EFFECT OF PROHEXADIONE-CALCIUM ON SPEARMINT (M*entha spicata* L.)

A Thesis Presented

by

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DEDICATION

To my parent and loving wife Sumaiya Sharmin.

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ABSTRACT

EFFECT OF PROHEXADIONE-CALCIUM ON SPEARMINT (M*entha spicata* L.)

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Prohexadione-calcium (Pro-Ca), a plant growth regulator used primarily in fruit trees to suppress excessive vegetative growth and to inhibit disease incidence, is known to inhibit dioxygenase enzymes and to inhibit GA biosynthesis. It induces genes for polyphenols synthesis. The objective of this project was to determine if the bioregulator Pro-Ca would alter the yield of essential oil, secondary metabolites, and growth in spearmint. Spearmint shoot cuttings from the same mother plant were used in this study. The plants were treated with 0, 125, 250, 375, and 500 mg/L a.i. of Pro-Ca over four weeks, and growth responses were measured every week and at harvest. Compared with the untreated control plants, plant height, branch length, number of nodes, and fresh weight were decreased with increased concentration of Pro-Ca treatment, and total phenolics accumulation increased. Rosmarinic acid and total chlorophyll content were reduced relative to control after treatment. Treatment with increased concentration of Pro-Ca altered the accumulation of flavonoids compounds. Increased concentration of catechin and eriodictyol-7-glucoside, and decreased concentrations of procyanidin and luteolin occurred compared with the untreated plant. Modification of newly formed flavonoid synthesis could be used as a new potential strategy in plant protection.

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CHAPTER 1

INTRODUCTION

Spearmint, *Mentha spicata* L. (syn. *M. viridis*) (Lamiaceae), is an herbaceous perennial plant grown for the content of aromatic and carminative oil produced by the plant. Originally from the Mediterranean area, this popular, aromatic plant now grows throughout the temperate climates. Due to the excellent aroma and colored flowers, fresh spearmint is one of the most liked of all the herbal mints, grown in the home garden , and an essential culinary spice commonly used as a food seasoning in rice, salads and desserts dishes (Anonymous, 2008b). The essential oil from spearmint is used widely as a flavoring agent for jellies, sauces, chewing gum, candy, iced tea, mouthwashes, toothpastes, and breath mints (Anonymous, 2006a) and as a medicine for inhibiting vomiting during pregnancy, diminishing colic in babies, and curing colds, flu, and gas (Anonymous, 2006b). The oil is also used as a scent in perfumes.

Recently, spearmint has been considered as a source of important antioxidant compounds, including rosmarinic acid and phenolics used in pharmaceutical and cosmetic industries (Shetty, 2001). Spearmint also contains other secondary metabolite compounds with industrial and medicinal value (Res, 2007; Zhao et al., 2008). The class of compounds known as flavonoids comprises a significant percentage of the polyphenols in spearmint.

Flavonoids are common in plant with over 6000 being described (Harborne and Williams, 2000). Biosynthetic pathways for flavonoid have been almost completely elicited in many model plants (Schijlen et al., 2004), such as maize, tobacco, and petunia. Currently, two types of genes are distinguished in the flavonoid pathway; structural genes encoding enzymes, that are responsible in the formation of flavonoids, and regulatory genes, that control the expression of the structural genes (Schijlen et al., 2004). Malonyl-CoA and *p*-coumaroyl-CoA, which come from carbohydrate metabolism and from the phenylpropanoid pathway, are common precursors for the synthesis of most flavonoids (Forkmann and Heller, 1999). The biosynthesis of flavonoids involved a series of enzymatic steps catalyzed by chalcone synthase, resulting in the yellow colored chalcone (Schijlen et al., 2004). Chalcone is frequently not the end-product in many plants, but latter other flavonoid pathways proceed in several enzymatic steps end to the production of flavonoids, such as flavanone, flavonols, dihydroflavonols, and anthocyanins.

Plant growth regulators, both natural and synthetic, are commonly known to modify plant growth and influence the production of metabolites and byproducts. Auxin, gibberellins, ethylene, cytokinin, and abscisic acid are naturally occurring plant growth regulators in plants. Auxin and gibberellins play somewhat similar roles in initiating cell elongation and stimulating cell division, but gibberellins have no direct inhibitory influence on vegetative growth of plants, such as alternative regulators (Davies, 1995).

Prohexadione-Calcium (*Calcium 3-Oxido-4-propionyl-5-oxo-3-cyclohexene-carboxylate,* Pro-Ca; BASF Corporation, Florham Park, NJ 07932) is a plant growth regulator used mainly on fruit trees and grasses (Evans et al., 1999). The proprietary formulation of Pro-Ca is known by the trade name Apogee® (27.5% a.i.) in USA, and

Regalis® (10% a.i.) in Europe. BAS 125 11W was the experimental code for this compound. Pro-Ca inhibits the synthesis of gibberellins in plants and is useful for reducing excessive vegetative growth in apples and for reducing the incidence of fire blight in apples (Buban et al., 2004; Evans et al., 1999; Roemmelt et al., 1999; Roemmelt et al., 2003b). Pro-Ca acts by reducing the levels of highly active $GA_{1,}$ increasing levels of precursors GA_{20} (inactive), and reducing longitudinal vegetative growth (Evans et al., 1999). The ring structure of Pro-Ca is similar to 2-oxoglutarate, and based on this property is able to inhibit dioxygenase enzymes (Rademacher, 2000), which involved in gibberellins biosynthesis and flavonoid biosynthesis (Forkmann and Heller, 1999). Consequently, Pro-Ca modifies flavonoid metabolism and interferes with the accumulation of secondary metabolites in apples (Rademacher and Kober, 2003; Roemmelt et al., 2003b). An alteration of flavonoid formation has been noted in young leaves of apples (Roemmelt et al., 1999) and pears (Roemmelt et al., 2003a) as 3-deoxycatechins. There change in flavonoid synthesis could act as antimicrobial agents and be related in plant disease resistance (Del Río et al., 2003; Yamamoto et al., 2000), and play a defensive role against mechanical injury (Feucht and Treutter, 1999; Schwalb and Feucht, 1998). However, Pro-Ca, a considerable alternative to antibiotics, is able to control of secondary incidence of diseases in pear and in apple. Less pathogen incidence occurred with this compound used against apple scab (*Venturia inaequalis*) and against grapevines grey mold (*Botryotis cinerea*) and in many other host pathogen systems (Bazzi et al., 2003). Luteoforol is suggested resistance against fire blight and other fungal diseases (Spinelli et al., 2005) as Pro-Ca altered eriodictyol, immediate precursors of

luteoforol (novel flavonoids) in young leaves of apple (*Musa* x *domestica*) (Roemmelt et al., 2003b).

Pro-Ca treatment increases levels of chlorogenic acid and naringenin-7-glucosides and decreases levels of catechin, procyanidin and flavonols (Roemmelt et al., 2003b; Ruehmann and Treutter, 2003). The 3-deoxyflavan pathway also mediates the alternate pathways for flavonoid flavan-4-ol and luteoliflavan. The synthesis and accumulation of unusual flavonoids, such as luteoliflavan, which is not observed in untreated fruit trees, was observed in trees treated with Pro-Ca (Roemmelt et al., 2003b). In a study regarding genetics of apple trees, Pro-Ca influenced the PR-10 gene transcript accumulation. The gene codes for a defense response against mechanical injury and also plays a role in allergen content that is important for binding protein in plant membranes (Poupard, 2006).

Mata et al. (2006) have reported that Pro-Ca influenced the concentration of anthocyanins and carotenoids in 'Fuji', but not in 'Royal Gala' apples. Pro-Ca have been demonstrated to increase color intensity, total anthocyanins, and total phenols, despite having minimal effect on crop yield (Giudice et al., 2004; Mata et al., 2006). The application of Pro-Ca to apple and pear trees increased the nectar secretion from flowers, but lowered sugar concentration (Spinelli et al., 2005). Application of Pro-Ca significantly reduced frost injury in apple (Albrecht et al., 2004) and slightly increased vegetative growth, fruit yield, and fruit qualities in pome fruit trees (Rademacher et al., 2004). In a study conducted with rose leaves, Pro-Ca inhibits flavonol- 3-hydroxylase and to alter the formation of luteoliflavan (Schlangen et al., 2003).

Higher N_2 nutrition of the plantlets lower total phenolics compound in apples leaves whereas lower N_2 supply favors flavonoid accumulation (Ruehmann and Treutter, 2003). Several enzymes are inhibited by the bioregulator Pro-Ca in the pathway of flavonoid metabolism. Detailed knowledge on their qualitative and quantitative changes in the effect of phenolic compounds in individual plants is prerequisite for the assessment of the bioregulator with regard of antimicrobial possibilities. In this study, we describe the changes of secondary metabolite and essential oil content and growth in spearmint induced by Pro-Ca.

Objectives of the Study

The purpose of this research was to determine if the bioregulator prohexadione-calcium would alter the yield of essential oil, secondary metabolites, and plant growth in spearmint.

CHAPTER 2

MATERIALS AND METHODS

Plant material

Spearmint (*Mentha spicata* L.) plants were used in these studies. The plants were grown from branch cuttings, originating from the same mother plant, rooted in a peat-based medium (MetroMix 360, SunGro Horticulture, Vancouver, Canada), and then transplanted to plastic pots (4-inch diameter) containing the same medium to establish a population of genetically identical, uniformly growing spearmint plants. Following transplanting, the pots were placed in a greenhouse (located on the University of Massachusetts campus, Amherst, MA 01003) with minimum temperature of 23°C and natural daylight during September through November.

Treatment

Prohexadione-calcium (Pro-Ca, BAS 125 11W, BASF Corporation, Florham Park, NJ 07932), as the proprietary formulation of apogee (27.5% a.i.), was used in these studies. A foliar application (using distilled water) of Pro-Ca in a distilled water solution at 0, 443, 886, 1329, and 1772 mg/L apogee (0, 125, 250, 375, and 500 mg/L a.i.) was applied, based on earlier studies (Evans et al., 1999; Roemmelt et al., 2003b). Regulaid® 0.1% (KALO Inc., Overland Park, KS 66213), a nonionic surfactant, was used to enhance wetting ability of the solutions (Czarnota and Thomas, 1997) at the rates of 1.01 ml/L to the Pro-Ca solutions. Quest (Crystal Quest®, Marietta, GA 30067), a commercial and industrial water conditioner used to remove traces of dissolved solids of calcium, iron, or

magnesium from solutions (Anonymous, 2008a), was added to each Pro-Ca solution as a water softener at 2.52 ml/L. To ensure that the constituents were completely dissolved into solution, the Pro-Ca, surfactant, Quest, and water were mixed together and vigorously shaken. The prepared Pro-Ca solutions were refrigerated until used.

