Effect of fertilization and type of pruning on quality of fresh and dehydrated *Stevia rebaudiana*

Efeito da adubação e tipo de poda na qualidade de *Stevia rebaudiana* fresca e desidratada

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ABSTRACT

Stevia rebaudiana is tropical and sub-tropical plant and has been received a large body of attention due to its potential as natural sweetener. In order to contribute to the optimization of the agricultural production in a coastal region in the Lisbon area, with mild climate, an on-farm study was conducted under a context of social agriculture. It was investigated the effects of two levels of pruning and two regimes of fertilization on the plant's biomass yield and quality, including accumulation of steviol glycosides. The quality differential of leaves was quantified after a customized solar drying coupled with a pre-heat abiotic stress procedure.

The results showed that, when growing under just basal fertilization, plants were higher, independently of pruning severity. Additional (foliar) fertilization in light-pruned plants lead to more leaves that in severe-pruned ones and higher leaf areas were observed under severe pruning. Glycosides concentration was not influenced by fertilization, but it was positively influenced by severe pruning resulted in increased amounts, in ca. 29.38% in case of leaves without additional fertilization. In the same time, severe-pruning resulted in higher increase of Stevioside, responsible for bitterer aftertaste, when compared to Rebaudioside A (Rebaudioside A: Stevioside ratio = 0.89).

The leaves of Stevia were subjected for 2h to pre-heating and 18h24m of active drying using a SOLAR DRYER until 10.00% of humidity, and with total soluble solids increased ca. 18% and pH decreased ca. 4%. The drying procedure increased Glycosides to 43.06 mg/g dry leaves, from 36.82 mg/g, suggesting secondary metabolites accumulation as a response to abiotic stress. The level of phenolic compounds increased in 100% and antioxidant activity was ca. 3.01 (ABTS) and 4.40 (DPPH) fold higher.

Key-words: Stevia, Glycosides, Solar drying, Secondary metabolites, Quality.

RESUMO

Stevia rebaudiana é uma planta tropical ou sub-tropical que despertou um grande interesse devido ao seu potencial como um adoçante natural. Com o objetivo de contribuir para a otimização da produção agronómica numa região costeira na zona de Lisboa, com clima ameno, um estudo “on-farm” foi realizado na Cercica, no âmbito do contexto da agricultura social. Foram estudados os efeitos de dois níveis de poda e dois regimes de adubação na produtividade e qualidade da planta, incluindo a acumulação de glicosídeos de steviol. Após secagem solar adaptada e com uma etapa de stress abiótico (pré-aquecimento) foi quantificado o diferencial dos parâmetros da qualidade das folhas.

Os resultados mostraram que, quando submetidas apenas à fertilização de instalação, as plantas foram mais altas, independentemente da severidade da poda. As plantas sob poda ligeira e com fertilização adicional (foliar) tiveram melhor rendimento em biomassa, enquanto as plantas com poda severa apresentaram maior área foliar. O teor de glicosídeos não foi influenciado pela fertilização, tendo sido positivamente influenciado pelo nível de poda, verificando-se um aumento de 29,38% no caso da poda severa. No entanto, a poda severa implicou níveis mais elevados de Steviosídeo, responsável por sabor mais amargo, quando comparado com o Rebaudiosídeo A (Rebaudiosídeo A: Steviosídeo = 0,89).

Folhas de Stevia foram submetidas a pré-aquecimento durante 2 horas com secagem de 18h24m realizada no SECADOR SOLAR até 10,00% de humidade, com um aumento de teor de sólidos solúveis totais de ca. 18%, e com a diminuição do pH de ca. 4%. O processo de secagem implicou um aumento do teor de Glycosídeos de 36,82 para 43,06 mg/g de folhas secas, sugerindo a acumulação de metabolitos secundários, como resposta ao stress abiótico. O teor de compostos fenólicos apresentou um aumente de ca. 100% e a atividade antioxidante em ca. 4,4 (ABTS) e 3,01 (DPPH).

Palavras-chaves: Stevia, glicosídeos, Secagem solar, metabólitos secundários, Qualidade.

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ABBREVIATIONS

MAP - Medical and aromatic plants

FMI - Future market insights

HFCS - High fructose corn syrup

ADI - Acceptable daily intake

EFSA - European food safety authority

SCF - Scientific committee on food

FAO - Food and Agriculture Organization

WHO - World Health Organization

TECFA - Expert committee on food additives

GRAS - Generally recognized as safe status

ANS - Panel on food additives and nutrient sources added to food

PAL - Phenylalanine ammonia-lyase

ISD - Indirect solar drying

REF - Unique reference sample

HPLC - High performance liquid chromatography

LSD Least significant difference

FDA - Food and Drug Administration

FAD - Flavin adenine dinucleotide

NOAEL No Observed Adverse Effect Level

EGA - Equivalent Gallic Acid

EAA - Equivalent Ascorbic Acid

ET - Equivalent Trolox

1. INTRODUCTION

1.1 FRAMEWORK OF THE TOPIC

Recognition of the importance of medicinal and aromatic plants (MAP) has grown in recent years, leading to an increased demand by consumers and consequent interest in production by farmers. A large number of species of MAP containing specific active compounds or unique relative proportions of active ingredients exist. The species *Stevia rebaudiana* (Bertoni) is original from tropical regions and its exploitation from the agricultural point of view is still incipient in Portugal. These plants produce diterpenes (steviol glycosides), which are natural sweetener recently approved for use as food ingredient, ca. 300 times sweeter than sucrose, low-calorie, water soluble, resistant to high temperatures and with favourable technological properties for the food industry. These compounds can be extracted, purified and dried using low-cost and simplified technologies.

Stevia is likely to become a major source of high-potency sweetener for the fast growing natural food market. Although replacing consumption of refined sugars by Stevia sweeteners can represent a universally healthy advantage, there are certain groups that are more likely to benefit from its remarkable sweetening potential. Among these, are included diabetic patients, those interested in decreasing caloric intake, and children (Goyal et al., 2010).

A Future Market Insights' (FMI's) recently published a report Global Stevia Market – Market Analysis and Opportunity Assessment, 2014 – 2020, sustains that hafting consumer preference for natural sweeteners is a major factor driving growth of a Stevia fresh market. Additionally, Stevia extracts are finding increasing application in soft drinks and juices, ice creams among other products. This is attributed to its high intensity natural sweetness properties. Due to these factors, the share of the Stevia market is expected to account for around 15% of the overall sweetener market by 2020. In fact, according to FMI (2014), "increasing demand for alternatives to synthetic or artificial sweeteners due to health concerns, coupled with rising demand for plant-based sweeteners is projected to fuel growth of the stevia market over the next five to six years."

