**Universidade de São Paulo
Escola Superior de Agricultura “Luiz de Queiroz”**

Agronomic and phytochemical aspects of cilantro and stevia crops

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Dissertation submitted in order to obtain the degree
of Doctor in Science. Area: Crop Science

**Piracicaba
2014**

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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DEDICATION

I am dedicating this dissertation to my beloved mother, Deize Lucidi Cordeiro Soares, for her love that was and will continue to nurture my life. Foremost, to my father Antonio Aparecido Donegá, *in memoriam*, whose love for me knew no bounds and, who taught me the value of hard work. Thank you so much.

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RESUMO

Aspectos agronômicos e fitoquímicos das culturas de coentro e estévia

O mercado de plantas medicinais, condimentares e aromáticas tais como coentro (*Coriandrum sativum*) e estévia (*Stevia rebaudiana*) vêm crescendo devido à demanda das indústrias alimentícias, químicas e farmacêuticas. As folhas *C. sativum* são utilizadas por diversos países no preparo de alimentos e na medicina popular. O óleo essencial das folhas tem potencial para utilização na indústria de alimentos como agente antimicrobiano e como condimento para mascarar cheiros e sabores indesejáveis. Estudos foram realizados para avaliar a atividade do óleo essencial de coentro contra a leishmaniose, e os resultados revelaram que o óleo essencial e seus compostos majoritários inibiram o crescimento *L. donovani* em suas diferentes formas, indicando potencial de uso dessa planta para a produção de novos medicamentos contra Leishmaniose. A partir desses resultados, outro estudo foi realizado com o objetivo de avaliar cinco materiais de coentro, uma cultivar e quatro acessos do germoplasma do Departamento de Agricultura norte americano quanto à qualidade da matéria prima para as indústrias de alimentos e farmacêuticas. Os materiais variaram entre si quanto à produção de biomassa, acúmulo de nutrientes e produção de aldeídos alifáticos tais como (E)- 2-decenal, (E)- 2-dodecenal. Os materiais que apresentaram maior potencial de uso foram AMES 18596 e PI193770, pois, produziram maior quantidade de massa seca e produção de compostos majoritários, que são os desejáveis pelas indústrias. Quanto à estévia, essa espécie apresenta elevado potencial para ser usado por pessoas diabéticas e obesas, pois é fonte de diterpenos glicosídicos usados como adoçantes naturais. Os adoçantes derivados de estévia são usados na produção de alimentos, sucos e refrigerantes em diversos países asiáticos, europeus e no continente americano. Os compostos responsáveis pelo dulçor das folhas de estévia são bem caracterizados pela literatura, entretanto, as boas práticas agronômicas ainda são pouco estudadas. Alguns países trabalharam no melhoramento dessa cultura e conseguiram alcançar cultivares mais produtivas. Na área de nutrição, o cálcio está entre os nutrientes mais importantes para a produção de esteviosídeo e produção de biomassa de estévia. Assim, um estudo preliminar foi conduzido com o objetivo de estudar o efeito do cálcio aplicado em solução nutritiva na produção de estévia, e de esteviosídeos e rebaudiosídeos, em sistema hidropônico. A produção de folhas, caule e parte aérea por plantas de estévia foi influenciada por níveis de Ca na solução nutritiva e foram máximos com as doses de 7,0, 5,7, 6,2 mmol L⁻¹ de Ca, respectivamente. A produção de esteviosídeo e rebaudiosídeo A foi máxima com a adição de 4,0 mmol L⁻¹ de Ca.

Palavras-chave: *Coriandrum sativum*; *Stevia rebaudiana*; Aldeídos alifáticos; Esteviol glicosídicos

ABSTRACT

Agronomic and phytochemical aspects of cilantro and stevia crops

The market of medicinal plants and herbs such as cilantro (*Coriandrum sativum*) and stevia (*Stevia rebaudiana*) has been growing due to the demand of the food, chemical and pharmaceutical industries. Leaves of *C. sativum* are used by many countries in the preparation of food and in folk medicine. The essential oil from the leaves has potential for use in the food industry as an antimicrobial agent and as a condiment to mask undesirable odors and flavors. Studies were conducted to evaluate the activity of the essential oil of cilantro against Leishmaniasis, and the results showed that the essential oil and its major compounds inhibited the growth of *L. donovani* in its different forms, indicating potential use of this plant for the production of new drugs against Leishmaniasis. From these results, another study was conducted to evaluate five materials of cilantro, a cultivar and four germplasm accessions from the North American Department of Agriculture regarding the quality of the raw material for the food and pharmaceutical industries. The materials with the highest potential to use were AMES 18596 and PI193770 for producing larger amount of dry biomass and the highest production of major compounds, which are desirable by industries. Stevia is a species with high potential to be consumed by diabetics and obese, it is a source of diterpene glycosides used as natural sweeteners. Sweeteners derived from stevia are ingredients in the production of foods, juices and soft drinks in Asia, Europe and in the Americas. The compounds responsible for the sweetness of stevia leaves are well characterized in the literature. However, good agronomic practices are still poorly studied. Some countries have worked on improving this culture and have achieved cultivars with higher yield. In the area of plant nutrition, calcium is one of the most important nutrients for the production of biomass and stevioside in stevia plants. Thus, a preliminary study was conducted to test the effect of calcium applied in nutrient solution in stevia yield, and stevioside and rebaudioside in hydroponic system. The leaf, stem and shoot biomass yield, by stevia plants were influenced by Ca rates in nutrient solutions and were maximal with 7.0, 5.7, 6.2 mmol L⁻¹ of Ca, respectively. The production of stevioside and rebaudioside A was maximal with addition of 4.0 mmol of Ca L⁻¹.

Keywords: *Coriandrum sativum*; *Stevia rebaudiana*; Aliphatic aldehydes; Steviol glycosides

1 INTRODUCTION

1.1 *Coriandrum sativum* L.

Cilantro (*Coriandrum sativum* L.) is an annual vegetable crop belonging to the family Apiaceae, its fresh leaves and dried fruits have been used in the cuisine of many countries such as China, India, United States, Mexico and Brazil. In addition to the use as spice, cilantro has been also cultivated for medicinal properties (PEDROSA; NEGREIROS; NOGUEIRA, 1984; FAN; SOKORAI, 2002).

The essential oil found in *C. sativum* leaves have become increasingly popular as alternative sources of natural preservative agents and functional foods (DELAQUIS et al., 2002; BURT, 2004; WONG; KITTS, 2006; BREWER, 2011). Furthermore, cilantro's essential oil has applications in the food industry as aroma and flavor to mask unpleasant odors since has a distinctive pungent, fatty, and aldehydic aroma. (MATASYOH et al., 2009; NEFFATI; MARZOUK, 2010). The main aromatic compounds found in the essential oil are aliphatic aldehydes (mainly C₁₀-C₁₆ aldehydes) with a fetid-like aroma in the fresh herb oil (POTTER, 1996). The most cited compounds in the cilantro essential oil are (*E*)-2-decenal, (*E*)-2-dodecenal, decanal, dodecanal, (*E*)-2-tridecenal and tetradecenal. These compounds have characteristic green, soapy, and are particularly important in the overall aroma of the *C. sativum* herb (CADWALLADER et al., 2005). There are reports in the literature on the efficacy of cilantro oil as an antimicrobial agent acting against bacteria, fungi and nematodes (DELAQUIS; STANICH, 2004; KUBO et al., 2002; KIM et al., 2008; MATASYOH et al., 2009; BEGNAMI et al., 2010). Therefore, cilantro, like other medicinal species, has been evaluated as a source of new compounds for pharmaceutical and chemical industry. The search for new molecules that exhibit efficacy and few side effects is a continuous discovery work due to the increasing of pathogens resistance.

The use of cilantro essential oil as a flavoring agent, food preservative and as a source of molecules of interest to the pharmaceutical and chemical industry shows the importance of this species as an agricultural commodity. Although the increased use *C. sativum* is a reality, the specie is still produced in empirical way (DIEDERICHSEN, 1996).

It is well known that genetic and environmental factors influence the biomass and essential oil production (EYRES et al., 2005; NEFFATI; MARZOUK, 2010).