Experimental

One month after transplanting, 30 similarly sized and uniformly developed plants (the same vegetative stage and 10 unfolded leaves) were selected from the population of plants for use in the trial. The plants were placed randomly in one of five treatment groups (six plants per treatment group) for treatment with the Pro-Ca solution or with water (control group), and the third youngest (most distal) leaves on each replicate branch was tagged, for following vegetative growth effects of Pro-Ca on plant growth and constituents. Treatments were applied to the spearmint plants with a hand-held mister (Ace Hardware Corp., Oak Brook, Illinois 60521) until the solution began dripping from the plant. Uniform coverage of the vegetative tissue was assured by misting the adaxial and abaxial surfaces of leaves for each treatment.

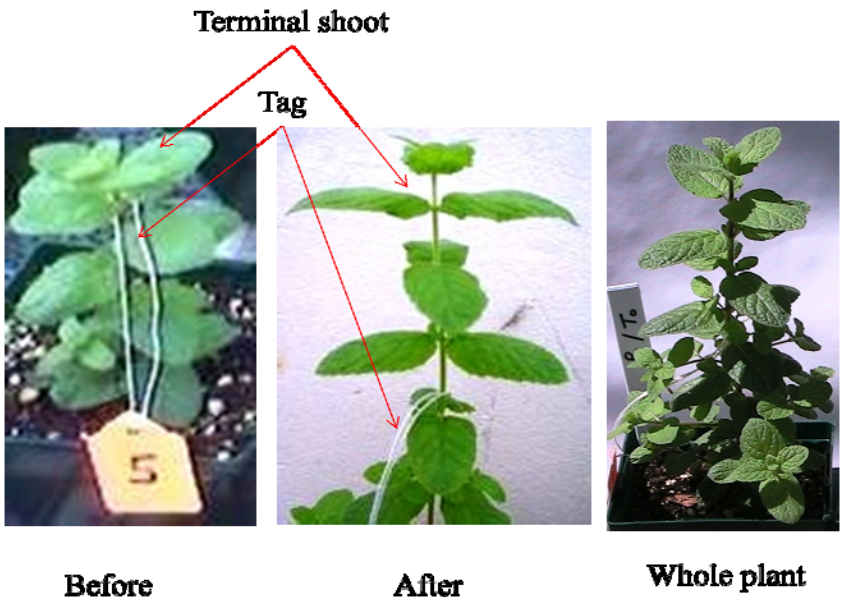

Figure 1. Branch development of spearmint treated with Pro-Ca

A tag was placed at the 2nd or 3rd node from the plant apical tip (Fig. 1) of each plant before treatment. This tag marked the zero point from which to monitor branch growth. A ruler was used to measure the distance from the tagged node to the branch tip weekly.

At three weeks after the first application of Pro-Ca, the terminal node leaves and associated stem tissue (that developed after treatment with Pro-Ca) (new tissue sample) were sampled (cut from plant by a sharp razor blade and sealed in a plastic zip-lock bags, and frozen at -80°C). The remaining portion of each spearmint plant (mature tissue sample) was harvested by cutting the plant stem with a razor blade at ground level, weighted to determine fresh weight sealed in zip-lock plastic bags, and frozen at -20°C until used for the extraction of essential oils and other constituents.

Sample Extraction and Analyses

Chlorophyll level was measured in the new tissue sample by extracting a 50 mg sample (fresh, frozen tissue) of each plant with 3 ml methanol (100%) in a 16 x 100 mm test tube. The tubes were covered with aluminum foil and incubated at 23°C for 2 h in the dark. Each sample was homogenized by a tissue grinder (Duall®, PTFE Pestle and Glass Tube, Kimble/Kontes, Vineland, NJ) and then filtered through Whatman No.1 filter paper. The absorbance of the extract was measured at 650 nm (Chl. A) and 665 nm (Chl. B) (Al-Amier et al., 1999). The concentration of chlorophyll was calculated using the formula of (Hipkins and Baker, 1986).

Rosmarinic acid was measured by extracting a second fresh, frozen tissue sample (50 mg) of new tissue from each plant (contained in 16 x 100 mm test tubes) by adding 3 ml of methanol (50%), incubating at 55°C for 2 h, homogenizing with the tissue grinder (as above), and filtering through Whatman No.1 filter paper. A sample of the methanol extract (1 ml) was placed subsequently in a 16 x 100 mm test tube, diluted with 5 ml of methanol (100%), and vortexed to ensure thorough mixing before measuring the absorbance of a 1 ml subsample at 333 nm (Al-Amier et al., 1999). The concentration of rosmarinic acid in diluted extract was calculated following the equation of $A=\Sigma bc$, where A = the absorbance of the solution, Σ = extinction coefficient (19,000 liter/mol-cm), b = the width of the cuvette (1 cm), and c = the concentration of rosmarinic acid.

The total phenolic concentration was determined by extracting a third sample (50 mg) of new tissue of each plant in 2.5 ml of 95% ethanol for a minimum incubation of 48 h. Each sample subsequently was homogenized and filtered through Whatman No. 1 filter paper, and 1 ml of the filtered extract was transferred to a 16 x 100 mm test tube for the

addition of 1 ml of 95% ethanol, 5 ml of distilled, deionized water and 0.5 ml of a 50% Folin Ciocalteu (Sigma-Aldrich Inc., St. Louis, MO) reagent. After 5 min, 1 ml of 5% Na_2CO_3 was added with mixing to stop the reaction, and the mixture was placed in the dark for 60 min before measuring the absorbance at 725 nm (Chandler and Dodds, 1983; Shetty et al., 1995; Singleton and Rossi, 1965). Absorbance values were compared with a standard curve of gallic acid (25-200 µg/ml in 95% ethanol) at various concentrations and converted to mg of total phenolics/g fresh tissue (Shetty et al., 1995).

Flavonoids were measured in the terminal (young and new tissues) branch (Fig. 1) stem and leaf (10 g) sample from each plant following extraction with 25 ml methanol (100%) (Roemmelt et al., 2003b; Wang et al., 2004). The samples were extracted three times with methanol (25 ml) and centrifuged at 1158 x g (Sorvall Super T21 Tabletop Centrifuge**,** Ramsey, MN 55303) for 10 min to remove residue. The extracts were combined in a 250 ml, round- bottom flask and then evaporated to dryness under vacuum at 45°C to 50°C using a rotary evaporator (Buchi Rotoevaporator-R200, HitechTrader.com, Mt. Holly, NJ 08060). The residues were dissolved in HPLC grade methanol, made to volume in a 10-ml volumetric flask with methanol, and filtered through a 0.22 µm × 13 mm syringe filter (Fisher brand Nylon Syringe Filters) in preparation for HPLC analysis.

The HPLC analysis was done on Perkin Elmer Nelson NCI 900, Network Chromatography Interface (Perkin Elmer, Waltham, MA 02451) system equipped with a Series 200 IC Pump, a Series 200 Diode Array Detector, an on line Series 200 Vacuum Degasser, a Series 200 Auto Sampler, a Series 200 Fluorescence Detector, and a Synergi Fusion RP-C18 boned silica column (5µ, 250 × 4.6 mm, 80Å) (Phenomenex, Torrance,

CA 9050). The gradient solvents were phosphoric acid and HPLC grade water for solvent A [2.45g phosphoric acid (98%) and 0.405g NaCl in 900 ml of pure water, pH 2.5 at 25°C] and HPLC grade acetonitrile for solvent B. The flow rate was 0.80 ml/min and injection volume was 10µl. Gradients condition were 85%A and 15% B for 1 min, 50% A and 50% B for 40 min, 50% A and 50% B for 10 min, 85% A and 15% B for 10 min. The absorbance of the effluent was measured at 220 nm and 360 nm for detection and characterized of flavonoids [(+)-catechin, caffeic acid, procyanidin, luteolin, and eriodictyol-7-glucoside] in the extracts that were identified by retention time and comparison with standards.

Essential oil was extracted from a whole plant sample collected at harvest of each treatment using stem distillation for 3 h in a lab Clevenger apparatus. The essential oil water dried over anhydrous Na_2SO_4 to remove traces of water and stored in sealed vials in the dark at 4 °C until analyzed (Özek et al., 2007).

Standard Compounds

Flavonoid compounds of the Pro-Ca treated spearmint plants were quantified by comparing with external standards [(+)-catechin hydrate ≥99.0% HPLC grade, procyanidin B2 ≥90% HPLC grade, luteolin ≥98% , caffeic acid ≥98.0% HPLC grade (Sigma-Aldrich Inc., St. Louis, MO)]. Eriodictyol-7-glucoside was quantified using eriodictyol-7-glucoside HPLC grade (ChromaDex Inc., Irvine, CA).

Statistical Analyses

The experiment was replicated six times, and was repeated three times (at different times of the year) to determine the effects of Pro-Ca. Plant height, branch length, and fresh weight data were analyzed as a two-way analysis of variance (main effect of treatment) using the SAS PROC GLM procedure (SAS 9.1.3, SAS Institute Inc., SAS Campus Drive, Cary, NC 27513). Mean separation was conducted by F test and Duncan's New Multiple Range Test (P=0.05).

Total phenolics, rosmarinic acid, total chlorophyll, and flavonoids concentration data were analyzed using SAS PROC GLM for the main effect of Pro-Ca. Mean separation was conducted by F test and Duncan's New Multiple Range Test (P=0.05), and regression analyses was performed. Polynomial comparison was performed by orthogonal polynomial test using PROC GLM and PROC IML. The data for essential oil also were analyzed using SAS PROC GLM. Mean separation was performed by F test and Duncan's New Multiple Range Test (P=0.05).

CHAPTER 3

RESULTS

Effect of Pro-Ca on Growth of Spearmint

Plant height of spearmint was significantly suppressed with increasing concentrations of Pro-Ca relative to the control, which received no Pro-Ca (Table 1). The application of Pro-Ca at 125 mg/L decreased plant heights by 15% relative to the control, whereas application of Pro-Ca at 250 mg/L, 375 mg/L, and 500 mg/L resulted in 22%, 24%, and 32% lower plant heights, respectively, compared with the controls, but no significant changes between 250 mg/L and 375 mg/L were observed. The highest concentration of Pro-Ca (500 mg/L) tested suppressed plant height more than lower concentrations. A negative cubic relationship was observed with increased concentrations of Pro-Ca (Fig. 2).

Terminal branch length of spearmint was suppressed with increased treatments of Pro-Ca significantly relative to the control (Table 1). The application of Pro-Ca at 125 mg/L decreased branch length about by 25% compared to the control, whereas application of Pro-Ca at 250, 375, and 500 mg/L resulted in respective 27%, 30%, and 33% suppressed branch length relative to the untreated plants. The results showed that all concentrations of Pro-Ca were effective in suppressing branch growth relative to the control. A negative cubic relationship occurred with increasing concentration of Pro-Ca (Fig. 3). However, the terminal branch length was suppressed with increasing concentration of Pro-Ca at every week after treatment (Fig. 4). The lower rate of Pro-Ca at 125 mg/L decreased branch length by 24%, 33%, and 19% at 1st, 2nd, and 3rd week

respectively, whereas, the highest rate (500 mg/L) suppressed branch length by 27%, 42%, and 42% within three weeks subsequently relative to the control.