This remarkable potential is therefore enhancing the importance of *S. rebaudiana* as an expanding or, in some regions, new agricultural crop to satisfy today's need for food ingredients of low calorie with nutritional, therapeutic and functional properties. Such goal requires adaptability studies and optimization of agronomic production practices in order to extract the full quality potential of the crop, while ensuring profit and economic and environmental sustainability. Consumers'

demand for herbal foods may encourage Stevia cultivation and production and may help those who have to restrict carbohydrate intake or reduce the glycemic index in the diet, to enjoy sweet taste with minimal calories (Roberto Lemus-Mondaca, et al., 2011).

The present study was conducted within the framework of a collaboration between the social institution *Cooperativa para a Educação e Reabilitação de Cidadãos Inadaptados de Cascais* (CERCICA), the private consulting company Consulai, Lda and the Portuguese research institute Instituto de Investigação Científica Tropical (IICT), developed under the project Aroma4Safe, financed by "Programa de Desenvolvimento Rural" (ProDeR). The work reported aimed at contributing to understand the potential adaptability and the best cultural practices for Stevia growing in the central-west littoral region of Portugal, by determining the effect of two types of pruning severity and two regimes of fertilization on the productivity and quality composition of *Stevia rebaudiana*, produced in a context of social agriculture using biological agriculture production approaches and solar drying.

1.2 OBJECTIVES

The main objective of this work was to investigate the impact of different cultivation treatments on the productivity and post-harvest quality of *Stevia rebaudiana* cultivated in littoral, central-west region of Portugal, 30 km north-west from Lisbon, in Sintra Municipality, village of Estoril.

Specific objectives of this work were to:

1. Investigate the impact of fertilization and pruning severity on plant production and leaf glycoside quantitative composition.
2. Design and construction a semi-active pilot Solar Dryer for processing stevia leaves.
3. Study the impact of drying on quality parameters of fresh Stevia leaves, including glycoside's concentration.
4. Evaluate if a social institution like CERCICA can gain low input agricultural production with value added and differentiation end products as a profitable alternative in their activity.

2. LITERATURE REVIEW

2.1 SWEETENERS

Early men would have exploited whatever food supplies were most readily available. Although in many cases, men became primarily carnivorous, gathering and eating the succulent and more attractive vegetable was also pursuit (Burton W., 1982).

The first recorded sweetener consumed by humans was honey, which was used in the ancient cultures of Greece and China (Bright G., 1999). Egyptian hieroglyphics dating back at least 3000 years indicate that honey was used as a sweetener, mixing it with various fruits, nuts, herbs, and spices in breads, cakes, and pastries (Tannahill, 1975). Pythagoras, born in BC 571, is said to have lived largely on honey and bread, and the bodies of his countrymen who died some distance from home were sometimes preserved in honey (Free, 1982). China is known to have imported honey and one official was granted on his retirement in AD 500 *a quart* of white honey each month (Crane, 1975). These amounts correspond a bit more than 19 kg of honey per year.

Honey was later replaced by sucrose, the common sugar, which was originally obtained from sugar cane. During the World Wars, sugar beets were the major source of sucrose.

Although standing for many decades as the world's most popular sweetener, sucrose has gathered in recent years most of the criticism when it comes to the obesity crisis (Ghosh, 2012). Nonetheless, the consumption of foods and beverages containing non-nutritive sweeteners has dramatically increased over the past few decades.

Based on the chemical groups (see Fig. 2.1), sweeteners have been classified as (Ghosh, 2012):

- Sulfamate (cyclamate)
- N-sulfamyl amides (acesulfame-K and saccharine)
- Dipeptides (aspartame, neotame)
- Ditepenoid glycosides (rebaudioside-A, stevioside)
- Halogeneted sugars (sucralose)
- Modified sugars (isomaltulose, tagatose)
- Polyhydric alcohols (manitol, sorbitol lactitol, xylitol)
- Citrus dihydrochalcone (neohesperidine dihydrochalcone)

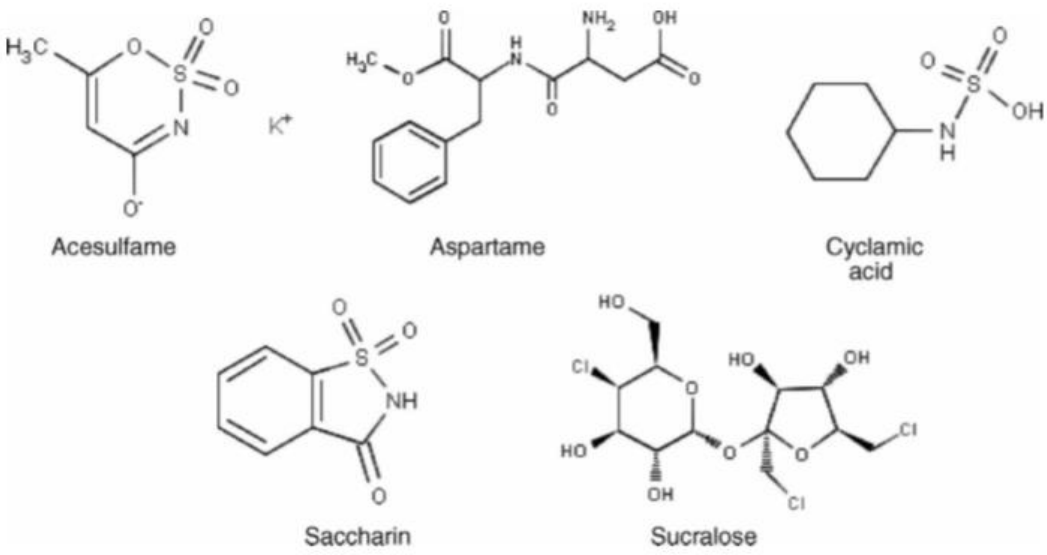


Figure 2.1 Chemical structural formula of intense sweeteners (Source: US National Library of Medicine. Environmental Health Information Programme)

The global sweeteners market is still dominated by synthetic or semi-synthetic molecules and most current products are touted as “alternative sweeteners” (Ghosh, 2011).

It is not possible to categorize sweeteners as either “natural” or “artificial”, as their presence in nature does not necessarily match their commercially manufactured route; and also their perception by consumers is much more complex (Bagley, 2013).

