	1.68 ± 0.46 a	5.56 ± 1.46	0.31 ± 0.05 a

[a] Values are reported in kg, and treatments that significantly differ from controls are denoted with the letter "a".

gibberellic acid metabolism and ethylene biosynthesis, we expected treatments with Pro-Ca late in the 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 of 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. Values of cone biomass, total biomass, and cone/total biomass ratios of Zeus and Willamette hop cultivars are shown in Table 1.

Previous experiments showed significant increases in total cone yield from Willamette plants treated with 50 ppm Pro-Ca at developmental stage I.[18] 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 3A,B). We used a ratio of yield/biomass to normalize cone yield values, because hop plants can be highly variable in size, even among clones within the same field. Whereas Zeus plants did not significantly change in either

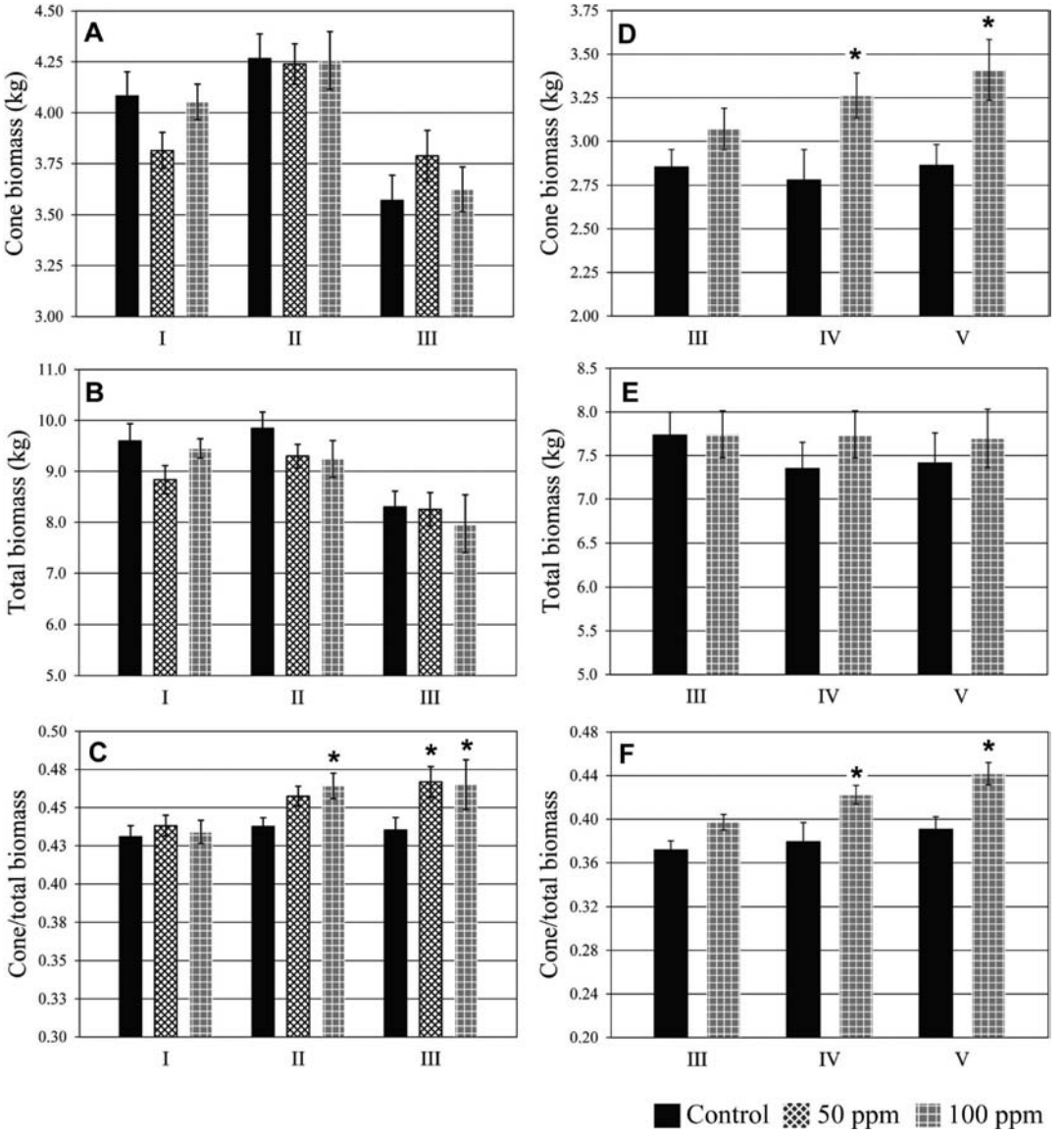

Figure 3. Changes in (A) yield, (B) biomass, and (C) yield/biomass ratios of Zeus plants after treatments with 50 and 100 ppm Pro-Ca at stages I, II, and III in season 07. Changes in (D) yield, (E) biomass, and (F) yield/biomass ratios in Zeus plants treated with 100 ppm Pro-Ca at stages III, IV, and V in season 09.