Nevertheless, few studies reporting the evaluation of different genotypes on the production of essential oil from the leaves.

Thus, the aim of this study was first analyzing the performance of five genotypes of cilantro for biomass production, essential oil yield, accumulation of nutrients (chapter 2), and second evaluate the potential of the cilantro essential oil as an antimicrobial agent (chapter 3).

1.2 *Stevia rebaudiana* Bert.

S. rebaudiana is a subtropical plant species. Its center of origin is South America, more precisely in the northwestern border with Paraguay and Brazil. Stevia is a cultivated crop for industrial purpose since its leaves contains a complex mixture of diterpene glycosides: stevioside, rebaudioside A, B, C, D, E, F, and dulcosideo A (GEUNS, 2003). These glycosides are used as natural sweeteners. The sweetness of these compounds was described as 200 to 300 times greater than the one produced from sucrose. This type of glycosides does not stimulate the formation of dental caries, thus there is a great applications in the dental industry as well as in the developing of products for dieting and diabetic patients. For therapeutical purposes, stevia glycosides have been used to treat patients diagnosed with metabolic syndrome (obesity, diabetes, cardiovascular disease and hypertension) while improve digestion and have antimicrobial properties (JEPPESEN et al., 2000; GONZALEZ et al., 2007).

Japan is the main consumer of stevia glycosides and has government approval for use in various foods including cereals, teas and sodas (RAMESH et al., 2006). Recently the use of stevia as a sweetener has increased globally including in Brazil, Argentina, Paraguay, China, Korea and the United States (VIRENDRA; KALPAGAM, 2008; ABOU-ARAB et al., 2010; LEMUS-MONDACA et al., 2012). The commercial market is demanding higher amount of stevia sweeteners, particularly for soda and juices companies, due of the need to reduce the level of sucrose and other artificial sweeteners in their products.

The biosynthesis of secondary metabolites, including diterpene glycosides from stevia, dependent on genetic, physiological, environmental and nutritional factors (BROWN et al., 2003). Besides breeding process, another factor that should be taken into account is the mineral nutrition of the species. Nutrients are essential for the development and growth of the plants, since they are directly linked to their

primary and secondary metabolism and without adequate nutrition plants do not reach their productive potential.

Among the macronutrients, calcium (Ca) was described as limiting the biomass and stevioside production in *S. rebaudiana* (LIMA FILHO et al., 1997; UTUMI et al., 1999). Ca is an essential element for plant development, as it participates in various physiological processes and biosynthesis (ALBINO-GARDUÑO et al., 2008). It is used in the mitotic spindle during cell division, is required for the normal functioning of plant membranes and has the role of a second messenger in several plant responses to environmental and hormonal signals (SANDERS; BROWNLEE; HARPER, 1999). Despite the importance of nutrients, studies reporting fertilization management to increase crop productivity are still lacking, especially in studies with improved varieties of stevia. Little is known about the effects of rates of nutrients in the stevia biomass and glycosides yields, especially rebaudioside A, which is the compound with the greatest interest for food industry.

Thus the purpose of this study was to investigate the influence of calcium (chapter 4) levels in the nutrient accumulation, on the biomass and on diterpene glycosides yield in stevia plants.

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2 NUTRIENT UPTAKE, BIOMASS YIELD AND QUANTITATIVE ANALYSIS OF ALIPHATIC ALDEHYDES IN CILANTRO PLANTS

Abstract

The objective of this study was to evaluate the nutrient uptake, biomass production and yield of the major compounds in the essential oil of five genotypes of *Coriandrum sativum* L. The treatments were composed for four accessions provided by the National Genetic Resources Advisory Council (NGRAC), U.S. Department of Agriculture's Agricultural Research Service (USDA/ARS) germplasm: ("AMES 18596"; "PI171592"; "AMES 4998" and "PI193770") and one commercially available cultivar, (Santo). Plants were harvested 50 days after sowing and evaluated for nutrient uptake, the number of leaves, fresh and dry weight of shoot per plant and yield of major compounds in the essential oil. Accessions AMES 18596 and PI171592 presented the following order of accumulation of macronutrients: K>N>Ca>Mg>P>S. For the accessions AMES 4998, PI 193770 and the cultivar Santo, the order K>N>Ca>P>Mg>S was observed. Micronutrients accumulation in cilantro shoot in descending order for all genotypes was: Fe>Zn>B>Mn>Cu. The highest number of leaves per plant was found in the accession AMES 18596 (9.94), while the others produced on average 7.24. Accessions AMES 18596 and PI 193770 produced 7.34 g shoot⁻¹ of fresh weight of shoots. Accession PI 171592 produced slightly higher yields (6.23 g shoot⁻¹) than the cultivar Santo and AMES 4998 (4.75 g shoot⁻¹). For dry weight of shoots, accessions AMES 18596 and PI 193770 produced the highest yields (0.58 g shoot⁻¹), followed by PI171592 (0.47 g shoot⁻¹) and the accession AMES 4998 and the cultivar Santo that had on average 0.35 g shoot⁻¹. The yield of the major compounds in the essential oil was higher for accessions AMES 18596 and PI 193770 (88.84 µg shoot⁻¹) following by AMES 4998 and Santo (38.95 µg shoot⁻¹) and PI171592 (23.31 µg shoot⁻¹). On average the yield of aliphatic aldehydes in fresh weight followed the order: (*E*)-2-dodecenal> decanal> (*E*)-2-decenal> tetradecenal>dodecanal>tetradecanal.

Keywords: Cilantro; *Coriandrum sativum*; Nutrient uptake; Quantitative analysis

2.1 Introduction

Coriandrum sativum L. is an annual herb commonly known as cilantro or coriander from the family Apiaceae. It is likely one of the first species used by humanity, being known since 5000 years before Christ. It is native to the western Mediterranean. The Romans spread cilantro across Europe and according to Diederichsen (1996) cilantro was one the first species to be introduced to the Americas. Cilantro has been cultivated worldwide as a spice and for its aroma and medicinal properties. The unique flavor and aroma attract consumers and the leaves are typically sold fresh, frozen or dried. The fresh leaves of cilantro are highly regarded in cuisine of China, Mexico, South America, India, and Southeast Asia (CARRUBA, 2009; FAN; SOKORAI, 2002; GIL et al., 2002; POTTER; FAGERSON, 1990).

The essential oils found in cilantro seeds and leaves have become increasingly popular as alternative sources of natural preservative agents and as functional foods (BREWER, 2011; BURT, 2004; DELAQUIZ et al., 2002; WONG; KITTS, 2006). In addition, cilantro's essential oil has been used in the food industry for its aroma and flavor, or to mask unpleasant odors of certain foods, due to its distinctive pungent, fatty, and aldehydic aroma. (MATASYOH et al., 2009; NEFFATI; MARZOUK, 2010). The main aromatic compounds are aliphatic aldehydes (mainly C₁₀-C₁₆ aldehydes) with a fetid-like aroma in the fresh herb oil (POTTER, 1996). The most cited compounds are (*E*)-2-decenal, (*E*)-2-dodecenal, decanal, dodecanal, (*E*)-2-tridecenal and tetradecenal. These compounds possess characteristic green, soapy, and cilantro-like aromas and are particularly important in the overall aroma of the *C. sativum* herb (CADWALLADER et al., 2005). Reports on cilantro have evaluated both environmental and genetic factors which influence biomass production of the leaves, quality, and yield of the essential oils (EYRES et al., 2005; NEFFATI; MARZOUK, 2010).

Lopez et al. (2008) described phenotypic, biochemical and molecular diversity in *C. sativum* populations maintained at the North Central Regional Plant Introduction Station. They assessed significant differences among populations for all the phenotypic traits. The main volatile compounds detected in cilantro seeds were measured from sixty populations. Results showed molecular evidences of phenotypic and biochemical subgroups. Variation attributed among subgroups and groups was very low, while variation among populations within groups was intermediate, and that within populations was large.

Our goal was to study the uptake of nutrients, biomass production and yield of the major compounds in essential oil in leaves of four accessions and one cultivar of cilantro.

2.2 Material and methods

2.2.1 Location

The study was conducted in the greenhouse of the United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Natural Products Utilization Research Unit in University, Mississippi, horticultural zone 7.