High fructose corn syrup (HFCS) is produced by converting corn starch to sugars through enzymatic processes. It has replaced the use of cane and beet sugar in food and beverage industry to a great extent. Sweet corn and HFCS together, position corn as an important sugar producing crop of the world. About one half of all sweeteners consumed in USA are derived from corn (Ishwar et al., 2014).

Premarket evaluation, beside safety matters, should take in consideration also relative costs for sweetener production and commercialization. Table 2.1 shows indicative relative costs compering to sucrose’s cost.

Table 2.1 List of comparative sweetening potential and relative cost for principal sweeteners (Lindsey B., The World of Food Ingredients, Feb 2013)

Sweetener	Sweetness potential (Multiplicity Relative to Sucrose	Theoretical Relative Cost of Sweetening a Beverage @ 10% Sucrose Equivalence (100.00)
Acesulfame K	x 200	2.50
Aspartame	x 200	5.60
Cyclamate	x 30	12.50
Neotame	x 8000	4.30
Saccharin	x 400	1.40
Sucralose	x 600	13.50
Stevia	x 250 (as Reb A)	60.00

Sweeteners approval process

Sweeteners are among the food additives that have created most debate. Sweeteners are considered to be potential high-consumption food additives because of their use in products consumed in large amounts, such as soft drinks, and ‘‘tabletop’’ sweeteners. Although the scientific evidence indicates that the sweeteners permitted for food use are safe, some individuals and organizations remain skeptical about long-term health risks due to their consumption (Mortensen, 2006).

Sweeteners can only be introduced into the food industry after a demanding approval by competent scientific institutions, later to evaluating toxicological, microbiological and safety aspects, and only that evaluation can detect any possible risks to the consumer. This means that before a sweetener is introduced into the composition of a product, it has to comply with all the established requirements (Teixeira et al., 2011).

The Scientific Committee on Food of European Commission issued in 11 July 2001 the *Guidance on Submissions for Food Additive Evaluation*.

For instance, as described by Magnuson (2007), before approval for use in foods and beverages, the comprehensive safety evaluation conducted included the following parameters: Safety = hazard × exposure

Assess potential hazard in toxicology studies:

- Acute, Sub-chronic, Long-term toxicity studies in multiple species
- Carcinogenicity
- Genetic toxicity
- Reproductive toxicity
- Teratogenicity
- Medical: blood chemistry, special populations such as diabetics

The risk assessment of sweeteners is performed following a general procedure for risk assessment of chemicals in food, a scientific process that requires expertise in toxicology and nutrition (for the intake assessment). The procedure consists of four steps: hazard identification, hazard characterization, exposure assessment and risk characterization (Renwick et al., 2003). As the result of hazard characterization an acceptable daily intake (ADI) has been established for each sweetener.

European Food Safety Authority (EFSA) considers ADI as the amount of a substance that people can consume on a daily basis during their whole life without any appreciable risk to health. ADIs are usually expressed in mg per kg of body weight per day (mg/kg bw/day).
Once authorised, these substances are compiled on an EU list of permitted food additives, which also specifies their conditions of use.

In December 2008, existing legislation was consolidated into four simplified regulations covering all so-called food improvement agents (i.e. food additives, food enzymes and flavourings). Regulation EC 1331/2008 introduced a common authorisation procedure for these agents. Regulation EC 1333/2008 on food additives established a Union list of authorised food additives, which was published in full in Regulation EU 1129/2011.

The use of sweeteners in the EU must be authorised by EFSA before they can be used in foods and is regulated by a framework regulation (Reg. (CE) 1331/2008) and a specific regulation (Reg. (CE) 1129/2011). The annexes to the specific regulations provide the information on which sweeteners are permitted in different foods or group of foods, together with the maximum allowed doses. Permitted sweeteners are listed in Table 2.2 which was established as a Union list of authorised food additives in Regulation EC 1333/2008 on food additives, and amended and published in full in Regulation EU 1129/2011.They must also comply with approved purity criteria laid down in Regulation EU 231/2012.

All sweeteners permitted for food use in the EU have been subjected to a comprehensive examination for potential toxicological effects according to the principles of toxicological testing of food additives, before accepting their safety in use. Their safety has been evaluated by the national authorities, by the EU's Scientific Committee on Food (SCF) and by the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA).

Table 2.2 EU Reg. 1129/2011, Appendix II, Part B, List of Sweeteners

E Number	Designation
E 420	Sorbitols
E 421	Manitol
E 950	Acesulfame K
E 951	Aspartame
E 952	Cyclamates
E 953	Isomalte
E 954	Saccharins
E 955	Sucralose

E Number	Designation
E 957	Thaumatin
E 959	Neo-hesperidine DC
E 961	Neotame
E 962	Aspartame-acessulfame salt
E 965	Maltitols
E 966	Lactitol
E 967	Xilitol
E 968	Eritritol

2.2 NATURAL SWEETENERS - SUGAR AND PLANTS CULTIVATED FOR SUGAR

Sugar and sweeteners consumption and supply overview

According to FAO, sugar and sweeteners includes sugar cane, sugar beet, honey and other sweeteners.

FAO's report in *Food and Nutrition (2014)*, refers that consumption of sugar has been growing rapidly in developing countries (Fig. 2.1), which now account for almost three-quarters of global consumption, up from just over half in the 1980s (FAO, 2014).

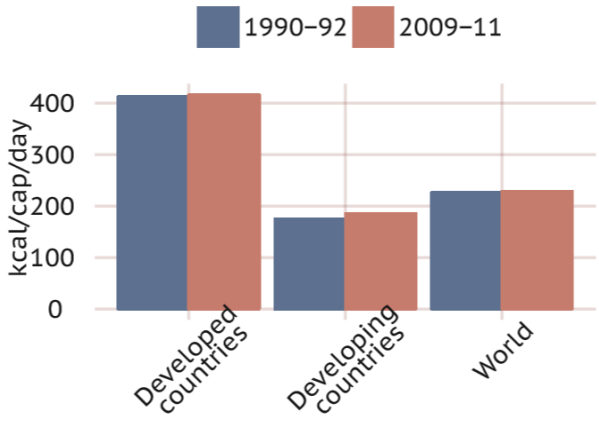


Figure 2.2 Evolution of Food supply of sugar and sweeteners in 1990-92 and 2009-11 (adapted from FAO - Food and Nutrition, 2014)

Consumption in high-income countries has stagnated (Fig. 2.2), partially as the result of the rapid expansion of corn-based sweeteners in the United States of America (FAO, 2014).