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 (Figure 3C).

In season 07, total cone yield increased from Willamette plants treated at stages I and III (Figure 4A). However, 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 4B). The above-ground biomass of Willamette plants also increased slightly after treatment 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 4C). Plants treated with either 50 or 100 ppm Pro-Ca at stage I also 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 (Figure 3D,E). Zeus plants treated at stage III increased in both cone yield production and yield/biomass ratios, but neither increase was statistically significant. Yield/biomass ratios from Zeus plants treated at stages IV and V also significantly increased (Figure 3F). In season 09, treatment of Willamette plants also induced increases in cone yield and yield/biomass ratios when plants were treated at stages III and IV (Figure 4D,F). Similar to Zeus, total above-ground biomass was not affected in Willamette plants.

Despite some variability among seasons for Zeus, results from both season 07 and 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

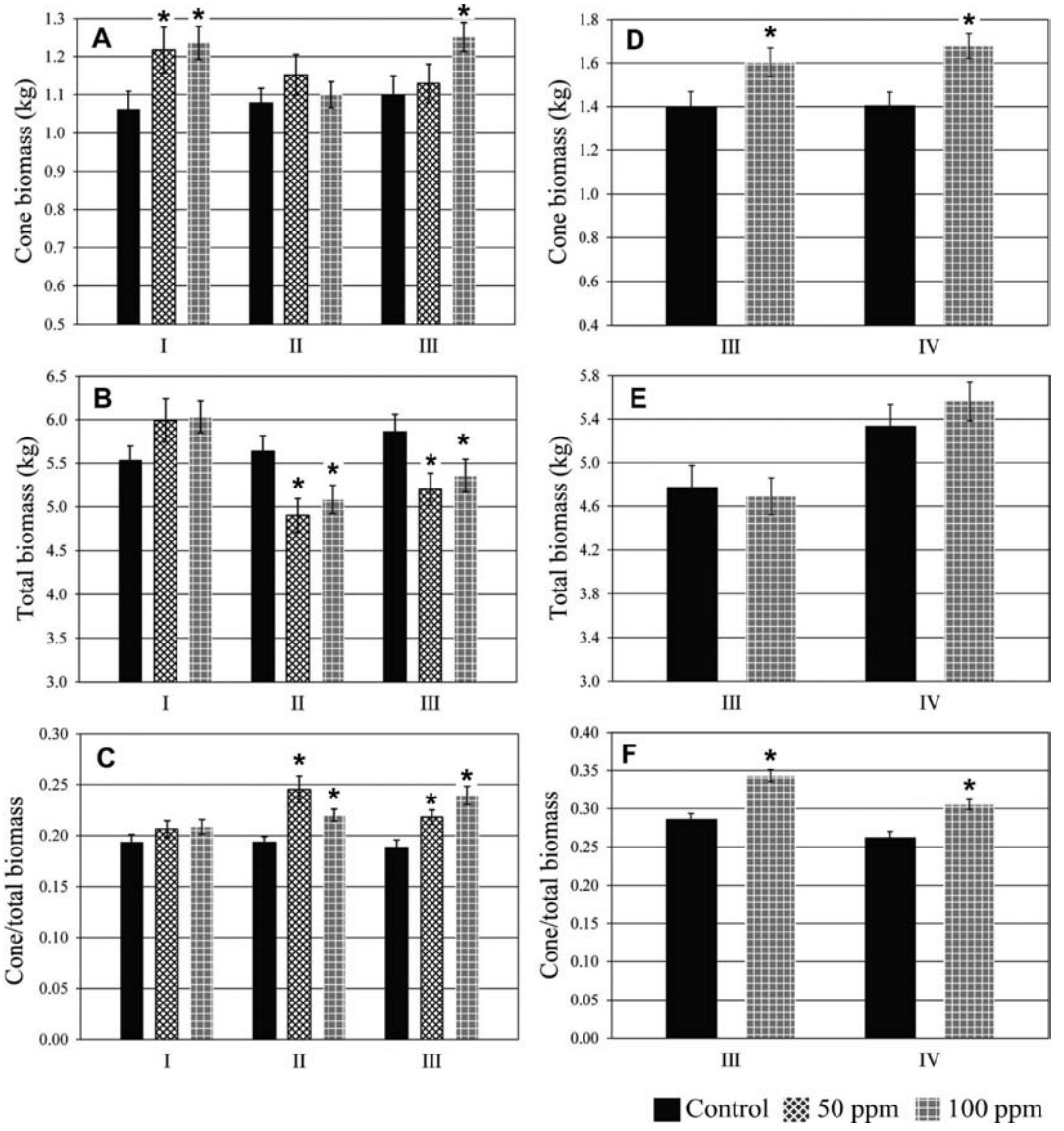

Figure 4. Changes in (A) yield, (B) biomass, and (C) yield/biomass ratios of Willamette plants after treatments with 50 and 100 ppm Pro-Ca at stages I, II, and III in season 07. Changes in (D) yield, (E) biomass, and (F) yield/biomass ratios in Willamette plants treated with 100 ppm Pro-Ca at stages III and IV in season 09.

stages. These late stages are described here as stages III, IV, and V according to the developmental index we previously described.[19]

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. Terpenophenolic contents are presented in Table 2 and expressed as milligrams of compound per gram of kiln-dried cone mass; kiln-dried cones contain, on average, 8−10% moisture by weight.

Statistical analysis of results 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 5A,B and 6A, B). In contrast to the large decreases in terpenophenolics following stage I treatments of Zeus plants in season 07, 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; all seven terpenophenolics increased in response to 50 and 100 ppm treatments.

Similar changes in terpenophenolic contents of Zeus were also observed in Willamette plants treated with 50 ppm Pro-Ca during season 07 (Figures 5D and 6D). Willamette plants treated at stage I with 50 ppm Pro-Ca produced cones with decreased levels of all seven terpenophenolics measured. Few changes occurred 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. Increases occurred in all terpenophenolics measured in response to stage III treatments in season 07, but the increases in α-acids adhumulone, cohumulone, and humulone were not statistically significant.

Whereas Willamette plants treated with 50 ppm Pro-Ca in season 07 experiments produced cones with significantly altered

Table 2. Quantities of Seven Terpenophenolics in Zeus and Willamette Cones following Pro-Ca Treatments Conducted over Two Seasons[a]