The cilantro accessions tested were AMES 18596; PI 171592; AMES 4998 and PI 193770 and one commercially available cultivar, Santo. The accessions were

donated by the National Genetic Resources Advisory Council (NGRAC), U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS) germplasm. After 20 days of sowing, cilantro seedlings were transplanted to 2.0 L pots filled with Metro-Mix® 300 commercial substrate formulated with vermiculite, bark, Canadian Sphagnum peat moss, coarse perlite, bark ash, starter nutrient and slow release nitrogen and dolomitic limestone (Bellevue, Washington). Pots were maintained for thirty days in a greenhouse. The experimental design was completely randomized with 19 replications. Each replication consisted of a pot with ten plants. Modified Hogland and Arnon (1950) nutrient solution was applied at a rate 0.2 L per pot, once a day adjusted to values maintaining the drainage percentage within a range of 15%.

Plants of eight pots per treatment were analyzed at 50 days after sowing to obtain the fresh and dry weight of shoots and the number of leaves per plant. The remaining plants of each treatment were used for essential oil extraction.

2.2.2 Plant tissue analyses

Dry shoot samples were sent to the Mississippi State University, Soil Testing and Plant Analysis Laboratory (Starkville, Mississippi) to perform a nutrient analysis to determine the plant tissue levels of N, P, K, Ca, Mg, S, Fe, Zn, B, Mn and Cu (ISAAC; JOHNSON, 1975; JONES; STEYN, 1973; NELSON; SOMMERS, 1972).

2.2.3 Essential oil isolation

Fresh leaves (100 g) were subjected to hydrodistillation for 90 min (MSAADA et al., 2007). Hydrodistillation was performed using a Clevenger apparatus containing n-pentane. Leaves were cut and placed into a 1 L round bottom flask followed by the addition of 500 mL of deionized water. The organic phase (n-pentane) was recovered and concentrated to 1.0 mL under a stream of dry nitrogen (POTTER, 1996).

2.2.4 Gas chromatography (GC)

GC analyses were performed using a Varian CP-3800 gas chromatograph equipped with a flame ionization detector (FID). The GC was equipped with a CP Sil 8 CB capillary column (30 m x 0.25 mm, with film thickness of 0.25 µm) operated using the following conditions: injector temperature, 240 °C, column temperature, 60-240 °C at 3 °C/min then held at 240 °C for 5 min; carrier gas, He; injection volume, 1 µL split, 50:1.

2.2.5 Quantitative analysis

Commercial standards decanal, dodecanal, (*E*)-2-decenal, (*E*)-2-dodecenal and tetradecanal were purchased from Sigma-Aldrich (St. Louis, MO) and BOC Sciences (New York, NY). Commercially available tetradecenal was not available; therefore the response factor determined for tetradecanal was used for quantitative analysis. With five concentration points, an external standard least squares regression was performed for quantification. All analytes were used to formulate separate calibration curves. Linearity was imposed by using response factors and regression coefficients independently. Response factors were calculated using the equation $RF = DR/C$, where DR was the detector response in peak area (PA) and C was the analyte concentration. The target peaks were confirmed by retention time and mass spectra, when needed. Confirmed integrated peaks were then used to determine the percentage of each chemical constituent in the sample. The R.F. of the target chemical constituent was used to determine the “percent” for each sample using the equation: $PA/R.F./C = \%$ (peak area / response factor / concentration) in the fresh weight. Percentage fresh weight was multiplied times 10,000 to provide $\mu g/g$ fresh weight.

2.2.6 Statistical analysis

Data were subjected to statistical analysis using the Statistical Analysis System – (SAS Institute Inc., Cary, NC). The analysis of variance (ANOVA) followed by the Scott-Knott test were employed and the differences between individual means were deemed to be significant at $p < 0.05$.

2.3 Results and discussion

2.3.1 Uptake of nutrients

Accessions AMES 18596 and PI171592 presented the following order of accumulation of macronutrients: $K > N > Ca > Mg > P > S$ (Table 1). For the Accessions AMES 4998, PI 193770 and Santo cultivar, the order of these changed because the plants absorbed more phosphorus than magnesium: $K > N > Ca > P > Mg > S$ (Table 1). Cilantro shoot has accumulated the elements in the following order: $K > Ca > N > Mg > P$ (GARG et al., 2004; GRANGEIRO et al. 2011).

Table 1 - Macronutrient uptake (mg shoot^{-1}) in four accessions and one cultivar of cilantro

Treatment	N	P	K	Ca	Mg	S
	 mg shoot^{-1}					
AMES 18596	32.92 b	3.32 b	52.08 a	4.68 a	3.40 a	1.81 c
PI 171592	28.57 c	2.94 b	40.58 b	4.31 a	3.05 a	1.23 b
AMES 4998	21.81 d	2.47 b	35.22 b	2.97 b	2.18 b	0.95 d
PI 193770	40.24 a	4.24 a	61.97 a	5.39 a	3.81 a	2.57 a
Santo	22.47 d	2.57 b	36.89 b	3.28 b	2.37 b	1.28 b

Means followed by the equal letter in the column are not significantly different by the Scott-Knott test $p < 0.05$

Potassium was the nutrient absorbed in the highest quantity by plants, as has been observed in many vegetables (FERREIRA et al., 1993). The higher demand for K is also observed in several species because the element is present in various plant functions such as regulation of the osmotic potential of cells, activation of enzymes involved in respiration and photosynthesis and translocation of assimilates (MEURER, 2006). However, crops have different requirements for potassium. The highest potassium extraction occurred in the accessions AMES 185 and PI 193770 ($52.2 \text{ mg shoot}^{-1}$) which have accumulated on average 34% more K than the accessions PI 171592, AMES 4998 and the cultivar Santo. Grangeiro et al. (2011) reported cilantro plant absorbed $25.43 \text{ mg of K shoot}^{-1}$.

The second most accumulated nutrient was nitrogen as it is responsible for the production of amino acids, proteins, nucleic acids, coenzymes, and other compounds (MARSCHNER, 1995). Nitrogen uptake ranged from 21.81 to $40.24 \text{ mg shoot}^{-1}$. Accession PI 193770 showed the highest accumulation ($40.2 \text{ mg shoot}^{-1}$) of this nutrient while accessions AMES 4998 and the cultivar Santo showed the lowest ($22.1 \text{ mg shoot}^{-1}$). Cilantro cultivated in soil accumulated $10.37 \text{ mg shoot}^{-1}$ (GRANGEIRO et al., 2011), half of the average value found in this study.

The accumulation of calcium was 4.68, 4.31 and $5.39 \text{ mg shoot}^{-1}$, respectively, for accessions AMES 18596, PI 171592 and PI 193770 and for the accession AMES 4998 and the cultivar Santo the accumulation was 2.97 and $3.28 \text{ mg shoot}^{-1}$, respectively. Magnesium accumulation ranged from 2.18 to $3.81 \text{ mg shoot}^{-1}$. Grangeiro et al. (2011) had found an accumulation of $20.4 \text{ mg plant}^{-1}$ of Ca and $11.15 \text{ mg shoot}^{-1}$ of Mg in cilantro.

Sulfur is found in two amino acids (cysteine and methionine) and is a constituent of several coenzymes and vitamins essential for plant metabolism (FAGERIA, 2009). In our study the concentration ranged from 0.94 to 2.57 mg shoot⁻¹.

Micronutrients accumulation in cilantro leaves in descending order was: Fe>Zn>B>Mn>Cu (Table 2). Kano et al. (2011) had found in lettuce the following order: Fe>Mn>Zn>B>Cu.

Accessions AMES 18596 and PI 193770 absorbed 36.67 and 35.52 µg Fe shoot⁻¹, and for other accessions the accumulation of iron ranged from 24.08 and 29.24 µg shoot⁻¹. Among the micronutrients, iron is required in the greatest abundance for plants. It functions in various important processes, including photosynthesis, respiration, and chlorophyll biosynthesis (KOBAYASHI; NISHIZAWA, 2012).