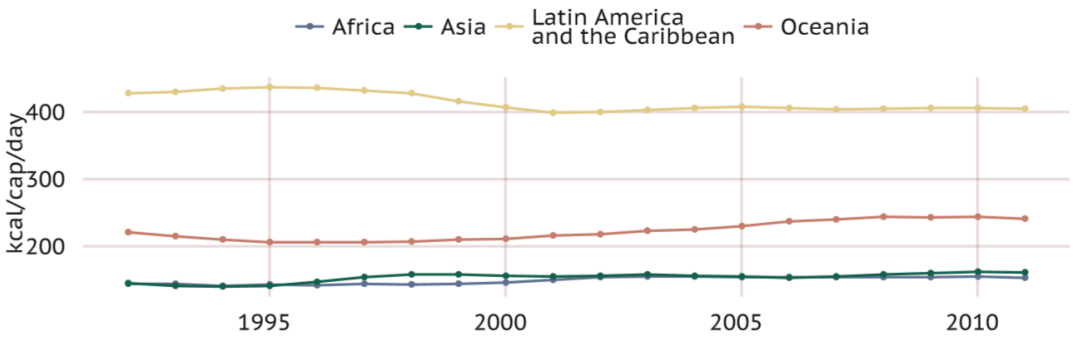


Figure 2.3 Evolution of Sugar and sweeteners world consumption in 1990-2010 (adapted from FAO - Food and Nutrition, 2014)

However, a high intake of low molecular mass carbohydrates is also associated with dental caries and diet-related health issues, and a reduction in the amount of free sugars in the human diet has already been recommended (WHO, 2004). A recently published meta-analysis (Morenga et al., 2012) endeavoured focus on primary contributory factors of obesity and demonstrated that, at similar levels of physical activity, a decrease in dietary sugar intake is highly associated with a decrease in body weight.

2.3 STEVIA REBAUDIANA

Among the 230 species that compose the genus *Stevia*, only the species *S. rebaudiana* and *S. phlebophylla* produce steviol glycosides (Brandle & Telmer, 2007). The leaves of Stevia, commonly referred to as honey leaf, candy leaf and sweet leaf, have functional and sensory properties superior to those of many other high-potency sweeteners. Stevia is likely to become a major sweetener source to attain the observed growing natural food market (Goyal et al., 2010). This plant's leaves contain different sweet tasting components, biochemically called steviol glycosides. There are many different types of steviol glycosides, but 11 of them are most abundant in a typical leaf (Borstel / KNAUER, 2009). Each of these steviol glycosides has its own unique taste profile and sweetness intensity and each can be 200 to 350 times sweeter than sugar – but all without calories (Borstel / KNAUER, 2009).

Presently, Stevia is well-known for its high content of sweet diterpene (about 4–20%) in dry-leaf matter (Ghanta, Banerjee, Poddar, & Chattopadhyay, 2007).

As stated, Stevia glycosides have recently been approved as sweeteners for a range of foods (Zahn, 2013). Toxicological studies have demonstrated that Stevioside does not have mutagenic, teratogenic or carcinogenic effects, and likewise, allergic reactions have not been observed when it is used as a sweetener (Pól et al., 2007). Recently, more studies on the general and reproductive toxicity of Rebaudioside A,

corroborates previous research carried out with purified steviol glycosides, demonstrating its safety even under high dietary intake levels (Carakostas, Curry, Boileau & Brusick, 2008).

2.3.1 Origins and History

Stevia has been used since ancient times for various purposes throughout the world (Goyal, Samsher, & Goyal, 2010). For centuries, the Guarani tribes of Paraguay and Brazil used Stevia species, primarily *S. rebaudiana*, which they called ka'a he'ê (''sweet herb''), as a sweetener in yerba mate and medicinal teas for treating heartburn and other ailments (Brandle & Telmer, 2007).

Stevia rebaudiana is a branched bushy shrub native to the Amambay region in the north east of Paraguay (Abdalbasit et al., 2014). It also occurs in the neighbouring parts of Brazil and Argentina (Soejarto, 2002).

The sweet molecule was first isolated in 1909 and only in 1931 the extract was purified to produce Stevioside, the chemical structure of which was established in 1952 and showed to be a diterpene glycoside (Asrul et al., 2013). Stevioside is described as a glycoside comprising three glucose molecules attached to an aglycone, the steviol moiety (Barriocanal et al., 2008). During the 1970s, other compounds were isolated, including Rebaudioside A, with a sweetening potency even higher than Stevioside (Barriocanal et al., 2008). Table 2.3 presents relevant events in history of Stevia.

Table 2.3 The Stevia Milestone (Adapted from Global Stevia Institute)

Year	Event
200 years ago	The stevia plant was first discovered by indigenous people in South America who used leaves of the plant to sweeten beverages or chew for the sweet taste.
1899	The stevia plant was first scientifically recorded as *Eupatorium rebaudianum* by Swiss botanist Moises Santiago de Bertoni, in Paraguay. He was the first to describe the sweet taste in detail.
1905	The stevia plant was later reclassified as *Stevia rebaudiana* a member of the sunflower (*Asterceae*) family and related to the Chrysanthemum.
1931	French chemists isolated the parts of the plant that it make it sweet. The sweet components are called steviol glycosides and are 200-350 times sweeter than sugar.
1971	Stevia sweetener is made first for commercial use in Japan.
1984	China began cultivating stevia plants.
2008	High-purity stevia extract was given "Generally Recognized as Safe" (GRAS) status by U.S.A. FDA, thus allowing the extract to be used in foods and beverages.
2011	EFSA approved the use of steviol glycosides as a sweetener in foods and beverages.

Stevioside, the major sweetener present in leaf and stem tissues of stevia, was first seriously considered as a sugar substitute in the early 1970s by a Japanese

consortium formed for the purpose of commercializing Stevioside and stevia extracts (Kinghorn and Soejarto 1985). Japan was the first country in Asia to market stevioside as a sweetener in the food and drug industry (Chatsudthipong & Muanprasat, 2009). Since then, cultivation of this plant has expanded to other countries in Asia, including China, Malaysia, Singapore, South Korea, Taiwan, and Thailand (Chatsudthipong & Muanprasat, 2009). Due to the increased importance, its cultivation has spread to other regions of the world, including Canada and some parts of Europe (Amzad-Hossain, *et al.*, 2003).