cultivar	season	treatment	stage	DMX	XN	Adhum	Cohum	Hum	Colup	Lup
Zeus	2007	0		0.7 ± 0.1	6.3 ± 0.3	25.9 ± 1.1	64.0 ± 2.6	95.6 ± 4.1	37.1 ± 1.4	28.6 ± 1.1
		50	I	0.1 ± 0.1 a	1.2 ± 0.1 a	5.4 ± 0.4 a	10.5 ± 0.8 a	18.9 ± 1.1 a	8.9 ± 0.3 a	7.5 ± 0.2 a
			II	0.8 ± 0.1	5.9 ± 0.5	32.4 ± 2.6 a	61.9 ± 5.0	96.8 ± 7.9	41.0 ± 3.3	34.1 ± 2.9 a
			III	1.1 ± 0.1 a	10.2 ± 0.4 a	48.6 ± 1.6 a	106.4 ± 3.1 a	161.5 ± 7.9 a	63.1 ± 0.9 a	48.1 ± 1.2 a
		100	I	0.2 ± 0.1 a	1.56 ± 0.1 a	7.9 ± 0.3 a	15.6 ± 0.5 a	26.4 ± 0.8 a	10.5 ± 0.3 a	8.6 ± 0.2 a
			II	0.7 ± 0.1	5.1 ± 0.6	28.5 ± 3.5	53.6 ± 6.7	83.6 ± 10.4	36.5 ± 4.6	29.0 ± 3.8
			III	1.2 ± 0.1 a	11.0 ± 0.3 a	41.5 ± 2.0 a	112.1 ± 2.5 a	161.3 ± 6.8 a	62.2 ± 0.9 a	47.5 ± 0.9 a
	2009	0		1.1 ± 0.1	4.9 ± 0.1	20.9 ± 0.2	58.6 ± 0.6	107.6 ± 1.2	36.1 ± 0.6	39.0 ± 0.7
		100	III	1.2 ± 0.1	5.0 ± 0.2	24.1 ± 0.5 a	65.8 ± 1.5 a	116.0 ± 2.7 a	40.4 ± 1.5 a	43.5 ± 1.6 a
			IV	1.3 ± 0.1 a	5.4 ± 0.3 a	21.1 ± 0.6	59.3 ± 1.6	110.5 ± 3.7	35.9 ± 1.4	38.1 ± 1.7
			V	1.1 ± 0.2	4.6 ± 0.5	19.7 ± 1.0	57.7 ± 3.3	97.4 ± 5.1 a	34.7 ± 2.4	34.6 ± 2.4
Willamette	2007	0		0.4 ± 0.1	3.8 ± 0.1	9.3 ± 0.3	16.4 ± 0.5	34.8 ± 1.1	23.5 ± 0.6	21.4 ± 0.6
		50	I	0.3 ± 0.1 a	3.2 ± 0.1 a	7.5 ± 1.0 a	13.2 ± 1.0 a	29.1 ± 1.6 a	17.0 ± 1.6 a	15.0 ± 1.4 a
			II	0.4 ± 0.1	4.6 ± 0.1 a	9.9 ± 0.3	16.4 ± 0.5	35.2 ± 1.2	26.7 ± 0.6 a	22.8 ± 0.6
			III	0.5 ± 0.1 a	4.6 ± 0.1 a	10.3 ± 0.3	17.9 ± 0.4	38.3 ± 1.1	29.9 ± 0.4 a	27.8 ± 0.5 a
		100	I	0.4 ± 0.1	3.6 ± 0.2	8.2 ± 0.5 a	14.9 ± 1.1	31.6 ± 2.1	19.7 ± 1.2 a	18.0 ± 1.1 a
			II	0.4 ± 0.1	3.9 ± 0.1	10.2 ± 0.3	18.2 ± 0.4	35.6 ± 1.2	24.5 ± 0.9	21.7 ± 0.9
			III	0.4 ± 0.1	4.1 ± 0.1	9.3 ± 0.4	15.4 ± 0.6	34.3 ± 1.3	24.0 ± 0.8	22.1 ± 0.7
	2009	0		0.5 ± 0.1	3.4 ± 0.1	10.0 ± 0.3	15.3 ± 0.6	27.2 ± 1.3	33.8 ± 0.6	26.7 ± 0.5
		100	III	0.5 ± 0.1	3.6 ± 0.1 a	9.9 ± 0.3	14.2 ± 0.4	26.3 ± 0.9	37.5 ± 0.5 a	31.0 ± 0.4 a
			IV	0.5 ± 0.2	3.2 ± 0.1 a	9.2 ± 0.3	13.3 ± 0.4 a	24.0 ± 0.9 a	32.5 ± 0.6	25.2 ± 0.4 a

[a] Values are reported in mg/g kiln dried tissue (10% moisture), and treatments that significantly differ from controls are denoted with the letter "a". Abbreviations: DMX, desmethylxanthohumol; XN, xanthohumol; Cohum, cohumulone; Hum, humulone; Adhum, adhumulone; Colup, colupulone; Lup, lupulone; C, control; I–V, treatments at stages I–V.

terpenophenolic contents, when plants were treated with 100 ppm Pro-Ca only a few changes were measured in terpenophenolic contents (Figures 5E and 6E). The results of season 07 experiments indicated that levels of all three groups of terpenophenolics, the prenylflavonoids, α-acids, and β-acids, significantly changed 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 similar 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 midlate season cone treatments, characterized as stage III.[19] We therefore conducted a second season (season 09) of experiments 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 stages during late cone development. 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 was characterized using a previously established index of developmental stages.[19]

Treatments of Zeus plants in season 09 induced significant increases in all terpenophenolics measured, but prenylflavonoids and α- and β- acids accumulated to variable levels, depending on the developmental stage at which treatments occurred (Figures 5C and 6C). Prenylflavonoids increased in cones from Zeus plants treated at stage IV, but prenylflavonoid content did not change in response to treatments conducted at stages III or V. Whereas α- and β-acids increased in cones from plants treated at stage III, 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 in cones from plants