Number of nodes in a branch was changed with increasing treatment of Pro-Ca relative to the control (Table 1). The cumulative number of nodes in a branch decreased by 7% at 125 mg/L of Pro-Ca treatment compared to the control, whereas increased concentrations of Pro-Ca at 250, 375, and 500 mg/L lowered the number of nodes by 10%, 10%, and 11% respectively in comparison with the control. All Pro-Ca concentrations were similarly effective. A negative, but non-significant linear relationship occurred with increasing application of Pro-Ca (Fig. 5).

Fresh weight of spearmint was changed effectively with increasing concentration of Pro-Ca (Table 1). The application of Pro-Ca at 125 mg/L reduced fresh weight by 24% compared to the untreated plants, whereas application of Pro-Ca at 500 mg/L resulted in 54% lower fresh weight relative to the control. The concentrations of 125 mg/L, 250 mg/L and 375 mg/L resulted at about similar effectives in suppressing fresh weight. The trend in fresh weight was a negative linear relationship with the increased concentration of Pro-Ca treatments (Fig. 6).

Effect of Pro-Ca on Chlorophyll Content of Spearmint

High concentrations of Pro-Ca reduced accumulation of total chlorophyll significantly relative to the control (Table 1). However, the application of 125 mg/L had no effect whereas, the concentrations of 250 mg/L, 375 mg/L, and 500 mg/L resulted in 10%, 16% and 24% lower total chlorophyll than the control. However, the concentration

with Pro-Ca at 250 mg/L was not different from the control. A negative linear relationship in chlorophyll concentration occurred with increasing concentration of Pro-Ca (Fig. 7).

Effect of Pro-Ca on Essential Oil of Spearmint, and Correlations

Essential oil of spearmint decreased with increased concentration of Pro-Ca (Table 1). The reduction was linear with increased Pro-Ca concentration (Fig. 8). The essential oil of spearmint was also effectively correlated with fresh weight as a reduction in fresh weight was followed with a reduction in the accumulation of essential oil (Tables 1 and 2).

The correlation coefficient between plant height and fresh weight was highly significant (Table 2). Fresh weights and plant heights decreased linearly with increasing Pro-Ca concentration. The correlation coefficient between branch length and number of nodes in a branch was highly significant (Table 2). Number of nodes and branch length decreased with increasing Pro-Ca concentration. Total phenolics and rosmarinic acid were significantly correlated (Table 2). The correlation coefficient between total phenolics and total chlorophyll and between rosmarinic acid and total chlorophyll were not significant (Table 2).

Effect of Pro-Ca on Secondary Metabolites of Spearmint

Pro-Ca treatments significantly influenced accumulation of total phenolics in spearmint (Table 1) in that low and intermediate applications of Pro-Ca reduced total

phenolics compared to the control; however, the higher rate of Pro-Ca had little influence on total phenolics. A quadratic relationship occurred with increasing concentration of Pro-Ca (Fig. 9).

Table 1. Effect of Pro-Ca on plant height, branch length, no. of nodes, fresh weight, essential oil, and total phenolics, total chlorophyll, and rosmarinic acid in spearmint.

	Measurement[yz]							
Treatment (mg/L)	Plant ht. (cm)	Branch length (cm)	No. of nodes	Fresh wt. (g)	Essential oil (ml/100 g)	Total Phenolics (mg/g FW)	Total Chlorophyll (µg/g FW)	Rosmarinic acid (mg/g FW)
0	28.5[a]	7.6[a]	4.5[a]	9.99[a]	0.17[a]	15.3[a]	3164[a]	2.31[a]
125	24.2[b]	5.9[b]	4.2[ab]	7.63[b]	0.16[ab]	10.9[b]	3169[a]	1.43[b]
250	22.2[c]	5.2[b]	4.1[b]	6.43[bc]	0.15[ab]	11.2[b]	2849[ab]	1.33[b]
375	21.7[c]	5.3[b]	4.1[b]	6.09[bc]	0.14[ab]	11.8[b]	2664[bc]	1.31[b]
500	19.5[d]	4.9[b]	4.0[b]	4.57[c]	0.12[b]	14.1[ab]	2351[c]	1.30[b]
Pro-Ca trendline	L[**], Q[*], C[*]	L[**], Q[**], C[*]	L[ns]	L[**]	L[*]	Q[**]	L[**]	L[**], Q[*]

[y] Mean

[z] Values with different letters within columns are significantly different at P= 0.05 (Duncan's -MR test). NS, *, ** Nonsignificant, and significant at P≤ 0.05, 0.01 respectively; L=linear, Q=quadratic, C=cubic.

All rates of Pro-Ca appeared to reduce rosmarinic acid in spearmint significantly relative to the control, but the differences among treatments from 125 to 500 mg/L was small (Table 1). A negative quadratic relationship was observed with increasing concentration of Pro-Ca (Fig. 10).

Catechin content in spearmint was significantly changed with increased concentrations of Pro-Ca relative to the control (Table 3). Curiously, Pro-Ca reduced catechin levels at low rates, whereas the high rates increased catechin. The trend of

catechin levels observed a quadratic relationship with increasing concentrations of Pro-Ca (Fig. 11).

Increased concentration of Pro-Ca decreased accumulation of procyanidin (Table 3). The response in concentration of procyanidin indicated that there was a great influence of Pro-Ca at the highest rates. A negative linear relationship occurred with increasing concentration of Pro-Ca (Fig. 12).

Pro-Ca had no significant influence on caffeic acid accumulation in spearmint (Table 3 and Fig. 13).

Table 2. Correlation coefficients on plant height, fresh weight, branch length, no. of node, essential oil, total phenolics, total chlorophyll, and rosmarinic acid in spearmint as a function of Pro-Ca treatment.

	Correlation co-efficient[yz]				
	Pearson's r- value				
Parameters	Plant ht.	No. of nodes	Essential oil	Total Chlorophyll	Rosmarinic acid
Branch length		0.921^{**}			
Fresh wt.	0.841^{**}		0.928^{*}		
Total Phenolics				0.015^{ns}	0.647^{**}
Total Chlorophyll					0.329^{ns}

[y] Pearson's r- value
[z] Correlation (r) value significantly different from critical value are indicated by ** (at P ≤ 0.01) and by * (at P≤ 0.05) by correlation co-efficient.
Non-significant correlations are indicated by ns.

Pro-Ca decreased accumulation of luteolin in spearmint with respect to the control (Table 3). Intermediate rates of Pro-Ca had greater influences than the high rates. A negative cubic relationship occurred with Pro-Ca concentration (Fig. 14).

Induction of Newly Formed Flavonoid after Pro-Ca Treatment

Fairly high concentrations of eriodictyol-7-glucoside were observed significantly with increasing concentration of Pro-Ca (Table 3). Pro-Ca at 375 mg/L was more effective than the highest concentration of 500 mg/L. Eriodictyol-7-glucoside is a precursor for the formation of flavonoid luteoforol, which is suggested to reduce the incidence of pathogens on fruit trees (Rademacher, 2004; Roemmelt et al., 1999). The accumulation observed a quadratic relationship with increasing concentration of Pro-Ca (Fig. 15).

Table 3. Effect of Pro-Ca on flavonoid concentrations measured by HPLC in spearmint.

Treatment	Flavonoid concentration (mg/g FW)				
(mg/L)	Catechin	Procyanidin	Caffeic acid	Eriodictyol-7-glucoside	Luteolin
0	0.113^{bc}	0.099^{a}	1.595^{a}	0.063^{c}	0.295^{a}
125	0.075^{c}	0.062^{b}	1.148^{a}	0.131^{b}	0.221^{b}
250	0.093^{c}	0.031^{c}	1.136^{a}	0.147^{b}	0.095^{d}
375	0.139^{ab}	0.030^{c}	1.970^{a}	0.209^{a}	0.082^{d}
500	0.158^{a}	0.025^{c}	2.011^{a}	0.144^{b}	0.162^{c}
Pro-Ca trendline	L^{**}, Q^{*}	L^{**}, Q^{**}	L^{ns}	L^{**}, Q^{**}	L^{**}, Q^{**}, C^{**}

Means followed by same letter are not significantly different (Duncan's MR Test, P = 0.05)
NS, *, ** Nonsignificant, and significant at P ≤ 0.05, 0.01 respectively; L=linear, Q=quadratic, C=cubic.

Correlations between Newly Formed Flavonoid and Others

There was a significant correlation found between newly formed flavonoid eriodictyol-7-glucoside and procyanidin, and luteolin (Table 4). However, no correlation was found between eriodictyol-7-glucoside and catechin, and caffeic acid. The

concentration of luteolin and procyanidin showed a fluent declined relative to the control, whereas, catechin and eriodictyol tended to accumulate comparatively. With increasing content of eriodictyol-7-glucoside decreasing procyanidin was appeared (Fig. 16).

Table 4. Correlation coefficients on catechin, procyanidin, caffeic acid, eriodictyol-7-glucoside, and luteolin in spearmint as a function of Pro-Ca treatment.

	Correlation co-efficient[yz]			
	Pearson's r- value			
Parameters	Procyanidin	Caffeic acid	Eriodictyol-7-glucoside	Luteolin
Catechin	0.394[ns]	0.618[*]	0.272[ns]	0.251[ns]
Procyanidin		0.177[ns]	0.725[**]	0.885[**]
Caffeic acid			0.215[ns]	0.098[ns]
Eriodictyl-7-glucoside				0.728[**]

[y] Pearson's r- value

[z] correlation (r) value significantly different from critical table are indicated by ** (at $P \leq 0.01$) and by * (at $P \leq 0.05$) by correlation co-efficient.

Non-significant correlations are indicated by ns.

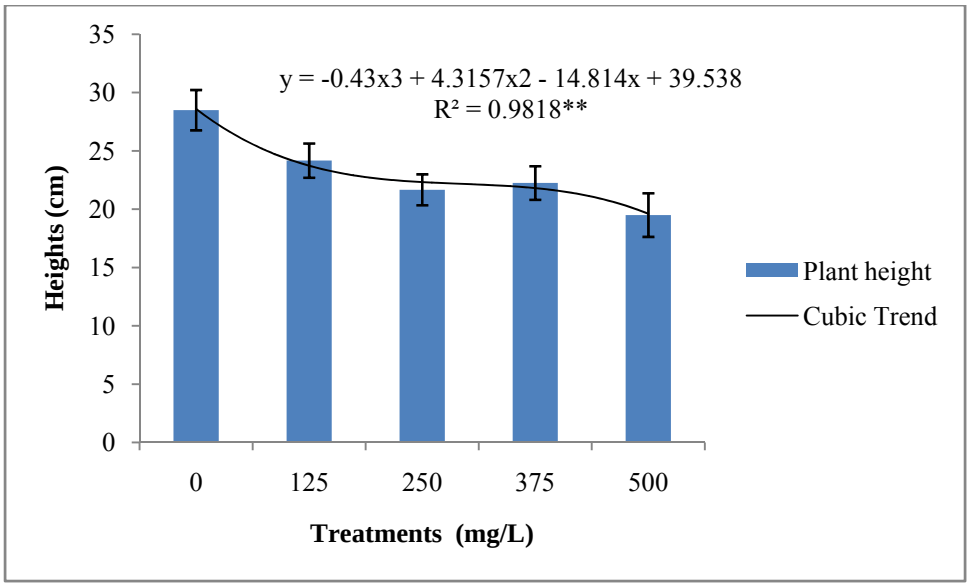

Figure 2. Changes in plant heights (cm), and regression co-efficient in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of six replicates.