2.3.2 Botanical characteristics

Stevia is a genus of about 200 species of herbs and shrubs in the *Asteraceae* family. The *Stevia rebaudiana* plant is a perennial herb with an extensive root system and brittle stems producing small, elliptic leaves (Shock, 1982). It can grow up to 1 m tall (Mishra, Singh, Kumar, & Prakash, 2010). This species was botanically classified in 1899 by Moisés Santiago Bertoni. Initially called *Eupatorium rebaudianum*, its name was changed to *S. rebaudiana (Bertoni)* Bertoni in 1905 (Lemus-Mondaca et al., 2012).

The leaves are sessile, 3–4 cm long, elongate, lanceolate or spatulate shaped with blunt-tipped lamina, serrate margin from the middle to the tip and entire below. The upper surface of the leaf is slightly granular pubescent. The stem is woody and weak-pubescent at the bottom. The rhizome has slightly branching roots. The flowers are pentamerous, small and white with a pale purple throat. They are composite surrounded by an involucre of epicalyx. The capitula are in loose, irregular, sympodial cymes. The tiny white florets are borne in small corymbs of 2–6 florets, arranged in loose panicles. The fruit is a five-ribbed spindle shaped achene (Blumenthal, 1996; Katayama et al., 1976).

2.3.3 Environmental requirements for distribution and growing

Stevia is a semi-humid subtropical plant that shows higher leaf production under conditions of high light intensity and warm temperature (NEDFI, 2007). Day length is more critical than light intensity, and long spring and summer days favour leaf growth whereas short days trigger blossoming (NEDFI, 2007).

Stevia suffers from cold and requires temperatures above 9ºC, although occasionally can tolerate temperatures near zero. For rapid growth, 20–24ºC are necessary (Singh & Rao, 2005).

Different climatic conditions would influence Stevia cultivation, so it is advisable to carry out trials in each planting zone to establish adequate plant population density for that particular area (Rahmesh, Singh, & Megeji, 2006). Edafoclimatic conditions

dramatically influence the crop behaviour. For instance, in Italy Stevia is plurennial (Ruta et al., 1999), while in Canada, for example, it is generally grown as an annual (Brandle and Rosa, 1992).

Stevia is a subtropical plant which, despite being very resistant variant the characteristics and soil types (Shock, 1982; Andrade, 2012), prefer moist soils, but not soggy, not very demanding in terms of texture (sandy and clay) with median levels of organic matter, characterized by good permeability and drainage. Allows a pH range of soil from 5.5 to 7.5 and also prefers a half-shadow for good agronomic performance (Abdullatif & Osman, 2012; Pande &Gupta, 2013; Cortés, 2012).

The soils where *Stevia rebaudiana* (Bert.) Bertoni grows spontaneously are typically of low fertility and a pH of 4 to 5, reaching a height between 0.6 and 0.7 m. When grown in soil where the conditions are more suitable for their development and growth, such as a soil pH slightly higher and good drainage, it can reach a height of about 1 meter or even more (Shock, 1982; Midmore & Rank, 2002).

Plant cannot tolerate excessive moisture in the soil, mainly due to fungal diseases (Landázuri & Tigrero, 2009; Cassacia, 2006).

However, Stevia will grow well on a wide range of soils if a consistent supply of moisture and adequate drainage is guaranteed (Shock, 1982).

Moreover, land should be disked and /or harrowed twice to prepare a fairly smooth, firm-planting surface (NEDFI, 2007).

Stevia is usually propagated by stem cuttings, which root easily (NEDFI, 2007). Cuttings should be 2-4 inches long, from leaf axils of current year growth with at least two leaf buds above ground. All the lower leaves are to be removed keeping 2 or 3 small leaves (NEDFI, 2007). Transplants from cuttings are placed in plug trays in the green house for a period of 7–8 weeks, in a rather expensive process. Stevia plug plants are then planted into the field on about 50 - 60 cm row spacing with a total plant density on the order of 100 000 plants per hectare (Rahmesh, Singh, & Megeji, 2006). According to Serio (2010), one planted hectare can produce between 1000 and 1200 kg of dried leaves that contain 60–70 kg Stevioside, which is a low yield compared to sugar cane or sugar beet. However, 70 kg Stevioside, which is 300 times sweeter than sucrose, is equivalent to a yield of 21 000 kg of sugar per hectare. Andolfi et al. (2006) found that the highest quantity of leaf dry matter produced was approximately 3.6 t/ha for the most productive genotype investigated in the initial 2 years, and then increased until the 6^{th} year reaching a peak of 6.1 t/ha.

Megeji et al. (2005) measured an average plant height of 0.45m in an experiment conducted in India at an altitude of 1300 m above sea level.

Planting density is an important parameter of cultivation method, because it directly affects the degree of primary and secondary branching, the height of the plants, and the diameter of the stem, the leaf area index, the number of flowering, and, therefore, the final yield and production (Katayama et al., 1976; Carneiro, 1990; Ruta et al., 1999).

Immediately after planting, the plant organs contain different amounts of the sweet glycosides, which decline in the following order: leaves, flowers, stem, seeds and roots. Roots are the only organs that do not contain Stevioside. The sweetness in the leaves is two times higher than that in inflorescence (Dwivedi, 1999). Sekaran et al. (2007) reported that individual tissues of stevia appear to differ significantly. As stated, the concentration of Stevioside in the leaves increases when the plants are grown under long day condition where vegetative period is longer and steviol glycoside yields will be higher (NEDFI, 2007).

Stevia has a remarkable water need, the leaves and stems can wilt rapidly, but recover rapidly if the stress is not prolonged; this is a limitation to the area suitable for its cultivation. It grows fast and can be grown as an annual herb during late spring and summer.

Hence, stevia cultivation needs irrigation. Generally, the plant requires frequent shallow irrigation and during summer irrigation at 3-5 days interval gives best results (NEDFI, 2007) and sprinkler irrigation (micro sprinklers) is found to be advantageous (Kaushik, Pradeep, Vamshi, Geetha, & Usha, 2010).

Harvest can be performed in a single cutting before blooming or more cuttings per year can be planned (Carneiro, 1990).

2.3.4 Biosynthesis of Steviol Glycosides

In higher plants a wide variety of secondary metabolites are synthesized from primary metabolites (e.g., carbohydrates, lipids and amino acids). According to Seigler, 1998, plant secondary metabolites are often referred to as compounds that have no fundamental role in the maintenance of life processes in the plants, but they are important for the plant to interact with its environment for adaptation and defence. However, it is now much more clear the crucial role played by them in plant growth and development.