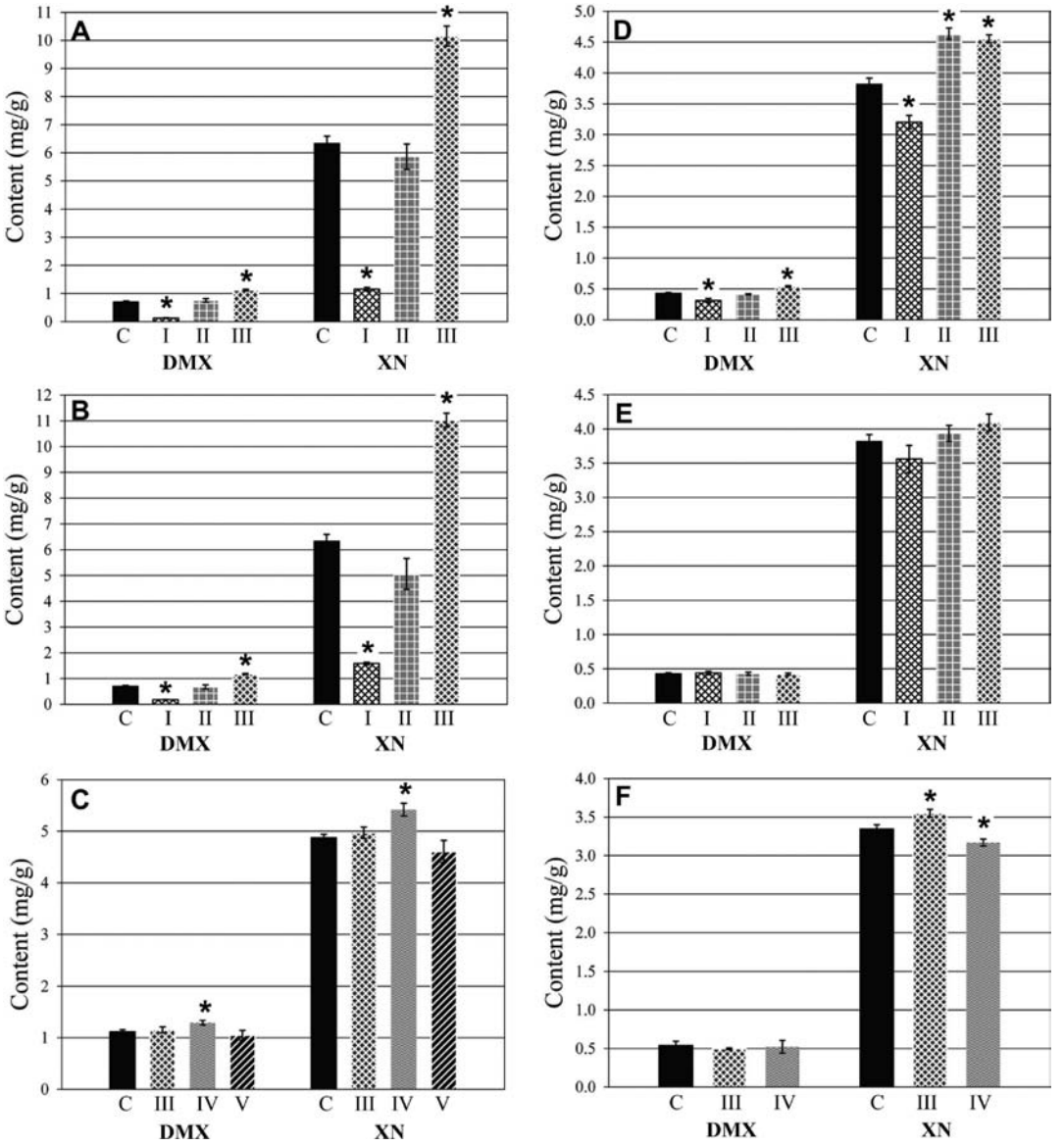

Figure 5. Changes in prenylflavonoids: season 07 Zeus hops treated with 50 ppm Pro-Ca (A) and 100 ppm Pro-Ca (B) and season 09 Zeus hops treated with 100 ppm Pro-Ca (C); season 07 Willamette hops treated with 50 ppm Pro-Ca (D) and 100 ppm Pro-Ca (E) and season 09 Willamette hops treated with 100 ppm Pro-Ca (F). Abbreviations: Cohum, cohumulone; Hum, humulone; Adhum, adhumulone; Colup, colupulone; Lup, lupulone; C, control; I—V, treatments at stages I—V.

treated at stage V. Prenylflavonoids and α- and β-acids 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, treatment of Willamette plants induced significant increases in levels of cone terpenophenolics. Changes in Willamette, as in Zeus, were 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. Adhumulone and colupulone also decreased in response to stage IV treatments, but the decreases were not statistically significant.