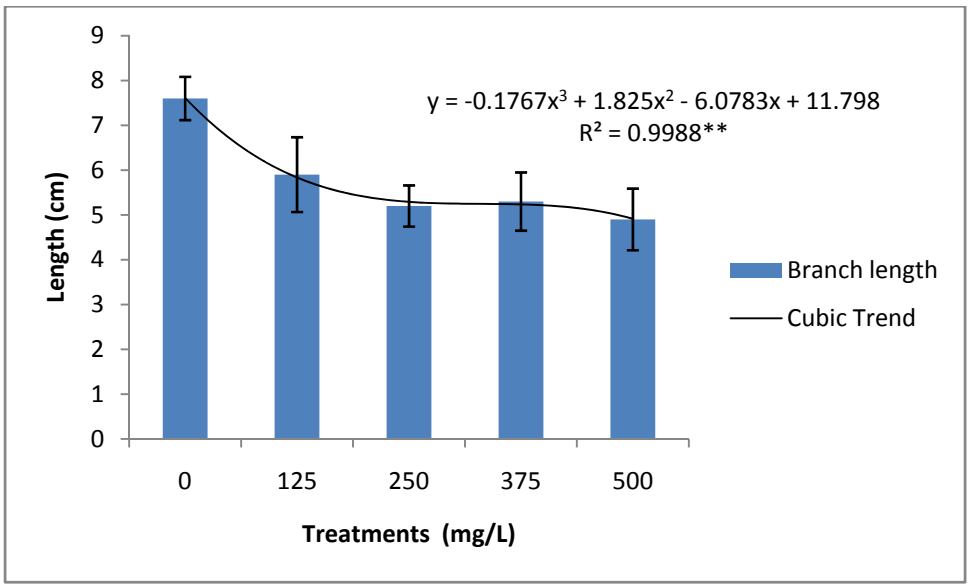

Figure 3. Changes in branch length (cm) and regression co-efficient in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of six replicates.

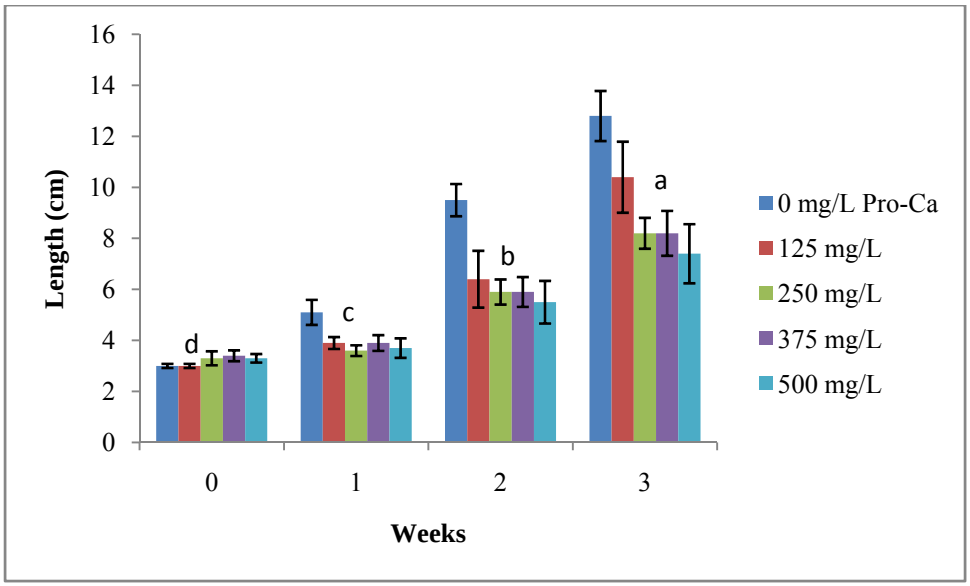

Figure 4. Changes of branch length as a function of Pro-Ca (0, 125, 250, 375, and 500 mg/L a.i.) during three weeks. The vertical lines represent the standard deviation from the mean of six replicates.

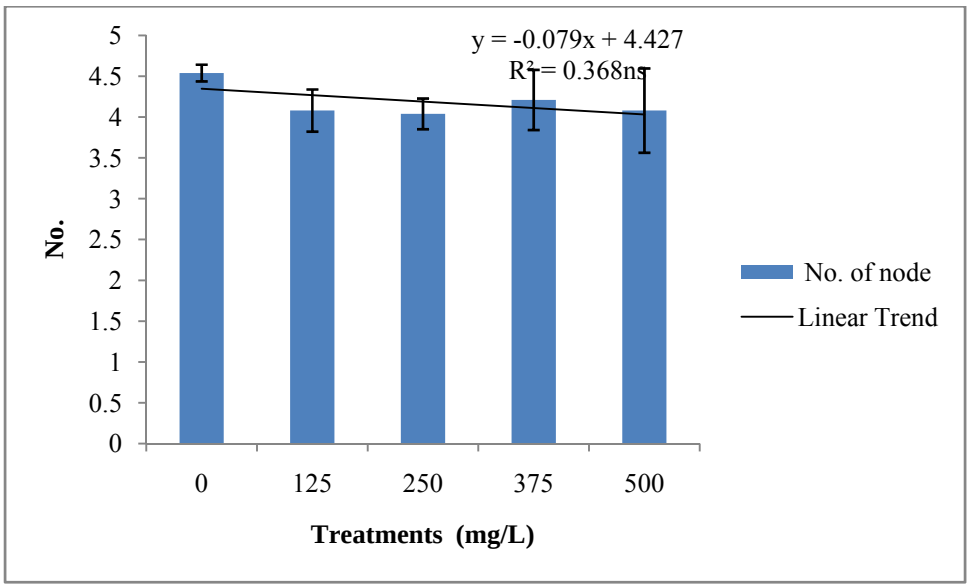

Figure 5. Changes in number of nodes in a branch and regression co-efficient in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of six replicates.

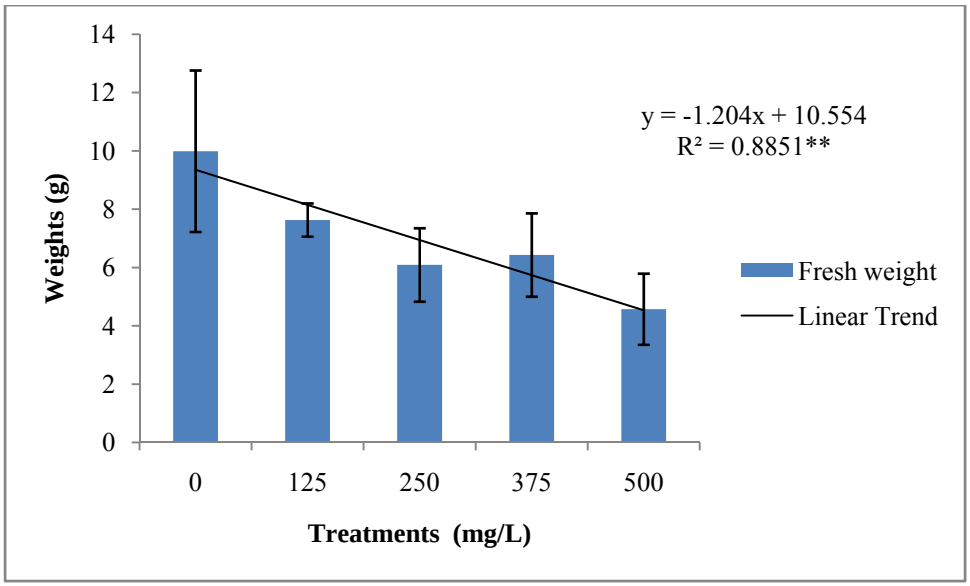

Figure 6. Changes in fresh weights (g) and regression co-efficient in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of six replicates.

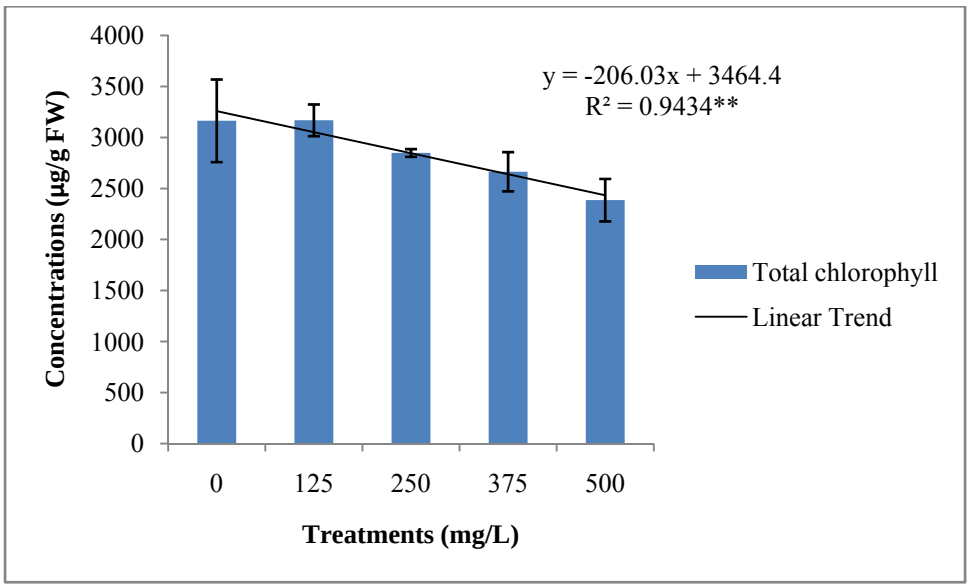

Figure 7. Changes in total chlorophyll (µg/g FW) and regression co-efficient in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of six replicates.