The steviol glycoside and gibberellin biosynthetic pathways diverge at kaurene (Kim et al., 1996). In Stevia, kaurene is converted to steviol, the ‘‘backbone’’ of the sweet glycosides (Fig. 2.4 A and B), then glucosylated or rhaminosylated to form the principle sweeteners. The purpose of these compounds to the plant is not yet clearly explained, with some authors suggesting that exists some evidence that Rebaudioside

B and Steviolbioside are not native constituents of *S. rebaudiana*, but are formed by partial hydrolysis during extraction (Prakash et al., 2008), being thus artefacts of the extraction procedure (Kennelly, 2002).

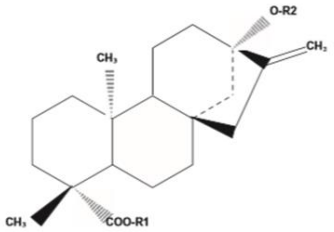
However, their high concentration in the leaf and the conservation of the pathway within the species indicate that, at some point in evolutionary time, their presence conferred significant advantage upon those individuals that possessed them. Some researchers feel that they act to repel certain insects and others speculate that it is an elaborate means of controlling levels of gibberellic acid (Smith and Van-Stadin, 1992).

Stevia is distinguished by the presence of the sweet diterpene glycosides (Fig. 2.5 C): Rebaudioside A, Rebaudioside C, Stevioside and Dulcoside in the leaf tissue (Ahmed, et al 1982) and in Stevia leave extracts fallowing concentrations could be found: Stevioside (5 – 10%), Rebaudioside A (2 – 4%), Rebaudioside C (1 – 2%) and Dulcoside A (0.5 – 1%) (Borstel / KNAUER, 2009).These are synthesized, at least in the initial stages, using the same pathway as gibberellic acid (Singh and Rao, 2005).

All steviol glycosides are high-potency sweeteners, and Stevioside has been reported to have a relative sweetness, compared to sucrose, between 210 and 300, depending on the protocol used (Crammer and Ikan, 1987; Kinghorn and Soejarto, 1986). Randi (1980) reviewed the potential uses of *Stevia rebaudiana*, which produces Stevioside, a non-caloric sweetener that does not metabolize in the human body. The sweet compounds pass through the digestive process without chemically breaking down, making stevia safe for those who need to control their blood sugar level (Strauss 1995). Unlike many low-calorie sweeteners, Stevioside is stable at high temperatures (100°C) and over a range of pH values (Kinghorn and Soejarto, 1985). It is also non-calorific, non-fermentable and does not darken upon cooking (Crammer and Ikan, 1986).

This natural variability could be partially due to the largely out-crossing nature of the species (Handro et al., 1993).

A)



B)

Compound	R1	R2
Steviol	H	H
Steviolbioside	H	β-Glc-β-Glc(2→1)
Stevioside	β-Glc	β-Glc-β-Glc(2→1)
Rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside B	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside C (Dulcoside B)	β-Glc	β-Glc-α- Rha(2→1) | β-Glc(3→1)
Rebaudioside D	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside E	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
Rebaudioside F	β-Glc	β-Glc-β-Xyl(2→1) | β-Glc(3→1)
Dulcoside A	β-Glc	β-Glc-α- Rha(2→1)

C)

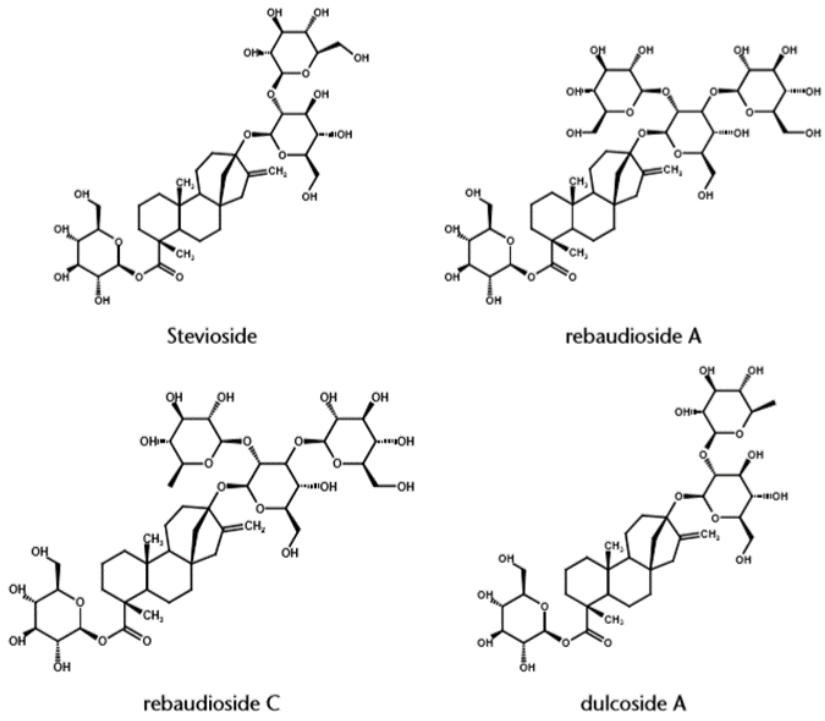


Figure 2.4 A and B) Structure of the major glycosides of Stevia rebaudiana leaves. Glc, Xyl, and Rha represent, respectively, glucose, xylose, and rhamnose sugar moieties (Geuns, 2003), C) Steviol Glycosides chemical structures (KNAUER, 2009)

Steviol is produced (Fig. 2.6) by the hydroxylation of (-)-kaurenoic acid at the C-13 position, but the gene for this FAD-dependent monoxygenase has not yet been isolated (Kim et al., 1996). The two oxygenated functional groups of steviol, the C-19

carboxylate and the C-13 alcohol, provide attachment points for the sugar side chains that determine the identity of the different glycosides. The C-13 alcohol is successively glycosylated, first yielding steviolmonoside then steviol-bioside, next the C-19 carboxylate is glycosylated, which forms Stevioside (Shibata et al., 1991, 1995). Rebaudioside A is formed by the glycosylation at C-13 Stevioside by the activity of enzyme UGT76G1 (Brandle and Telmer, 2007), as the end of pathway. Rhamnosylated glycosides can also be formed by the addition of a UDP rhamnose moiety to steviolmonoside (Richman et al., 1999).