Zinc was the second most accumulated micronutrient. Accessions AMES 18596, PI 171592 and PI 193770 had an average of 26.9 µg shoot⁻¹, and thus the accession AMES 4998 and cultivar Santo had an average of 19.3 µg shoot⁻¹. Zinc is necessary in plants for producing chlorophyll and forming carbohydrates (FAGERIA, 2009).

Boron accumulation for accessions AMES 18596 and PI 193770 was the highest (19.46 and 20.76 µg shoot⁻¹), and the lowest accumulation was found in the accession AMES 4998 and the cultivar Santo (11.41 and 12.51 µg shoot⁻¹). B is essential for cell elongation, nucleic acid synthesis, wall formation, protein formation and sugar translocation (BROWN et al., 2002; TAIZ; ZEIGER, 2004).

Table 2 - Micronutrient uptake ($\mu\text{g shoot}^{-1}$) in four accessions and one cultivar of cilantro

Treatment	Fe	Mn	Zn	Cu	B
	 $\mu\text{g shoot}^{-1}$				
AMES 18596	36.67 a	15.71 a	24.70 a	0.56 a	19.46 a
PI 171592	29.24 b	14.11 a	26.27 a	0.64 a	14.93 b
AMES 4998	25.47 b	9.11 b	17.80 b	0.70 a	11.41 c
PI 193770	35.52 a	14.20 a	29.73 a	0.61 a	20.76 a
Santo	24.08 b	10.14 b	20.93 b	0.51 a	12.51 c

Means followed by the equal letter in the column are not significantly different by the Scott-Knott test $p < 0.05$.

Manganese accumulation was 15.71, 14.11 and 14.20 $\mu\text{g shoot}^{-1}$ for accessions AMES 18596, PI 171592 and PI 193770, respectively. For accessions AMES 4998 and the cultivar Santo, the accumulation was 9.11 and 10.14 $\mu\text{g shoot}^{-1}$. This micronutrient is important in the photosynthetic reaction through which oxygen is produced from water and also as a cofactor for a number of enzymes that catalyse redox, decarboxylation and hydrolytic reactions (MARSCHNER, 1995; WHITE; BROWN, 2010).

There was no difference among treatments for copper absorption and the accumulation was on average 0.60 $\mu\text{g shoot}^{-1}$. Copper is important in photosynthesis as well as protein and carbohydrate metabolism (FAGERIA, 2009).

The accumulation of micronutrients was on average 30.20, 12.65, 23.88, 0.60 and 15.81 $\mu\text{g shoot}^{-1}$, respectively, for Fe, Mn, Zn, Cu and B. Similar results had been found in cabbage: 12.62, 12.23, 4.41, 0.33 and 11.01 $\mu\text{g plant}^{-1}$ Fe, Mn, Zn, Cu and B (ZANÃO JÚNIOR et al., 2005).

2.3.2 Biomass yield

Results described in Table 3 show that there were significant differences among genotypes for the number of leaves, fresh, and dry weight of shoots per plant under the same growing conditions. The AMES 18596 accession produced the highest number of leaves per plant (9.94), while the others produced on average of 7.24. The highest leaf numbers per plant also translated into more fresh weight of shoots per plant, thus AMES 18596 and PI 193770 (7.34 g plant^{-1}). Accession PI 171592 produced a slightly higher yield (6.23 g plant^{-1}) than the cultivar Santo and AMES 4998 (4.75 g plant^{-1}).

Table 3 - Biometric analysis of four cilantro accessions and one commercial cultivar. The analyzed parameters were number of leaves (LN), leaf fresh weight (FWL), leaf dry weight (DWL) and yield of major compounds (decanal, (*E*)-2-dodecenal, (*E*)-2-decenal, tetradecenal, dodecanal, tetradecanal) in essential oil (YMC)

Treatment	LNg plant ⁻¹	FWLg plant ⁻¹	DWLg plant ⁻¹	YMC µg plant ⁻¹
AMES 18596	9.94 a	7.31 a	0.55 a	83.35 a
PI 171592	7.21 b	6.23 b	0.47 b	23.31 c
AMES 4998	7.33 b	4.46 c	0.33 c	39.27 b
PI 193770	7.64 b	7.37 a	0.61 a	94.34 a
Santo	6.77 b	5.04 c	0.38 c	38.63 b

Means followed by the equal letter in the column are not significantly different by the Scott-Knott test $p < 0.05$

Leaf dry weight ranged from 20% to 40% among the accessions. The accessions AMES 18596 and PI 193770 produced the highest yields (0.58 g plant⁻¹), followed by PI171592 (0.47 g plant⁻¹) and the accession AMES 4998. Grangeiro et al. (2011) obtained 0.92 g of dry weight in cilantro plants grown in the soil.

The accessions AMES 18596 and PI 193770 had a higher yield of major compounds in the essential oil (88.84 µg plant⁻¹) following by AMES 4998 and Santo (38.95 µg plant⁻¹) and PI171592 (23.31 µg plant⁻¹). The yield of major compounds in the essential oil for AMES 18596 and PI 193770 was 56% higher than AMES 4998 and Santo and 73% higher than PI171592 (Table 1).

2.3.3 Quantitative analysis of major constituents

There were significant differences among the genotypes studied for the concentration of decanal, dodecanal, tetradecanal, (*E*)-2-decenal, (*E*)-2dodecenal, and tetradecenal (Figure 1). Decanal concentration in fresh weight of accessions ranged from 13.82 to 61.76 µg g⁻¹ and was higher for accession PI 193770. Msaada et al. (2007) and Fan and Sokorai (2002) described, respectively, 0.26 µg g⁻¹ and 98.21 µg g⁻¹ as the concentration of decanal. The cultivar Santo had the highest dodecanal content (7.60 µg g⁻¹), followed by the accessions AMES 18596, AMES 4998 and PI 193770 that did not show differences among them, and accession PI 171592 with 2.85 µg g⁻¹. This concentration of dodecanal was higher than those reported for Msaada et al. (2007) and Fan and Sokorai (2002) in cilantro essential oil 0.08 µg g⁻¹ and 1.42 µg g⁻¹, respectively. Tetradecanal production was between 0.38 to 0.97 µg g⁻¹. The accession AMES 4998 and the cultivar Santo had the highest

production (0.95 and $0.97 \mu\text{g g}^{-1}$ respectively). The accession AMES 18596 had $0.70 \mu\text{g g}^{-1}$, and accessions PI 171592 and PI 193770 did not show difference and had, respectively, (0.51 and $0.38 \mu\text{g g}^{-1}$ of tetradecanal). A concentration much higher for tetradecanal was found by Fan and Sokorai (2002) that obtained a value of $3.15 \mu\text{g g}^{-1}$.

Figure 1 - Concentration in $\mu\text{g g}^{-1}$ fresh weight of decanal, dodecanal, tetradecanal (A) and (*E*)-2-decenal, (*E*)-2-dodecenal, tetradecenal (B) in cilantro genotypes

Regarding the unsaturated aldehydes, (*E*)-2-decenal concentration ranged from 5.75 to 58.75 $\mu\text{g g}^{-1}$. The accession AMES 18596 had 58.75 $\mu\text{g g}^{-1}$, followed by accessions PI 193770 (47.45 $\mu\text{g g}^{-1}$), AMES 4998 (22.50 $\mu\text{g g}^{-1}$), Santo (6.89 $\mu\text{g g}^{-1}$) and accession PI 171592 (5.75 $\mu\text{g g}^{-1}$), but the last two genotypes did not show significant differences among them. The concentration of (*E*)-2-decenal in accession PI 171592 and the cultivar Santo was higher than those reported for Msaada et al. (2007) (3.37 $\mu\text{g g}^{-1}$). For the accession AMES 18596, the concentration of (*E*)-2-decenal was similar to that described by Fan and Sokorai (2002) in cilantro plants (60.27 $\mu\text{g g}^{-1}$). (*E*)-2-dodecenal concentration ranged from 14.50 to 40.43 $\mu\text{g g}^{-1}$. For this compound, the accessions can be divided in four groups: group I with the highest production, accession AMES 18596 (40.43 $\mu\text{g g}^{-1}$); group II the accession AMES 4998 and cultivar Santo with an average of 36.25 $\mu\text{g g}^{-1}$, group III accession PI 193770 with 31.05 $\mu\text{g g}^{-1}$ and group IV accession PI 171592 with 14.50 $\mu\text{g g}^{-1}$. (*E*)-2-dodecenal was found in a higher concentration for Msaada et al. (2007) because they described 85.5 $\mu\text{g g}^{-1}$ in cilantro leaves. On the other hand, Fan and Sokorai (2002) reported 9.42 $\mu\text{g g}^{-1}$ of (*E*)-2-dodecenal. The concentration of tetradecenal varied among 9.88 $\mu\text{g g}^{-1}$ (accessions PI 171592 and PI 193770) to 24.3 $\mu\text{g g}^{-1}$ (accessions AMES 185961, AMES 4998 and Santo cultivar). The concentration found in this study was higher than that found for Msaada et al. (2007). These authors described 0.68 $\mu\text{g g}^{-1}$ as a concentration of tetradecenal in cilantro.