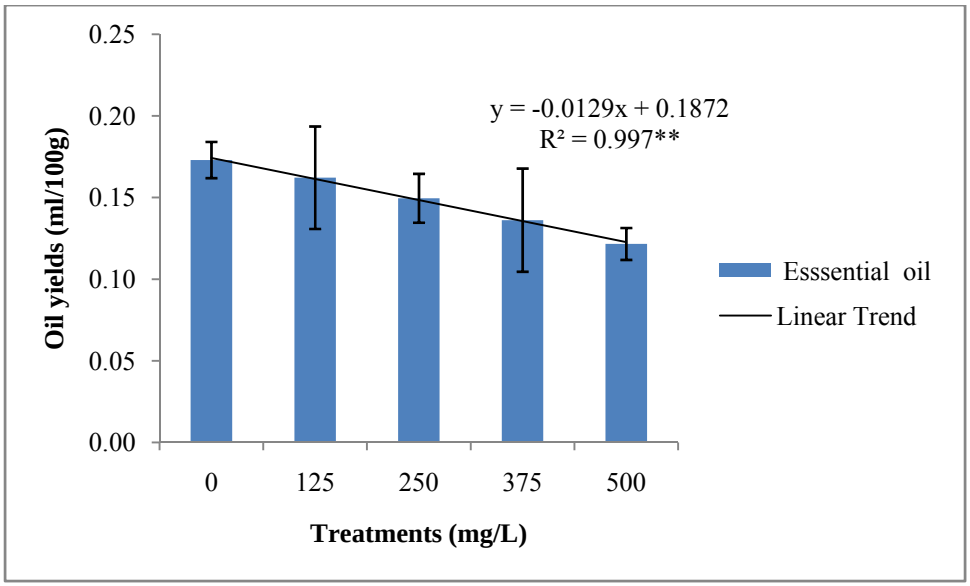

Figure 8. Changes in essential oil (ml/100 g FW) and regression co-efficient in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of three replicates.

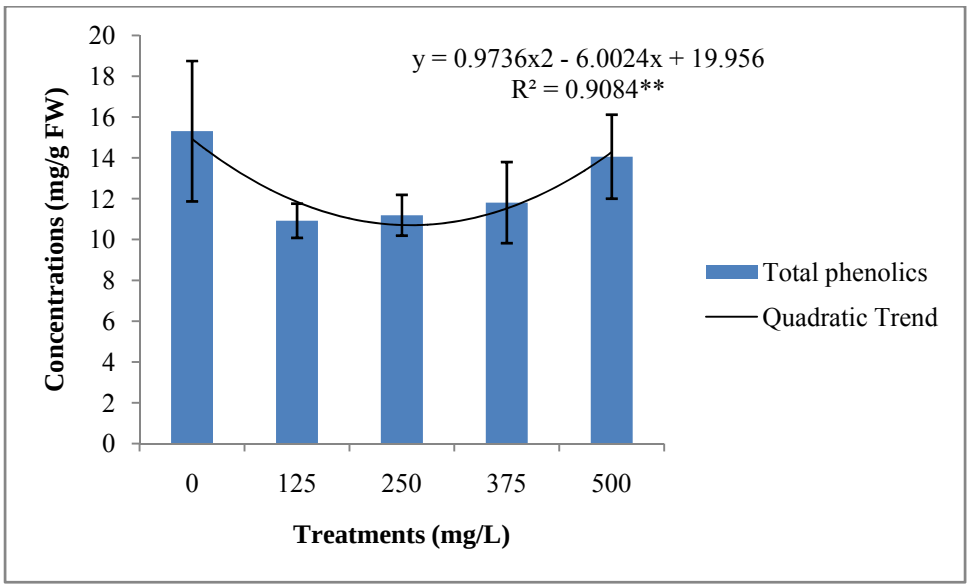

Figure 9. Changes in total phenolics (mg/g FW) and regression co-efficient in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of four replicates.

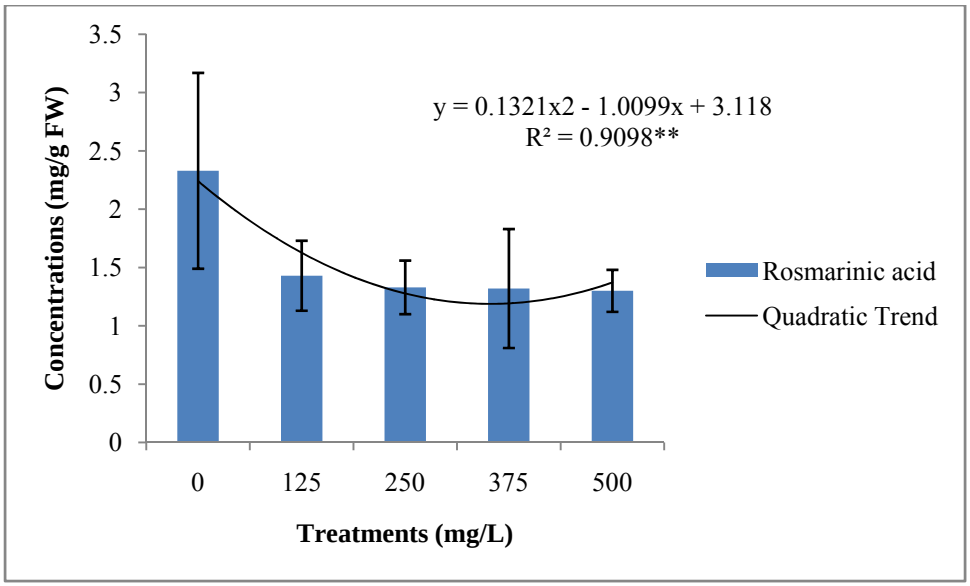

Figure 10. Changes in rosmarinic acid (mg/g FW) and regression co-efficient in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of four replicates.

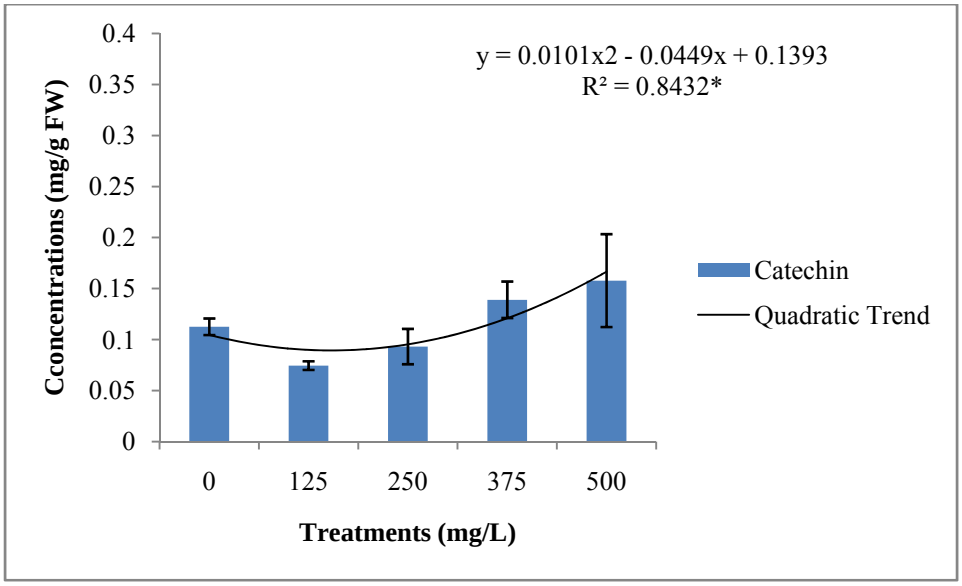

Figure 11. Changes in catechin mean concentrations (mg/g FW) in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of three replicates.

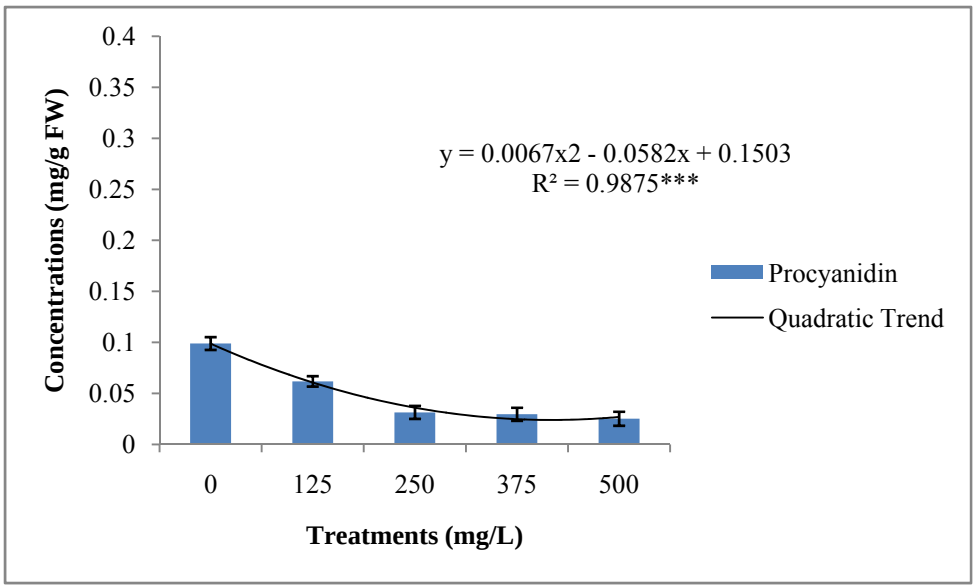

Figure 12. Changes in procyanidin mean concentrations (mg/g FW) in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of three replicates.

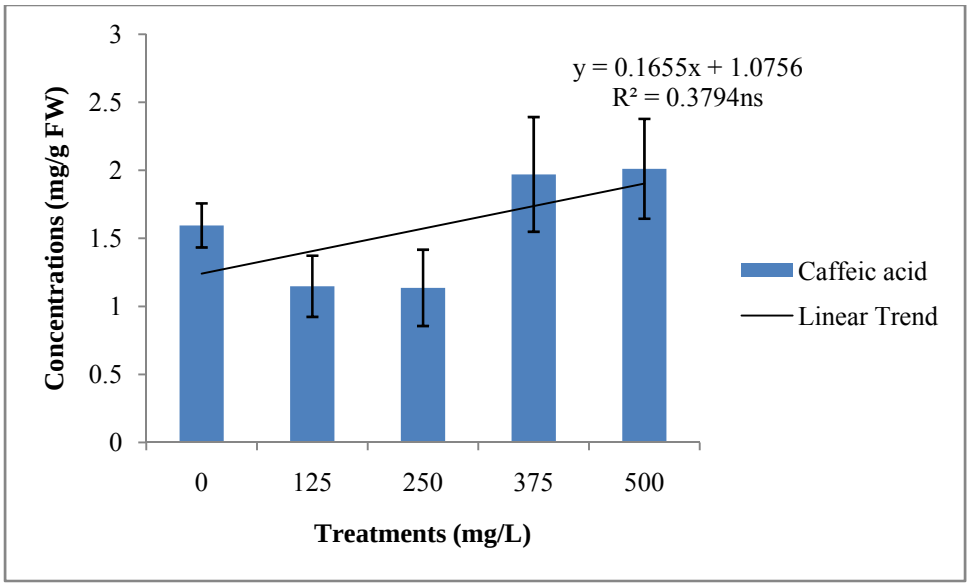

Figure 13. Changes in caffeic acid mean concentrations (mg/g FW) in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of three replicates.