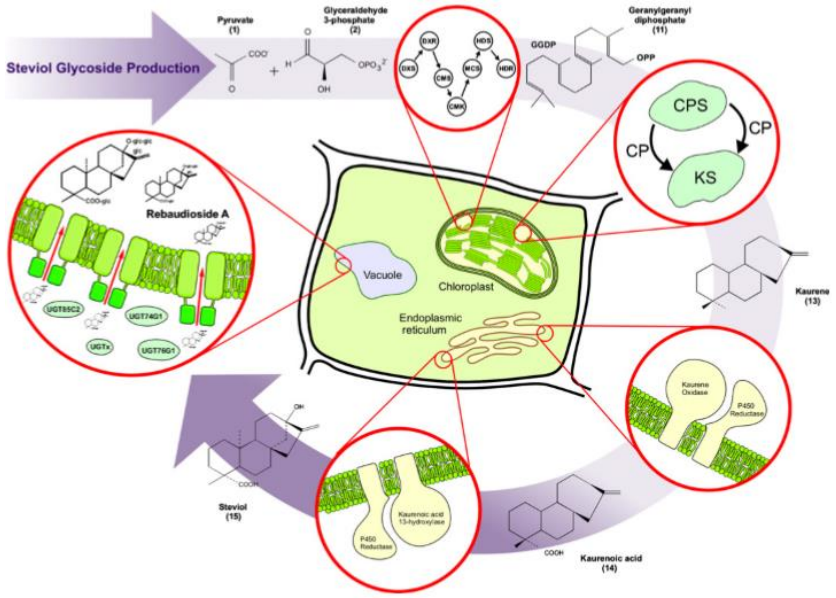


Figure 2.5 Cycle of Biosynthesis of Rebaudioside A ending in plant cell vacuole (Brandle and Telmer, 2007)

2.3.5 Influence of agricultural practices on yield and quality

The yield of sweetening compounds in leaf tissue can vary according to method of propagation (Tamura et al., 1984), daylength (Metivier and Viana, 1979) and agronomic practices (Shock, 1982). There are reports of stevioside content (total glycosides) ranging between 4 and 20% on a dry weight basis, depending on the cultivar and growing conditions (Kennely, 2002; Starrat et al., 2002).

2.3.5.1 Management of soil fertility

Soil management can be comprehended as a systematic approach which should enhance all capacity in reaching fullest potential of a soil in its role of supporting the growth of high-yielding, high-quality, and healthy crops.

The soil should be viewed as a dynamic body consisting of mineral and organic materials, gases and various living organisms (Leeper and Uren, 1993; Van Veen et al., 1997), some of which are beneficial for soil fertility and plant nutrition, while others,

including various pathogens, nematodes, insect larvae and weed seeds, can cause serious injury to plant growth and productivity.

Stevia plants were reported to have low nutrient requirements. Stevia plants prefer low nitrogen (N), but high levels of phosphorus (P) and potassium (K) (NEDFI, 2007). Slow release N sources are better due to steady release of N from source. Since the feeder roots tend to be quite near the surface, addition of organic matter for extra nutrients is beneficial (NEDFI, 2007).

Under conventional production, at the point of maximum dry matter accumulation, Stevia plants consist of 1.4 % N, 0.3% P and 2.4 % K (NEDFI, 2007). When biomass production is 7500 kg/ha, it is constituted by 26 % roots, 35 % stems and 39 % leaves (NEDFI, 2007). Such biomass would require about 105 kg N, 23 kg P and 180 kg K from both soil and fertilizer. Therefore, the actual rate of application will vary according to soil type and production environment, and needs to be optimized for each specific situation (NEDFI, 2007).

2.3.5.2 Pruning

Pruning is an agronomic practice aiming at stimulating regrowth, which occurs in proportion to pruning severity.

Generally, the reason of pruning is to control the plant growth, to manipulate branching, control flower and fruits production in different season or year round. Many researchers have underlined the outcome of pruning on flowering and its effects on the subsequent fruit growth, quantity and quality (Calatayud et al., 2007). In non-pruned plants, the less growth of shoots may be caused a short-term reduction of cell activity of older shoots, causing a decline in yield production (Zieslin and Mor, 1981).

The level of plant recovery relies on several factors, particularly pruning-position, pruning-height, and the timing of plant phase (time in plant's life cycle when pruning happen) (Li et al., 2009; Zieslin and Mor, 1981). Pruning can act by modifying the light distribution within its canopy and change their photosynthetic capacity and quantum yield of leaves (Stitt et al., 1990; Hossain et al., 2007).

Ravichandran (2004) and Yilmaz et al. (2004) have shown that pruning leads to enhanced branching and hence it rejuvenates the tea plants resulting in a greater number of tender leaves for healthier and better quality tea plants and Mohammed Saifuddin, et al. (2010) observed significant higher values of quantum yield in all kinds of pruning leaves whereas, the photosystem of leaves, measured by quantum yield referred lower values in the non-pruning leaves.

As by research from Hossain and Fusao (2008), flower buds, sugar content and N, P, and K content in leaf were higher in pruned trees than unpruned peach trees.

2.3.5.3 Effect of mineral and wounding stresses in metabolite accumulation in plants

Plants, when exposed to unfavourable environments, such as high and low temperature, drought, alkalinity, salinity, UV stress, oxygen deficiency, air pollution, or pathogen infection, experience some degree of stress and express differentially the plants' genetic potential (Seigler, 1998). See different abiotic stress signals in Table 2.4. Plants may adapt to unfavourable conditions through genetically determined stress resistance (Drew 1998).

The secondary metabolites contribute to plant defence against herbivores and pathogens and often confer protection against environmental stresses (Seigler, 1998; Jochum et al., 2007; Tanveer et al., 2012). Induction of abiotic stresses can be used at pre-harvest to enhance the quality and yield of products (Kalt et al., 2001). In fact, elicitation has been widely used to increase the production or to induce *de novo* synthesis of secondary metabolites, as proved for instances in *in vitro* plant cell cultures (Dicosmo and Misawa, 1985).

Table 2.4 Different abiotic stress signals creating stress in plants (adapted from Mahajan and Tuteja 2005)

Abiotic stress signals

TEMPERATURE		SALINITY (SALT)	WATER	RADIATION	CHEMICAL STRESS	MECHANICAL STRESS
HEAT	COLD		Drought,	Light, UV,	Mineral salts	Wind
	Chilling		Flooding	Ionization	Gaseous toxins	Soil Movement
	Frost			radiation	Pollutants,	Submergence
					Heavy metals,	
					Pesticides and	
					Aerosols	

Based on the same assumption of affectation the levels of secondary metabolites, induction of abiotic stresses is also used in postharvest activities as traditional tools to extend the shelf life of products, focusing mainly on colour, texture, and flavour quality changes (Kader, 1992). Among the stresses used are wounding for fresh-cut (Saltveit, 1997), altered O_2 and CO_2 levels in controlled and modified atmospheres, or C_2H_4 gassing for ripening and degreening (Kays, 1991). For example, anthocyanin accumulation due to abiotic stress induced at ripening has been reported for thornless blackberries (Sapers et al., 1986), apples (Curry, 1997, Faragher and Brohier, 1984; Arakawa 1991), and strawberries (Given et al., 1988).