On average, in our study, the yield of aliphatic aldehydes in fresh weight of cilantro followed the order: (*E*)-2-dodecenal > decanal > (*E*)-2-decenal > tetradecenal > dodecanal > tetradecanal. MacLeod; Islam (1976) report that there is no doubt that the aroma of crushed cilantro leaves is unique in nature, and perhaps nearly unique as such. Furthermore, it is probably in this mixture that the two most abundant compounds (dodecanal and 7-dodecenal) are also the most important in coriander leaf aroma. Cadwallader et al. (2005) described the (*E*)-2-alkenals: (*E*)-2-decenal, (*E*)-2-undecenal, (*E*)-2-dodecenal, (*E*)-2-tridecenal and tetradecenal which possess characteristic green, soapy, and cilantro-like aromas are particularly important in the overall aroma of the herb *C. sativum*. Therefore, they concluded that

among the alkenals, (*E*)-2-dodecenal is believed to be the predominant cilantro-like aroma component in *C. sativum* leaves.

Our results show that there is a difference among genotypes to accumulation of nutrients, biomass yield and production of aliphatic aldehydes when grown in a greenhouse. These differences among genotypes are important because it may offer the opportunity for production of cilantro to meet the market requirements, such as biomass production, cilantro essential oil or production of individual compounds such as (*E*)-2-decenal and (*E*)-2-dodecenal.

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3 PHARMACOLOGICAL ACTIVITIES OF CILANTRO'S ALIPHATIC ALDEHYDES AGAINST LEISHMANIA

Abstract

Leishmaniasis is a chronic infectious disease caused by different *Leishmania* species. Global occurrences of this disease are primarily limited to tropical and subtropical regions. Treatments are available; however, patients complain of side effects. Different species of plants have been screened as a potential source of new drugs against Leishmaniasis. In this study, we investigated the antileishmanial activity of cilantro essential oil, and its main components: (*E*)-2-undecenal, (*E*)-2-decenal, (*E*)-2-dodecenal, decanal, dodecanal and n-tetradecanal. Cilantro's essential oil achieved 98% inhibition of *Leishmania donovani* promastigotes. The aliphatic aldehydes (*E*)-2-undecenal, (*E*)-2-decenal and (*E*)-2-dodecenal, are effective inhibitors of in vitro cultures of *L. donovani* promastigotes and axenic amastigotes. Cilantro compounds showed no toxicity against *L. donovani* in THP1 macrophages cultures.

Keywords: *Coriandrum sativum* L.; Essential oil; (*E*)-2-undecenal; (*E*)-2-decenal; (*E*)-2-dodecenal

3.1 Introduction

Leishmaniasis is a disease that occurs in many tropical and sub-tropical areas, causing significant morbidity and mortality in Africa, Asia and Latin America (ROCHA et al., 2005; RONDON et al., 2011). There are two million new cases annually, and 350 million people are living at risk of being infected (MANZOTE, 2009). Species of the genus *Leishmania* are protozoan parasites belonging to the family *Trypanosomatidae*. They are the causative agents of human Leishmaniasis, which has a reservoir in mammal species, and it is transmitted by mosquitoes of the genera *Lutzomia* and *Phlebotomus* (MANZOTE, 2009; GALLEGU, 2004).

The treatment of leishmaniasis is difficult due to the intramacrophagic infection form (ROCHA et al., 2005). There are around 25 compounds and formulations useful in the treatment of this disease. Usually, the treatments are based on amphotericin B, pentamidine, miltefosine and aminosidine. These drugs have disadvantages such as intravenous administration, long duration therapy, toxic effects and high cost (MANZOTE et al., 2007; MANZOTE, 2009). Efforts to improve the therapeutic arsenal against this disease have prompted the search for new and less expensive drugs (MARQUES, et al., 2011). Medicinal plants have been investigated as a potential source of new therapeutic compounds with better antileishmanial properties and fewer side effects (SILVA et al., 1995; PATRICIO et al., 2008).

Cilantro (*Coriandrum sativum* L.) belongs to the Apiaceae family. The species is cultivated worldwide as a spice and for its aroma and medicinal properties. The food industry has used cilantro's essential oil for aroma and flavor to mask unpleasant odors of certain foods, due to its distinctive pungent aroma. The essential oils found in cilantro seeds and leaves are becoming increasingly popular as functional foods and natural preservative agents (WONG; KITTS, 2006; MATASYOH et al., 2009; NEFFATI; MARZOUK, 2010; BREWER, 2011). Few studies showed cilantro's antimicrobial properties, namely antibacterial, antifungal and antinematocidal. The aim of this study was to investigate the antileishmanial activity of cilantro essential oil major compounds.

3.2 Material and Methods

3.2.1 Essential oil extraction

Cilantro (2.0 kg) was purchased in a retail store in Oxford, MS, transported to the laboratory in the Natural Products Utilization Research Unit – USDA–ARS, where healthy leaves were selected. Leaves were cut and placed into a 1 L round bottom flask followed by the addition of 300 mL of deionized water. Fresh leaves were subjected to hydro-distillation for 90 min (MSAADA et al., 2007). Hydro-distillation was performed using a Clevenger apparatus containing 10 mL of n-pentane. The organic layer of the four distillations was combined and dried under a stream of dry nitrogen, resulting in a yield of 500 mg.

3.2.2 Fraction of essential oil

The essential oil (500 mg) was fractionated using Biotage (Charlotte, NC) XP-Sil, 100 g, SNAP cartridge (40-63 μ m, 60 Å, 40 x 150 mm) running at 40 mL min⁻¹ using a hexane:acetone step gradient beginning with 100:0 to 0:100 over 2200 mL followed by 0:100 over 600 mL. Twenty-two mL portions were collected in 16 x 150 mm test tubes. Based on Thin Layer Chromatography (TLC) similarities, the content of the tubes were combined providing three fractions with the following weights: **1**: 2.1 mg; **2**: 51.0 mg; **3**: 34.0 mg.

3.2.3 Analysis by gas chromatography – mass spectrometry (GC-MS)

The essential oil and the fractions were analyzed by GC-MS on a Varian (now Agilent, Santa Clara, CA) CP-3800 GC coupled to a Varian Saturn 2000 MS/MS. The

GC was equipped with a CP Sil 8 CB capillary column (30 m x 0.25 mm, with film thickness of 0.25 μ m) operated using the following conditions: injector temperature, 240°C, column temperature, 60-240°C at 3°C/min then held at 240°C for 5 min; carrier gas, Helium (He); injection volume, 1 μ L, split, 50:1. The MS mass ranged from 40 to 650 m/z, filament delay of 5 minutes, target TIC of 30,000, a prescan ionization time of 100 μ sec, an ion trap temperature of 150°C, manifold temperature of 60°C, and a transfer line temperature of 170°C.

Compounds identification was obtained using a mixture of hydrocarbon inject under same conditions, comparing mass spectra, retention time and Kovats indexes.

3.2.4 Chemicals

Commercial standards decanal, dodecanal, n-tetradecanal, (*E*)-2-decenal, (*Z*)-4-decenal, (*Z*)-7-decenal, (*E*)-2-undecenal, (*Z*)-8-undecenal, (*E*)-2-dodecenal were purchased from Sigma-Aldrich (St. Louis, MO) and/or BOC Sciences (New York, NY).