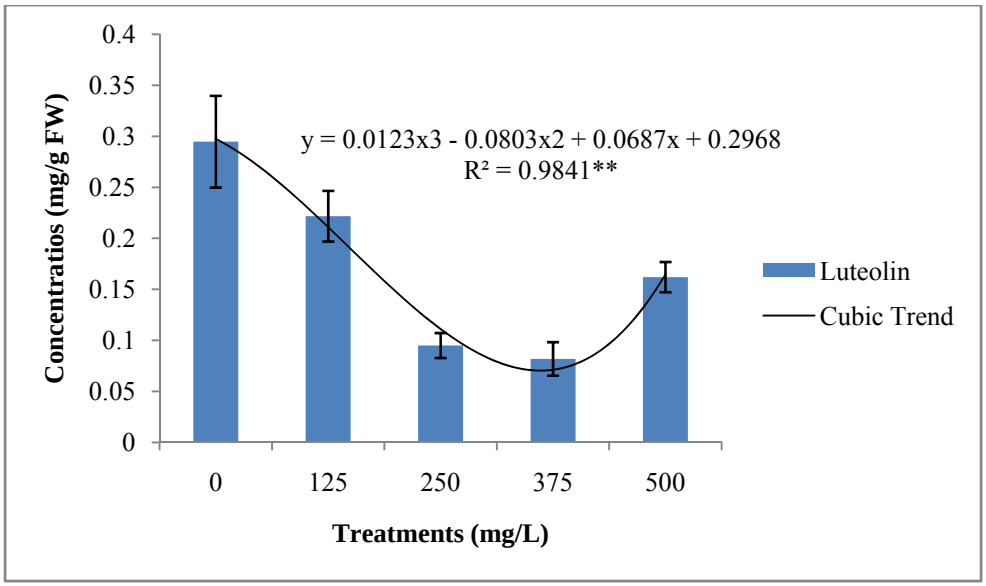

Figure 14. Changes in luteolin mean concentrations (mg/g FW) in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of three replicates.

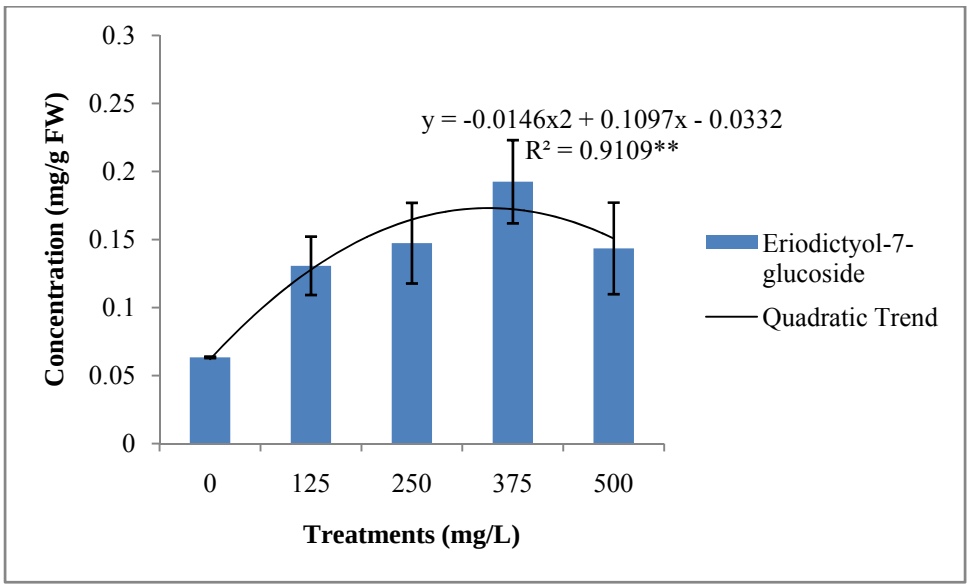

Figure 15. Changes in eriodictyol-7-glucoside mean concentrations (mg/g FW) in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation from the mean of three replicates.

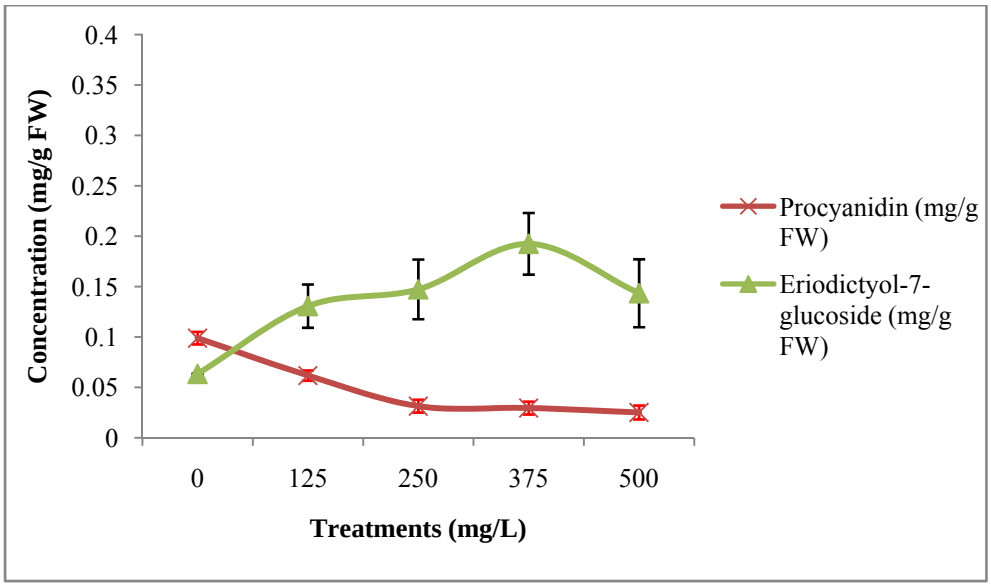

Figure 16. Correlation between procyanidin and eriodictyol-7-glucoside concentrations (mg/g FW) in spearmint with increasing concentration of Pro-Ca (0, 125, 250, 375, 500 mg/L a.i.). The vertical lines represent the standard deviation.

CHAPTER 4

DISCUSSION

Prohexadione-calcium (Pro-Ca) a plant growth regulator, inhibits biosynthesis of gibberellin (Rademacher et al., 2004) and provides effective control of vegetative growth in spearmint plants, reduces cell elongation (Rademacher, 2000), and decreases total plant weight (Karhu and HytÖNen, 2006). Gibberelic acid (GA_1) causes longitudinal branch growth and cell elongation (Rademacher, 2000). The foliar application of Pro-Ca reduces the level of GA_1 (highly active) and enhances accumulation of the immediate precursor GA_{20} (inactive) (Fig. 17). Therefore, Pro-Ca treated plants are likely to have an induced reduction in GA_1, which could account for the observed suppressed plant height, branch length, and total plant weight relative to untreated control plants. The number of nodes in the branch would be lowered because of the decreased branch length under Pro-Ca treatment. May be the shorter branch length is due to restricted elongation of internodes.

A reduction of phenolic compounds in the Pro-Ca treated spearmint tissues compared with untreated plants was noted with low concentrations of Pro-Ca, but accumulation increased with the highest Pro-Ca treatment (Fig. 9). This difference is probably due to the Pro-Ca treated tissues inhibiting flavanone 3-hydroxylase (FHT) and flavonol synthase (FLS) at low Pro-Ca levels and inducing the same enzymes at a higher concentration at a certain point (Fig. 18). In this respect, accumulation of rosmarinic acid declined in the treated plants relative to amounts in the untreated control plants (Fig. 10) because of the inhibition of enzymes like FHT and FLS after treatment. Although the

exact mechanism responsible for this inhibition after treatment is unknown, generally some effective inhibition after treatment occurred, probably due to enzymatic action lowering rosmarinic acid concentrations (Fig. 10 and 18). For treated spearmint leaves, a lower concentration of total chlorophyll content was observed (Fig. 7) than in untreated leaves, an action that may affect nutrient uptake and photosynthesis in plants (Kura-Hotta et al., 1987).

Some dioxygenases involved in flavonoids metabolism and other compounds are affected by Pro-Ca (Rademacher, 2000; Roemmelt et al., 2003b). The accumulation of flavonoids (Fig. 11 and 15) and the reduction of flavonoids (Fig. 12 and 14) in the Pro-Ca treated leaves showed that the enzymes FHT and FLS, which act as a 2-oxoglutarate-dependent dioxygenases (Forkmann and Heller, 1999), are inhibited by Pro-Ca (Fig. 18). This result was observed earlier in apples (Gosch et al., 2003; Roemmelt et al., 2003b) and pears (Peterek, 2004). Synthesis of catechin is relatively low at low levels of Pro-Ca, but treatment of the tissue at 375 mg/L Pro-Ca caused a substantial accumulation afterward in leaves compared to untreated plants (Fig. 11). We assume that in the presence of a strongly active FLS, which is responsible for the conversion of dihydroflavonols to flavonols (catechin) dominates over dihydroflavonols 4-reductase (DFR) and leucoanthocyanidin reductase (LAR) route of the pathway until a certain level of treatment that reconstitutes the flavonols (catechin) afterward. This action may require a high concentration of treatment level to reduce the enzyme inhibition or accumulate catechin from leucocyanidin and other flavonoids by LAR (Fig. 18). Other research (Fischer et al., 2003) shows activity of the enzyme DFR, which is responsible for the formation of dihydroflavonols to leucoanthocyanidins (Gollop et al., 2002). However,

procyanidin and luteolin are synthesized to a low extent until the treatment level reaches 500 mg/L in leaves (Fig. 12 and 14). In this context, I suggest that with the active presence of enzyme FLS, the modification of dihydroflavonols to flavonols (procyanidin and luteolin) strongly dominates over DFR and LAR in the flavonoids pathway (Fig. 18). This result may provide an explanation as to why procyanidin and luteolin concentrations in leaves (Fig. 12 and 14) are affected by Pro-Ca treatment. As caffeic acid concentration is accumulated to a low extent until 250 mg/L, and increased afterward (Fig. 13), we suggest that an enzyme inhibition of the caffeic acid formation pathway occurred at a low treatment level. A possible accumulation may occur at higher treatment levels in the phenylpropanoid pathway by induction of DFR enzyme. Blocking FLS (Fig. 18) may have directed metabolites toward the synthesis of flavan -3-ols (catechin and procyanidin) and thus may have compensated for the loss due to the partially inhibited FHT activity.

The formation of 3-deoxyflavonoids in the Pro-Ca treated spearmint tissues is formed by a postulated flavanone 4-reductase (FNR) enzyme, which catalyzes the reduction of the eriodictyol to luteoforol (Fig. 18). Eriodictyol is the unstable immediate precursor of luteoliflavan (Bate-Smith, 1969; Stich and Forkmann, 1988). We assume that the luteoforol is formed from eriodictyol by FNR or DFR. Luteoforol is a novel flavonoid present only in the Pro-Ca treated plants and has antibacterial activity (Roemmelt et al., 2003a; Roemmelt et al., 1999). This observation shows that at the substrate affinity of the FNR since the FHT inhibition also leads to the positive accumulation of pentahydroxyflavanone (Fig. 18). However, the formation of corresponding 3-deoxyflavonoids was not lowered by the accumulation of

pentahydroxyflavanone. In this study, we measured only the amount of eriodictyol, the immediate precursors (Bate-Smith, 1969) of luteoforol after treatment. We believe the more eriodictyol induced after treatment, the more reduction of 3-deoxyflavonoids (Fig. 15 and 18).