In both season 07 and 09, the effect of Pro-Ca on hop terpenophenolic contents 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 that occurred during midinflorescence development, denoted here 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 changed similarly in response to Pro-Ca treatment; in several cases both groups of compounds either increased or decreased in the same direction and to the same extent. Although these two groups of compounds are prenylated terpenophenolics and present in glandular

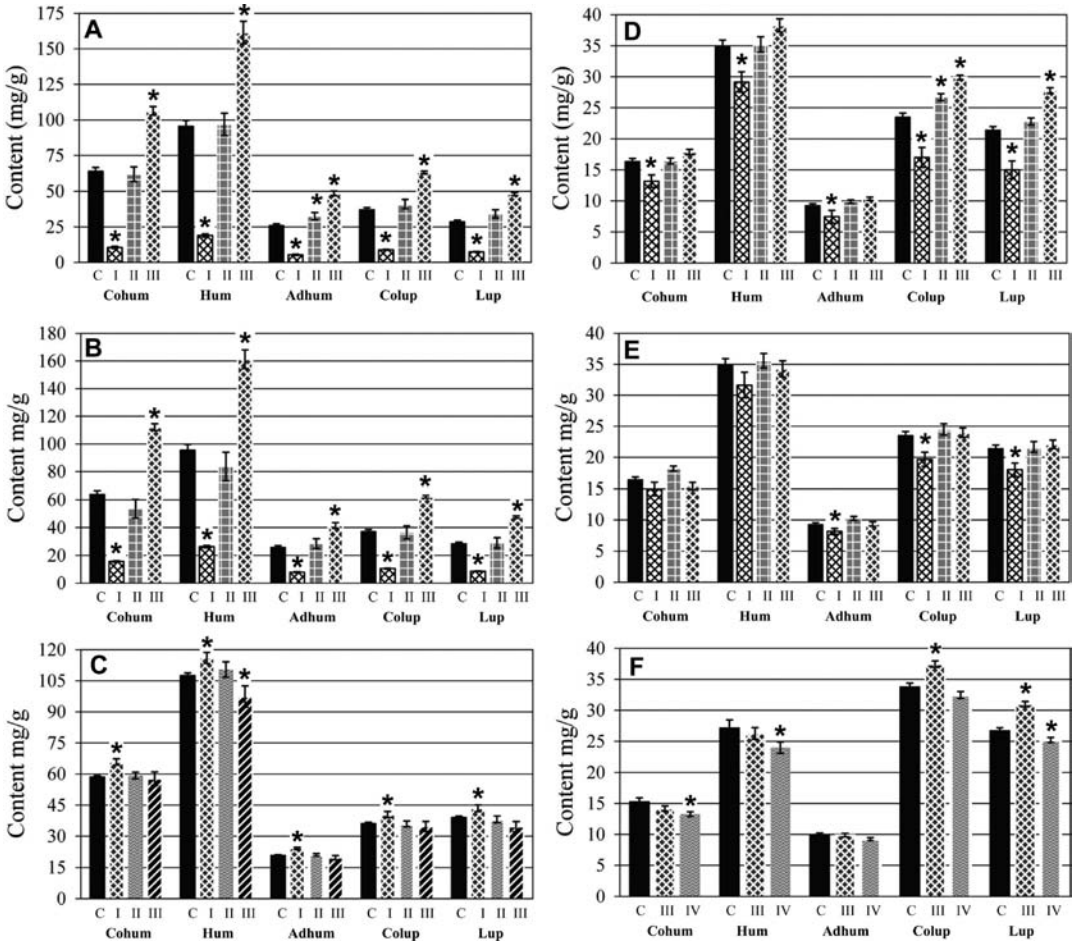

Figure 6. Changes in α- and β-acids: season 07 Zeus hops treated with 50 ppm Pro-Ca (A) and 100 ppm Pro-Ca (B) and season 09 Zeus hops treated with 100 ppm Pro-Ca (C); season 07 Willamette hops treated with 50 ppm Pro-Ca (D) and 100 ppm Pro-Ca (E) and season 09 Willamette hops treated with 100 ppm Pro-Ca (F). Abbreviations: DMX, desmethylxanthohumol; XN, xanthohumol; Cohum, cohumulone; Hum, humulone; Adhum, adhumulone; Colup, colupulone; Lup, lupulone; C, control; I—V, treatments at stages I—V.