3.2.5 *In vitro* assay for antileishmanial activity

The *in vitro* antileishmanial assay was done on a culture of *Leishmania donovani* promastigotes and axenic amastigotes by Alamar Blue assay (MIKUS; STEVERDING, 2000). The promastigotes culture was maintained at 26°C in RPMI 1640 pH 7.4 with 10% FBS. The axenic amastigotes were cultured at 37°C and 5% CO₂ in RPMI-1640 fortified with 4-Morpholineethanesulfonic acid (MES) (4.88 g/L), L-glutamine (298.2 mg/L), Adenosine (26.7 mg/L), Folic acid (10.1 mg/L), BME Vitamin mix, Sodium Bicarbonate (352.8 mg/L) and 10% of FBS. The pH of the culture medium was 5.5. In a 96 well microplate, the samples with appropriate dilution were added to the leishmania promastigotes/axenic amastigote culture (2x10⁶ cell/mL). The compounds were tested at six concentrations ranging from 40 to 0.0128 μ g/ml. The plates were incubated for 72 hours at 26°C and 37°C, respectively for promastigotes and axenic amastigote. The growth of leishmania promastigotes/amastigote was determined. Inhibitory concentration (IC₅₀ and IC₉₀) values were computed from the dose response curves.

3.2.6 In vitro macrophage amastigote assay

A recently developed promastigote rescue assay was used (JAIN et al., 2012). The THPI 1 cells (Human acute monocytic leukemia cell line) were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were prepared prior to each assay and suspended in RPMI1640 medium with 10 % FBS at the cell density of 2.5×10^5 cells/mL. Phorbol-12-myristate-13-acetate (PMA) was added to the cell suspension to achieve final concentration of 25 ng/mL and cells were seeded onto a clear flat-bottom 96 well plate with 200 μ L (5×10^4 cells) into each well. Plate was incubated in a 5% CO₂ incubator at 37°C for at least 12 hours for differentiation of the THP1 cells to adherent macrophages. After overnight incubation the medium from each well was discarded and adherent cells were gently washed at least twice with serum free RPMI1640 medium. The *L. donovani* promastigotes culture was harvested at the stationary phase (metacyclic infective stage) and suspended into RPMI1640 medium with 2% FBS at the density of 2.5×10^6 cells/mL. The 200 μ L of promastigotes (5×10^5) culture was added to each well. The plate was further incubated in a 5% CO₂ incubator at 37°C for at least 24 hours to allow infection of macrophages with the leishmania parasites. After 24 hours the non-adherent macrophages and unattached leishmania promastigotes were washed off with serum free RPMI1640 medium. The infected macrophages were further incubated at 37°C and 5% CO₂ in 200 μ L RPMI1640 medium and 2% FBS with different concentrations of standard antileishmanial drugs (pentamidine & amphotericin B) or the test compounds for 48 hours. The control wells with medium, uninfected THP1 cells, infected cells without drugs or test compounds were also set up simultaneously. The cultures were washed off with serum free RPMI1640 and treated for 30 seconds with 20 μ L of 0.05% sodium dodecyl sulfate in RPMI1640 medium for the release of amastigotes from the infected macrophages. To each well 180 μ L of RPMI1640 medium with 10% FBS was added and the plate was further incubated at 26°C for 48 hours to allow transformation of released amastigotes to promastigotes. To each well 20 μ L of Alamar blue was added, the plates were incubated at 26°C for 24 hours and read on a BMG Fluostar micropate reader (BMG Lab Technologies) at excitation wavelength of 544 nm and emission wavelength of 590 nm. Each compound was tested in duplicates at six concentrations, IC₅₀ and IC₉₀ values were computed from the dose response curves.

3.3 Results and Discussion

In our study, cilantro's essential oil had a remarkable effect against *L. donovani* in vitro, achieving 98% inhibition. The IC₅₀ and IC₉₀ values for the cilantro essential oils were much higher than those found for the standards pentamidine and amphotericin B (Table 1). As we obtained this result, the essential oil from *C. sativum* leaves were fractioned and three fractions were obtained. One of the fractions had IC₅₀ and IC₉₀ values around 2.0 and 1.5 times lower than cilantro essential oil, respectively, suggesting the presence of the bioactive constituent (Table 1).

Table 1 - *In vitro* activity of cilantro's essential oil and its fractions against *Leishmania donovani* promastigotes

	<i>L. donovani</i> IC ₅₀ (µg mL ⁻¹)	<i>L. donovani</i> IC ₉₀ (µg mL ⁻¹)
Essential oil	26.58	33.03
Fraction 1	NA*	NA
Fraction 2	13.57	22.76
Fraction 3	NA	NA
Pentamidine	0.73	1.92
Amphotericin B	0.13	0.24

*NA=No Activity

Using GC/MS and Kovats indices technique it was possible to identify the main constituents of the active fraction as aldehydes with linear chains ranging from 10 to 14 carbon atoms. The major aliphatic aldehydes found were decanal, dodecanal, n-tetradecanal, (*E*)-2-undecenal, (*E*)-2-decenal and (*E*)-2-dodecenal. Aliphatic aldehydes found in the active fraction are well known as major compounds in cilantro essential oil (POTTER, 1996; MATASYOH et al., 2009). Since the active fraction had aliphatic aldehydes, the commercially available standards of (*E*)-2-undecenal, (*Z*)-8-undecenal, (*Z*)-4-decenal, (*Z*)-7-decenal, decanal, dodecanal, (*E*)-2-decenal, (*E*)-2-dodecenal and n-tetradecanal were purchased and tested against *L. donovani* promastigotes. Results showed that (*E*)-2-undecenal had the lowest IC₅₀ and IC₉₀ values followed by (*E*)-2-dodecenal, (*E*)-2-decenal, n-tetradecanal and dodecanal. Decanal and the isomers of (*E*)-2-undecenal ((*Z*)-8-undecenal) and (*E*)-2-decenal ((*Z*)-4-decenal and (*Z*)-7-decenal) had no activity against *L. donovani* promastigotes (Table 2).

Table 2 - *In vitro* activity of compounds present in cilantro's essential oil against *Leishmania donovani* promastigotes

Compound	<i>L. donovani</i> IC ₅₀ (µg mL ⁻¹)	<i>L. donovani</i> IC ₉₀ (µg mL ⁻¹)
Decanal	NA*	NA
Dodecanal	35.81	NA
n-tetradecanal	32.00	36.41
(<i>E</i>)-2-decenal	7.85	10.04
(<i>Z</i>)-4-decenal	NA	NA
(<i>Z</i>)-7-decenal	NA	NA
(<i>E</i>)-2-undecenal	2.81	4.71
(<i>Z</i>)-8-undecenal	NA	NA
(<i>E</i>)-2-dodecenal	4.35	5.97
Pentamidine	1.12	2.11
Amphotericin B	0.16	0.32

* NA=No Activity

In a second stage, (*E*)-2-alkenals were evaluated in axenic amastigotes and macrophage amastigotes in cultures of *L. donovani*. (*E*)-2-decenal, (*E*)-2-undecenal and (*E*)-2-dodecenal were highly active against axenic amastigotes, but less active in macrophage amastigote. When these compounds were tested, they showed no toxicity against *L. donovani* in THP1 macrophage cultures (Table 3).