The application of Pro-Ca to spearmint potentially is a tool for changing flavonoid composition. Because of the phenolic compounds that often enhance plant resistance, modification of the flavonoid metabolism using elicitation can be considered as a new potential mechanism of plant protection. The 3-deoxyflavonoids luteoforol, which have antimicrobial properties, were effective against *Erwinia amylovora* in pome fruits (Spinelli et al., 2005). Thus, susceptibility might be reduced in spearmint by Pro-Ca treatment.

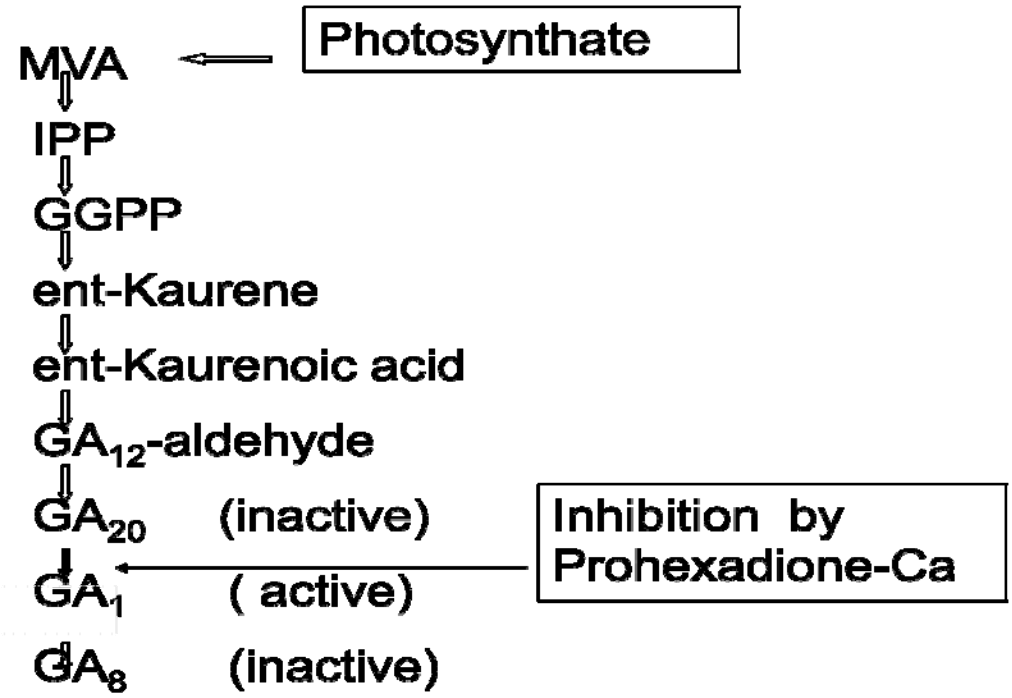

Figure 17. Simplified way of GA biosynthesis and main point of inhibition by Pro-Ca (MVA, mevalonic acid; IPP, isopentenylsphosphate; GGPP, geranylgeranylbisphosphate) in spearmint after reference (Rademacher, 2000).

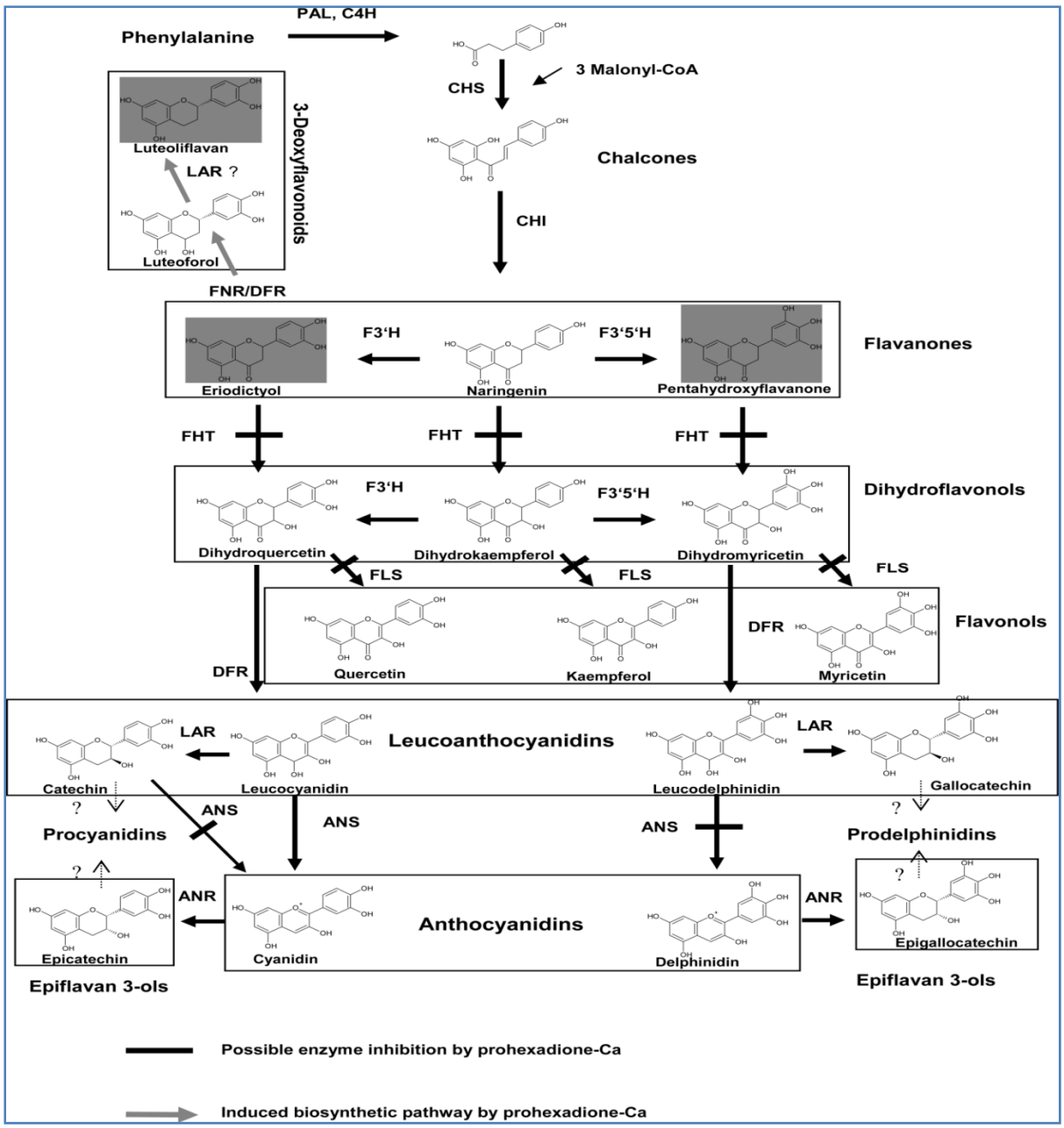

Figure 18. Simplified schematic overview of the major phenylpropanoid pathway in spearmint modified after references (Bogs et al., 2005; Forkmann and Heller, 1999) (CHI, chalcone isomerase; CHS, chalcone synsthase; FNR, flavanone 4-reductase; DFR, dihydroflavonols 4-reductase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3'5'-hydroxylase; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; PAL, phenylalanine amonialyase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; C4H, cinnamate 4-hydroxylase).

CHAPTER 5

CONCLUSIONS

Increasing concentration of Pro-Ca in spearmint decreased the plant height, branch length, number of nodes in a branch, and fresh weight relative to the control. The results were showed reduced plant height, branch length, and fresh weight with increasing treatment due to the inhibition of gibberellin biosynthesis. An accumulation of total phenolics, rosmarinic acid, and total chlorophyll were reduced with increased treatment courses relative to control. However, total phenolics content was showed increased accumulation after 125 mg/L and continued until 500 mg/L. The result may concluded some enzymes inhibited accumulation of phenolic compounds firstly then another enzymes promoted accumulation with highest concentration of Pro-Ca treatment afterward.

The application of Pro-Ca concentration was decreased flavonoid compounds relative to the control with limited exception. Here, the catechin content was observed elevated accumulation with higher concentration of Pro-Ca. The result concluded specific enzymes like DFR and LAR contributed accumulation after treatment at higher concentration. However, procyanidin and luteolin were decreased after treatment due to the enzymes inhibition. On the other hand, caffeic acid was found elevated accumulation with higher concentration of Pro-Ca that may caused for enzymes activation occurred on higher course of treatment. Eriodictyol-7-glucoside was also found elevated accumulation after treatment relative to control. There was an enzyme (F3´H) responsible for this accumulation that did not observe in control plant. Eriodictyol is the immediate precursor

of luteoforol (3-deoxyflavonoids) that reduced the disease incidence to apple, pear and some fruit trees. Here, the result concluded an alteration of such flavonoids metabolism occurred with increasing concentration of Pro-Ca that was eriodictyol-7-glucoside, the immediate precursor of luteoforol, which occurred by the activation of enzymes FNR or DFR or Both.

Essential oil was reduced by the increased treatment courses respectively relative to the control. Reduced fresh weight after treatment was observed that was mainly cause of reduced accumulation of essential oil.

Further investigation might be required for enzymatic behavior in respect to genetics role related to their metabolism after treatment of Pro-Ca. However, we observed mostly positive response based on the reviews of literature.

APPENDIX A

PROHEXADIONE-CALCIUM

Prohexadione-calcium (Pro-Ca) is a plant bioregulator first registered in USA on April 27, 2000 as a replacement of daminozide. Pro-Ca (Fig. 19) is patented by Kumai Chemical Industries Co., Tokyo, Japan that was registered for control growth of rice (*Oryza sativa* L). Recently, Pro-Ca has been got EPA registration in USA under the trade name of apogee for use of apple, commercially marketed by BASF Chemical Co. USA as apogee (27.5% a.i.), which belonged molecular formula: $C_{10}H_{10}O_5Ca$, molecular weight: 250.26 g/mol, and IUPAC name: calcium 3-oxido-5-oxo-4-propionylcyclohex-3-enecarboxylate. Mode of action of Pro-Ca is to inhibit the biosynthesis of gibberellin, which is the natural hormone that causes cell elongation and enhances longitudinal branch growth (Roemmelt et al., 2003b). Inhibition of gibberellin therefore reduces longitudinal branch growth. More recently Pro-Ca draws attention of researchers for alteration of flavonoids that has role to minimize the incidence fire blight in fruit trees.