trichomes, their biosynthetic origins are quite different. α- and β-acids are produced via the prenylation of polyketides derived from branched-chain amino acid degradation products, whereas prenylflavonoids are produced by the prenylation of naringenin chalcone, a polyketide derived from phenolic acid biosynthesis.[20] The changes in these compounds of different biosynthetic origin may indicate multimechanistic effects of Pro-Ca on hop metabolism and terpenophenolic accumulation. Pro-Ca has been shown to inhibit the 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.[8,16] Naringenin chalcone, a polyketide derived from the phenolic acid 4-coumaric acid, has been shown to be the precursor compound to prenylflavonoid biosynthesis in hops.[1,20] Therefore, an inhibition of F3H in hop cones may influence the contents of phenolic acids and naringenin chalcone; however, changes in these substrates would not likely have a direct effect on terpenophenolic biosynthesis, because the compartmentalizations 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.[20] More specifically, degradation products of valine and leucine provide precursor compounds for production of α- and β-acids. Whereas 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 was suggested that Pro-Ca inhibits 2-ODDs by competitive inhibition, possibly due to its structural similarity to 2-oxoglutaric acid.[21,22] 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. Although it is possible that Pro-Ca can influence any process involving 2-oxoglutarate, the effects that have been observed are nontoxic, 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 complicate 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, naringenin chalcone, and other precursor metabolites related to terpenophenolic biosynthesis.

The effects of Pro-Ca treatment on hop cone biomass production and terpenophenolic content may be related to inhibition of enzymes within the gibberellic acid (GA) and ethylene biosynthetic pathways. When apple trees were treated with Pro-Ca during early vegetative development, the activation of the inactive gibberellic acid GA_{20} was inhibited and decreased internode length was observed.[22] However, when apple trees were treated in late summer, when vegetative development slows, the inactivation of the active gibberellic acid GA_1 was inhibited, and an increase in internode growth and plant vigor was observed.[23,24] We have also observed significant decreases in internode elongation in young hops treated with 500 ppm Pro-Ca (Figure 2), although no changes in internode lengths were observed when hop plants were treated with 50 or 100 ppm Pro-Ca (data not shown). 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.[25−27] 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.[27] We hypothesize that changes in cone biomass and terpenophenolic content may be related to increased levels of the active gibberellic acid GA_1, in response to Pro-Ca inhibition of GA_1 inactivation. Methods for gibberellic acid quantitation have been developed for hops,[28] and future experiments include comparison of morphological and phytochemical effects of Pro-Ca-treated hops with active gibberellic acids.

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.[8] We have previously reported the production of hop cones with increased density in response to Pro-Ca treatment.[18] 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.[19] 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. Whereas no studies of ethylene in hops have been published, ethylene is well-known to be a hormone that initiates flowering and senescence; 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 been shown to delay the onset of flower senescence.[29] The increase of cone yield and of cone/biomass ratios, as we observed in Pro-Ca-treated hop plants, may be attributed to delayed 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. Our data suggest that the most agronomically favorable results occur when hop plants are treated with Pro-Ca during stage III, as harvested hops produced increased cone biomass and terpenophenolic contents. 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.[22] Hop flower and cone development may vary due to seasons, cultivars, and individual plants.[30] 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. To apply Pro-Ca for yield gain in hops, application must be conducted at a precise time point. Although our results suggest stage III is the best approximate treatment time, further agronomic evaluations are required. Future experimental work at an agronomically relevant scale is needed to ensure efficient use of Pro-Ca treatment for hop crop improvement, including treatments of additional varieties over multiple seasons under commercial operating procedures. Further work on the mechanisms and interactions of Pro-Ca effects on the 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.

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Funding Sources

This work was funded by Hopsteiner-S.S. Steiner, Inc., and the City University of New York Center for Advanced Technology.

ACKNOWLEDGMENT

We thank Wilhelm Rademacher (BASF, Germany) for his advice on the use of prohexadione-calcium. We also thank Danny Hallman and J. R. Ozuna (Golden Gate Ranches, Hopsteiner-S. S. Steiner, Inc.) for direction in field procedures and Dr. Dwight Kincaid (Lehman College, CUNY) for his contributions to statistical analysis in this study.

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