Table 3 - Activity of (*E*)-2-Alkenals against axenic amastigotes, macrophage amastigotes and cytotoxicity in transformed THP1 cells

Compound	Axenic amastigotes		Macrophage amastigotes		Cytotoxicity (Transformed THP1 cells)	
	IC50	IC90	IC50	IC90	IC50	IC90
(<i>E</i>)-2-undecenal	<1.6	2.07	5.65	>10	>10	>10
(<i>E</i>)-2-decenal	2.47	3.49	>10	>10	>10	>10
(<i>E</i>)-2-dodecenal	4.78	5.2	9.6	>10	>10	>10

To our knowledge this is the first report on the antileishmanial properties of cilantro's essential oil and its major compounds. There are, however, reports on the effectiveness of cilantro oil as an antimicrobial agent, acting against bacteria, fungi and nematodes but not against *Leishmania*. (MATASYOH et al., 2009; DELAQUIS; STANICH, 2004; KIM et al., 2008; BEGNAMI et al., 2010). Some studies have demonstrated that aliphatic aldehydes from cilantro's essential oil (alkanals and alkenals) have antibacterial and antifungal properties (KUBO et al., 2003, 2004). Although our study does not explain the mechanism of how aliphatic aldehydes kill *Leishmania*, Fujita and Kubo (2002), carried out a mode of action study of such

aldehydes on fungi and bacteria. They reported that alkanals and alkenals disrupt microbial cell membranes. This capacity is correlated with the hydrophobic alkyl (tail) chain length from the hydrophilic aldehyde group (head). The activity related to the number of carbon chain molecule depends on the microorganism and its form. For *Salmonella choleraesuis*, the activity increased with the increasing carbon chain length up to (*E*)-2-dodecenal (FUJITA; KUBO, 2002).

In our case, leishmania forms responded differently to aliphatic aldehydes. For *L. donovani*, promastigotes (*E*)-2-undecenal (C11) was more efficient, followed for (*E*)-2-dodecenal (C12) and (*E*)-2-decenal (C10). Against axenic amastigotes, (*E*)-2-undecenal (C11) was also more effective, followed for (*E*)-2-decenal (C10) and (*E*)-2-dodecenal (C12).

Our study showed that alkenals ((*E*)-2-decenal, (*E*)-2-undecenal and (*E*)-2-dodecenal) were more effective against *L. donovani* promastigotes than alkanals (decanal, dodecanal and n-tetradecanal). The results may be explained by double bonds present in alkenals, since this characteristic is associated with enhancing of antimicrobial property (KUBO et al., 2004).

The results showed that essential oil of *C. sativum* leaves and its major compounds may represent a valuable source of new drugs against Leishmaniasis.

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4 RESPONSE OF STEVIA TO CALCIUM IN HYDROPONIC SYSTEM

Abstract

Stevia rebaudiana Bert. is a crop that has been used worldwide as a potential source of non-caloric and natural sweeteners. There are many studies in the literature that describe diterpene glycosides present in stevia leaves, however, few studies describe good agronomic practices in the stevia growth. Good agronomic practices are the first step to reach raw material with high potential for food, chemical and pharmaceutical industries. It is essential to understand how plant nutrition and fertilization can contribute for the biomass production and yield of stevioside and rebaudioside A. The objective of this study was to evaluate the influence of calcium (Ca) supply on *S. rebaudiana* growth, biomass production and yields of stevioside and rebaudioside A. Plants grew in pots filled with sand in a greenhouse at the United States Department of Agriculture, Agricultural Research Service, at the University of Mississippi Campus. The experimental design was completely randomized with four replications and six treatments. The treatments were Hoagland's nutrient solution modified with calcium applied at rates of 0, 2, 4, 6, 8 and 12 mmol L⁻¹. The leaf, stem and shoot biomass yield, by stevia plants were influenced by Ca rates in nutrient solutions and were maximal with 7.0, 5.7, 6.2, respectively. The results indicate that stevia can be produced with 4 mmol L⁻¹ of Ca to reach higher yield of stevioside and rebaudioside A.

Keywords: *Stevia rebaudiana*; Nutrient solution; Calcium; Rebaudioside

4.1 Introduction

Stevia (*Stevia rebaudiana* Bertoni) is a species that belongs to the Asteraceae family. This South American plant is the commercial source of potent non-caloric and non-synthetic sweeteners. *Stevia* leaves produce a complex mixture of natural sweet diterpenes, called steviol glycosides and identified specifically as: stevioside, steviolbioside, rebaudiosides (A, B, C, D, E and F) and dulcoside A, which have been reported as 200 to 450-fold sweeter than saccharose (SINGH; RAO, 2005; CARNEIRO, 2007). These molecules are highly stable in aqueous solutions within a broad range of pH and temperature and are also non-fermentative, which make stevia glycosides a natural sweetener to replace sucrose in food processing and household uses. The use of steviol glycosides has been approved in Brazil, Argentina and Paraguay as well as in China, Korea and Japan (VIRENDRA; KALPAGAM et al., 2008; ABOU-ARAB et al., 2010; LEMUS-MONDACA et al., 2012).

The biosynthesis of secondary metabolites in plants depends on genetic, physiological, environmental and nutritional factors (BROWN, 2003). Nutrients are essential for biomass growth and yield of secondary compounds such as diterpene glycosides. Among the nutrients, calcium was described as limiting of biomass and stevioside production in *S. rebaudiana* (LIMA FILHO et al., 1997a; UTUMI et al., 1999). Ca is an essential element in plant growth and is part of several physiological processes, including mitotic fuse during cell division (ALBIÑO-GARDUÑO et al., 2008). It is also required for cell membrane functionality, playing a role as secondary messenger in plant responses to environmental and hormonal signals (SANDERS et al., 1999). As a second messenger, Ca connects with calmodulin forming a complex to regulate many metabolic processes (TAIZ; ZEIGER, 2004). Despite the importance of nutrients, the studies reporting fertilization management to increase crop productivity are still lacking, especially in studies with improved varieties of stevia. Little is known about the effects of rates of nutrients in the stevia biomass and glycosides with emphasis on rebaudioside A, which is the compound with the greatest interest for food industry.

Thus, the purpose of this study is to investigate the influence of calcium levels on nutrient accumulation, in the biomass on diterpene glycosides production in stevia plants.

4.2 Material and Methods

4.2.1 Plant material and general conditions of stevia growth

The experiment was conducted from April 9 to June 6 of 2012 at the United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Natural Products Utilization Research Unit on the campus of The University of Mississippi. The temperature and humidity were kept at 25°C and 70% in the greenhouse environment.

Shoot tips of Crazy Sweet® stevia variety were used as explants for production of *in vitro* micropropagated plants following the protocol described by Jain et al. (2009). Rooted plantlets were transplanted to trays using the commercial substrate Metro-Mix® 300 (Bellevue, WA). Trays were covered with plastic chambers to maintain humidity close to 100% for plant acclimatization during two weeks. After that, the plastic chamber was removed and stevia plants remained in the planting

trays until six pairs of leaves were fully expanded. They were kept at field capacity watered with 20% Hoagland's solution.

4.2.2 Plant growth on Hoagland's solution

Thirty days old acclimatized plants were planted in 3.78 L pots filled with sand (Pavestone, Atlanta-GA). The pots were placed in the greenhouse leaving 30 cm between them. Daily nutrient solutions containing different levels of calcium were applied at a rate 0.2 L per pot with 15% of drainage. Twenty-five percent Hoagland's solution was applied during a 14 days period, followed by a gradual increase on nutrient concentrations to half-strength for 11 days and full-strength solution for the remaining time of the experiment, 30 days period.

The experimental design was completely randomized with four replications. Each replication consisted of a pot with one plant. The treatments were composed by Hoagland's nutrient solution (Hoagland; Arnon, 1950) modified with 0, 2, 4, 6, 8 and 12 mmol L⁻¹ of calcium: 1 mmol L⁻¹ NaH₂PO₄, 4 mmol L⁻¹ KNO₃, 2 mmol L⁻¹ MgSO₄, 0.5 mmol L⁻¹ KCl, 1 mmol L⁻¹ NH₄NO₃, 1 mmol L⁻¹ of iron solution (Fe-EDTA, FeSO₄·7H₂O,) and 1 µmol L⁻¹ micronutrient solution (H₃BO₃, MnCl₂·4H₂O, ZnCl₂, CuCl₂ and H₂MoO₄·H₂O). The source of Ca was calcium chloride. Solutions were prepared with distilled water. The pH and electrical conductivity (EC) of nutrient solutions were measured using a pH meter and EC meter, respectively. The mean values of electrical conductivity were 1.7, 2.1, 2.5, 2.8, 3.4, 4.1 dS m⁻¹, for corresponding nutrient solutions at 0, 2, 4, 6, 8 and 12 mmol L⁻¹ Ca, respectively.