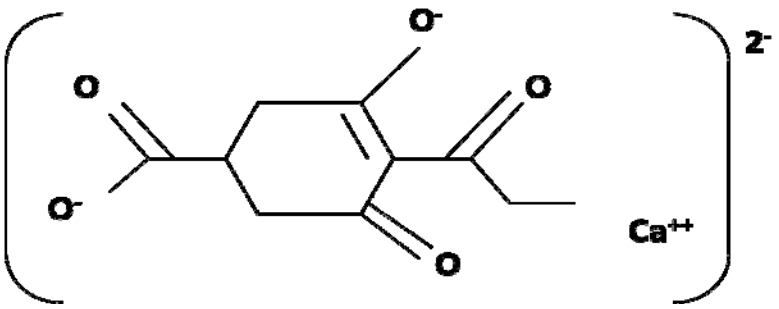

Figure 19. Chemical structure of prohexadione-calcium (Pro-Ca)

APPENDIX B

HPLC CHROMATOGRAM

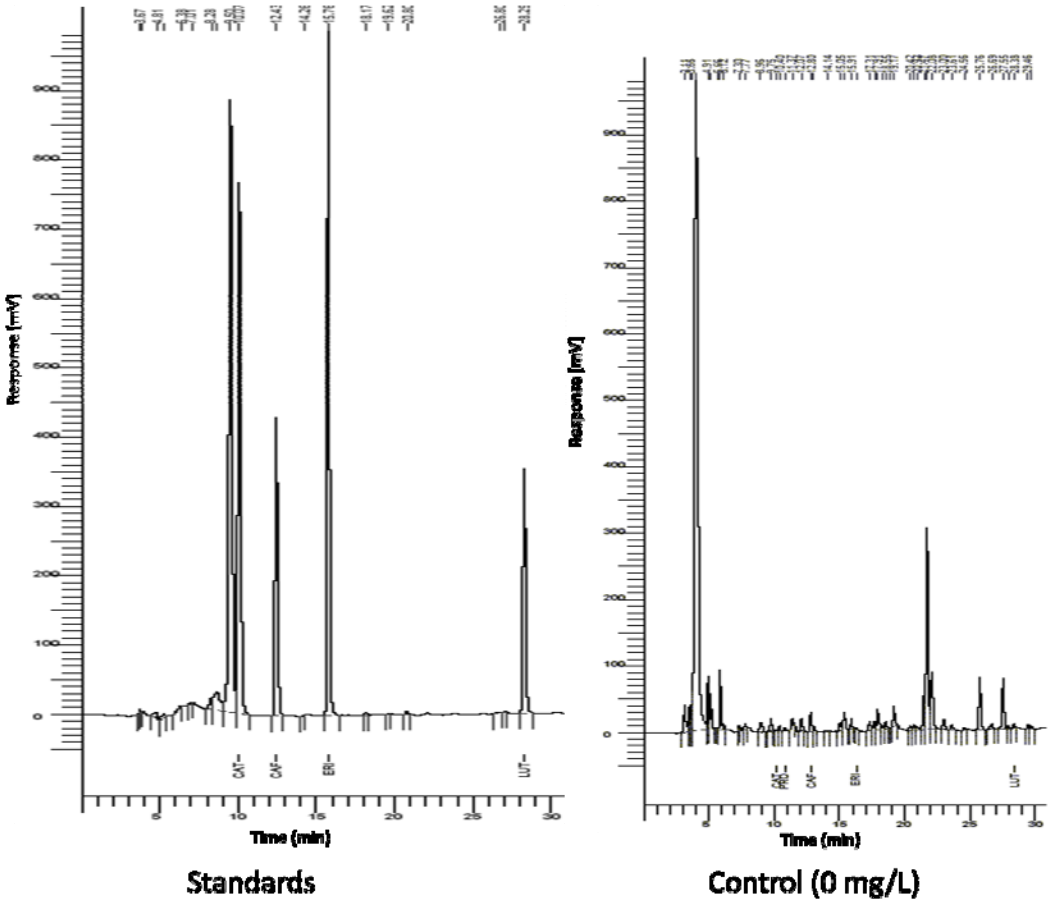

Figure 20 A. Chromatogram of standards and plant sample (control) for flavonoid content during HPLC analysis.

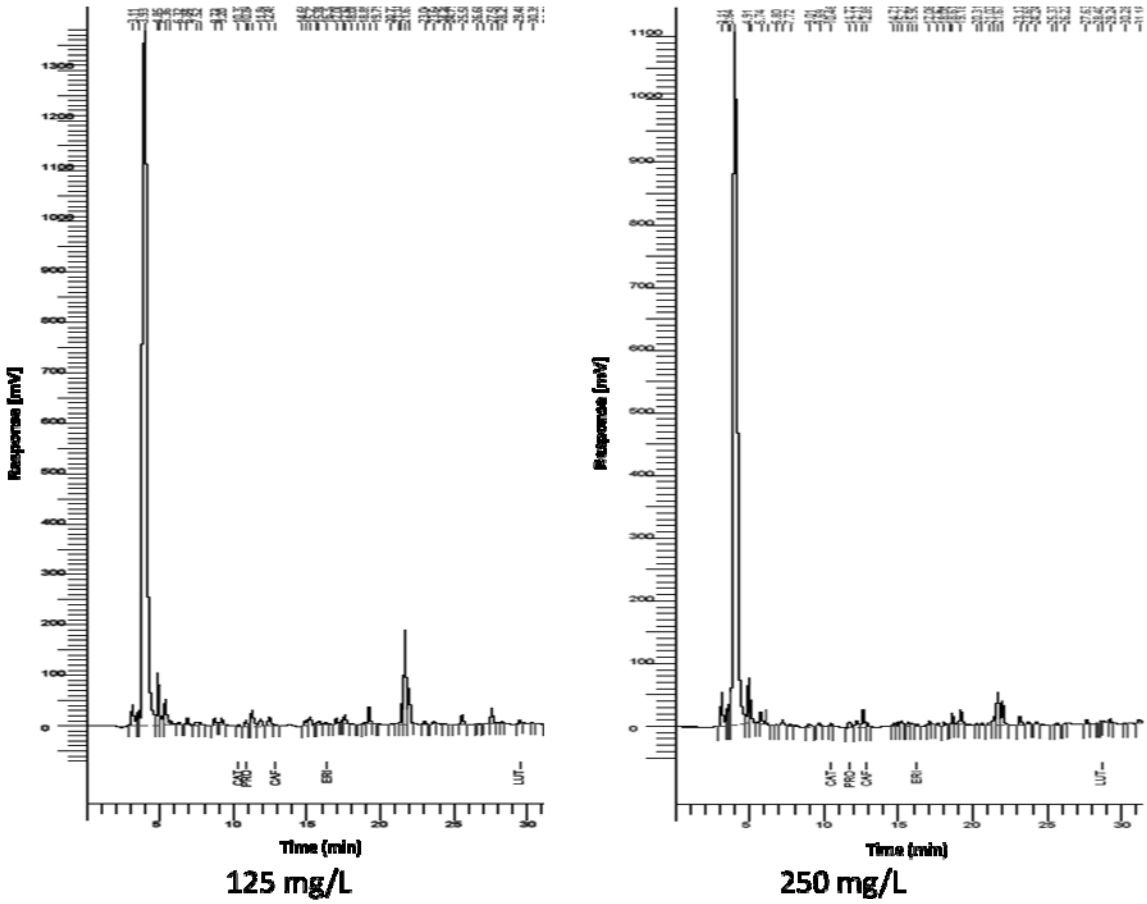

Figure 20 B. Chromatogram of plant samples (125 and 250 mg/L of Pro-Ca) after prohexadione-Ca treatment for flavonoid content in spearmint during HPLC analysis.

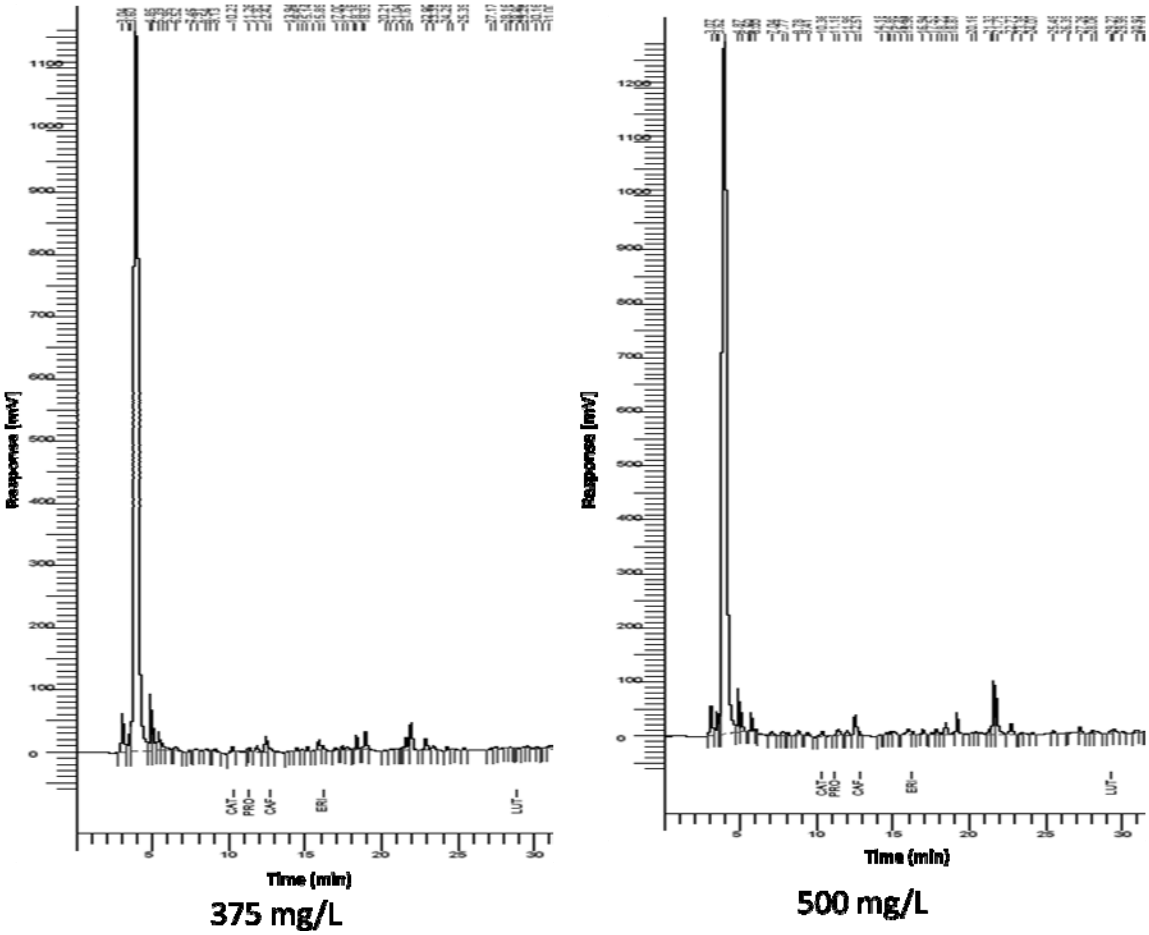

Figure 20 C. Chromatogram of plant samples (375 and 500 mg/L of Pro-Ca) after prohexadione-Ca treatment for flavonoid content in spearmint during HPLC analysis.

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