4.2.3 Nutrient analysis

Plants were harvested 55 days after transplant, separated into leaves and stem and dried in an oven maintained at 65-68°C with forced air to obtain dry weight of each part. Dry leaves and stem were weighed, ground and analyzed to determine N, P, K, Ca, Mg, S, Fe, Zn, B, Mn and Cu concentrations following Nelson; Sommers, (1972); Jones; Steyn, (1973) and Isaac et al. (1975) methodology at the Mississippi State University, Soil Testing and Plant Analysis Laboratory (Starkville, MS).

4.2.4 Extraction of sweet diterpene glycosides from *S. rebaudiana*

Dried and ground leaf samples (1 g) were placed in a 250 mL Erlenmeyer flask filled with 100 mL of EtOH 70% (w/w). Flasks were incubated in a 70 °C water bath circulator for 30 min under shaking conditions. After the extracts were cooled, they were filtered and analyzed by HPLC using an NH₂ column (250 × 4.6 mm) and a mixture of acetonitrile/water (80:20, v/v) as mobile phase, pH 5.0 adjusted with acetic acid. The detection was in the UV range at 210 nm (0.04 AUFS) following the method described by Kolb et al. (2001).

Quantitation was performed by means of an external standard calibration curve for each analyte at four concentration points (0.15, 0.3, 0.6 and 1.0 mg mL⁻¹). It was obtained from standard solutions of pure stevioside and rebaudioside A purchased from ChromaDex (Irvine, CA). Each compound was used to formulate separate calibration curves. Linearity was imposed by using response factors and regression coefficients independently. Response factors were calculated using the equation $RF = DR/C$, where DR was the detector response in peak area (PA) and C was the analyte concentration. The target peaks were confirmed by retention time and mass spectra. Confirmed integrated peaks were then used to determine the percentage of each chemical constituent in the sample. The R.F. of the target chemical constituent was used to determine the percent for each sample using the equation: $(PA/R.F./C) \times 100 = \% \text{ in the dry weight}$. Percentage of dry weight was multiplied times ten to provide dry weight expressed in g kg⁻¹.

4.2.5 Statistical Analyses

The data were interpreted by analysis of variance (ANOVA), followed by regression analysis, using the General Linear Model procedure of SAS (Statistical Analysis System, 1999-2000, SAS Institute, Cary, NC). The choice of the regression equation was considered by checking the significance of coefficients ($p < 0.05$), and the coefficient of determination (R^2).

4.3 Results and Discussion

Dry biomass production (stem leaves and shoot) responded significantly to Ca levels in the hydroponic nutrient solution. Increasing the Ca rate from 0 to 12 mmol L⁻¹ caused a highly statistically significant quadratic trend in the leaves, stem and shoot dry weight with the optimum values recorded at 7.0, 5.7 and 6.2 mmol L⁻¹ of Ca,

respectively (Figure 1). Adding calcium from 0 to 6.2 mmol L⁻¹ promoted an increase of 40% on shoot dry biomass.

Figure 1 - Dry weight of leaves, stem and shoot of stevia grown under Ca rates in nutrient solution

Ca levels affected the accumulation of other nutrients in the leaves. B and Mg concentrations in leaves were significantly influenced by Ca applications. Accumulation of Ca in the leaves increased with application rate up to 8.3 mmol L⁻¹, above this rate it showed a gradual decline. Mg concentration followed a similar trend with peak concentration at 2.9 mmol L⁻¹ of Ca. B concentration decreased linearly with increase of Ca rates (Figure 2). Concentrations of N, P, K, S, Cu, Fe, Mn and Zn in the leaves were not significantly affected by Ca rates in nutrient solutions. The mean concentrations for N, P, K, S, Cu, Fe, Mn and Zn were 49.1 g kg⁻¹, 5.8 g kg⁻¹, 21.5 g kg⁻¹, 2.7 g kg⁻¹, 7.1 mg kg⁻¹, 453.6 mg kg⁻¹, 518.3 mg kg⁻¹ and 39.8 mg kg⁻¹, respectively. Compared with mineral composition of stevia reported by Carneiro et al. (1997), the means of leaf nutrient concentrations showed similar values for N and P. However, the K and S concentrations were 75% and 290% higher than those obtained by the authors. Nutrients concentrations in plant tissues vary with the growth medium composition.

Figure 2 - Concentration of Ca, Mg and B in the leaves under Ca rates in the nutrient solution

Ca application increased and decreased linearly the Ca and N concentrations in the stem, respectively (Figure 3). The concentrations of P, K, Mg, S, Cu, Fe, Mn, Zn and B in the stem did not respond significantly to Ca levels in the nutrient solution. The mean values were 3.0 g kg⁻¹, 42.0 g kg⁻¹, 1.5 g kg⁻¹ and 4.3 g kg⁻¹, for P, K, Mg and S, respectively. The mean concentration of micronutrients were 9.2 mg kg⁻¹ for Cu, 25.2 mg kg⁻¹ for Fe, 86.9 mg kg⁻¹ for Mn, 16.7 mg kg⁻¹ for Zn and 11.8 mg kg⁻¹ for B.

Figure 3 - Concentration of N and Ca in the stem of stevia under Ca rates in the nutrient solution

The uptake of N, P, K, Ca, Mg and B by shoot increased quadratically and was maximal with 5.5, 5.9, 6.2, 7.8, 4.8 and 5.1 mmol L⁻¹ of Ca, respectively (Figure 4). The S, Cu, Fe, Mn and Zn uptake in the shoot was not affected by Ca rates in the nutrient solutions. The mean values were 42.8 mg plant⁻¹ S, 99.3 µg plant⁻¹ Cu, 2850.7 µg plant⁻¹ Fe, 3644.1 µg plant⁻¹ Mn and 337.7 µg plant⁻¹ Zn, respectively. K was the most absorbed nutrient, followed by N, Ca, Mg, P and S. Other studies show similar findings, however, in a different order of accumulation: N, Ca, P, Mg and S (LIMA FILHO et al., 1997b; UTUMI et al., 1999). The descending order of micronutrient accumulation was: Mn, Fe, B, Zn and Cu. It has been reported in other study that plants of *S. rebaudiana* accumulated the micronutrients in the following order: Fe > Mn > B > Cu > Zn (LIMA FILHO et al., 1997c).

Figure 4 - Nutrient uptake in the shoot of stevia grown under Ca rates in the nutrient solution

Rebaudioside A and stevioside content in the leaves were highly influenced by Ca rates in nutrient solution. Rebaudioside A and stevioside concentrations decreased linearly with increase of Ca rates. However, the amounts of these compounds expressed per plant increased quadratically and yields were maximal with 4.0 mmol L⁻¹ of Ca (Figure 5).

Figure 4 - Concentration (g kg⁻¹) and yield (mg plant⁻¹) of rebaudioside and stevioside in leaves of stevia grown under Ca rates in the nutrient solution

High and low levels of any nutrient may adversely affect plant growth. Such harmful impacts occur due to nutrient deficiency or osmotic stress, from lower and higher concentrations in the nutrient solution, respectively (SAVVAS; ADAMIDIS, 1999; ROUPHAEL et al., 2012). Furthermore, the adverse effect caused by high concentrations of Ca on dry biomass, nutrient uptake and yield may be due to the antagonistic relationship between Ca and other cations. Thus, increased calcium fertilization relative to potassium, magnesium and ammonium reduced the uptake and accumulation of these cations. The degree of the antagonistic effect, however, varied with Ca rates and the level of these nutrients in the nutrient solution. The osmotic stress associated with antagonism or competition between nutrients, may have contributed to the decrease in the growth and yields of stevia glycosides. Studies reported by Colla et al. (2006, 2008, 2010, 2012) showed plant growth reduction in different crops influenced by osmotic stress.

The results of our study support the use of hydroponic cultivation on the production of quality stevia biomass and the influence of calcium in stevia growth and development. In addition, data show that leaves, stem and shoot biomass yield, and accumulation of stevioside and rebaudioside A by stevia plants were influenced by Ca rates in nutrient solutions and were maximal with 7.0, 5.7, 6.2, 4.2 and 4.1 mmol L⁻¹ of Ca, respectively. The results indicate that stevia can be produced with 4 mmol L⁻¹ of Ca to reach higher yield of these diterpenes.

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