2.3.6 Biochemical and nutritional aspects of Stevia

Carbohydrates

The benefits associated to Stevia leaf are mainly due to their nutritional composition (Table 2.5), which disclose a good source of carbohydrates, protein and crude fibre that promotes wellness and reduces the risk of a number of important diseases (Braz de Oliveira et al., 2011).

In *S. rebaudiana* roots and leaves, inulin-type fructooligosaccharides, a naturally occurring plant oligofructose with important functional properties related to prebiotics, dietary fibre, role lipid metabolism and diabetes control, have been isolated by Braz de Oliveira et al. (2011).

Table 2.5 Proximate analysis reported for dried Stevia leaves (g per 100g dry weight basis) – (adapted from R. Lemus-Mondaca et al., 2012)

Component	References						
	Mishra et al. (2010)	Goyal et al. (2010)	Serio (2004)	Savita et al. (2004)	Abou-Arab et al. (2010)	Tadhani and Subhash (2006)	Kaushik et al. (2010)
Moisture	7	4.65	n.d.	7	5.37	n.d.	7.7
Protein	10	11.2	11.2	9.8	11.40	20.4	12
Fat	3	1.9	5.6	2.5	3.73	4.34	2.7
Ash	11	6.3	n.d.	10.5	7.41	13.1	8.4
Carbohydrate	52	n.d.	53	52	61.90	35.2	n.d.
Crude fibre	18	15.2	15	18.5	15.50	n.d.	n.d.

n.d. – not determined

Proteins

The protein quality of a food is determined by its total content and the composition and proportion in amino acids present (Latham, 2002). Mohammad, Sher, Habib, and Iqbal (2007) identified nine amino acids in Stevia leaves, namely glutamic acid, aspartic acid, lysine, serine, isoleucine, alanine, proline, tyrosine and methionine. Abou-Arab et al. (2010) found additionally the presence of other amino acids in the Stevia leaves as shown in Table 2.6. Altogether seventeen amino acids were determined and classified as essential and non-essential amino acids (Table 2.6).

Table 2.6 Amino acid composition of Stevia rebaudiana leaves (Abou-Arab et al., 2010)

Essential amino acid g / 100g d.m.		Non-essential amino acid g / 100g d.m.	
Arginine[a]	0.45	**Aspartate**	0.37
Lysine	0.70	**Serine**	0.46
Histidine	1.13	**Glutamic**	0.43
Phenyl alanine	0.77	**Proline**	0.17
Leucine	0.98	**Glycine**	0.25
Methionine	1.45	**Alanine**	0.56
Valine	0.64	**Cycteine[b]**	0.40
Threonine	1.13	**Tyrosine[b]**	1.08
Isoleucine	0.42		
Total	7.62	**Total**	3.72

[a] Not considered as indispensable amino acid in Technical Report FAO/WHO/UNU (WHO, 2007).

[b] Considered indispensable under specific situations

According to the report of a joint FAO/WHO/UNU Expert Consultation (WHO, 2007), the indispensable amino acids are leucine, isoleucine, valine, lysine, threonine, tryptophan, methionine, phenylalanine and histidine. According to this knowledge, Stevia leaves contained almost all the indispensable amino acids, including tyrosine and cysteine and only tryptophan is not present. Their content can match the protein requirements recommended by the World Health Organization (WHO, 2007). As a result, after extraction of Stevioside from the leaves, the residue could be a valuable source of indispensable amino acids for health products.

Minerals

Manish and Rema (2006) showed in mineral analysis that the high content of K, Ca, Mg, P, Na and S, which are nutritionally important, were found in Stevia leaves. The high concentration of these minerals would be very beneficial to human health (Choudhary & Bandyopadhyay, 1999). The mean concentration of macro and micro elements that have been quantified in dried Stevia leaves are shown in Table 2.7.

Table 2.7 Mineral content (mg /100 g) of dried Stevia leaves

Minerals	References					
	Mishra et al. (2010)	Goyal et al. (2010)	Serio (2004)	Tadhani and Subhash (2006)	Kaushik et al. (2010)	Abou-Arab et al. (2010)
Calcium	464.4	544	600	1550	722	17.7
Phosphorous	11.4	318	318	350	ND	ND

Minerals	References					
	Mishra et al. (2010)	Goyal et al. (2010)	Serio (2004)	Tadhani and Subhash (2006)	Kaushik et al. (2010)	Abou-Arab et al. (2010)
Sodium	190	89.2	ND	160	32.7	14.93
Potassium	1800	1780	1800	2510	839	21.15
Iron	55.3	3.9	3.9	36.3	31.1	5.89
Magnesium	349	349	500	ND	ND	3.26
Zinc	1.5	1.5	ND	6.39	ND	1.26

n.d. – not determined

The high content of potassium determined in all studies is remarkable. The exception is the amount of potassium found by Abou-Arab et al. (2010) that seems to be very low compared to that of the other studies and may be explained by different growth conditions, as suggested by Rahmesh, Singh, and Megeji (2006). Zinc and iron are found in foods of plant and animal origin and are also present in Stevia leaves. According to Wu et al. (2005), zinc is a mineral that acts as a non-enzymatic antioxidant, so that its consumption would help in preventing oxidative damage of the cell. The main biological function of iron is the transport of oxygen to the body and consequently a lack of this mineral in the diet leads to anaemia. The high amount of iron in Stevia leaves could again be helpful in contributing to the maintenance of a normal haemoglobin level in the body. Furthermore, Stevia leaves could also be used to prepare various sweet preparations to combat iron deficiency in anaemia which is a major nutritional disorder in developing countries (Abou-Arab et al., 2010).Manganese (Mn) is also reported to be detected by Abou-Arab (2010) and zinc and manganese are considered as antioxidant micro nutrients and their presence could therefore boost the immune system (Jimoh and Oladiji, 2005) and in prevention of free radical mediated diseases. Regarding copper (Cu), also reported by Abou-Arab (2010), its trace content can be important to healthy diets due to its role in growth and development of human organism.

Lipids

In the leaf oil of Stevia, Tadhani and Subhash (2006a) identified six fatty acids. Among the identified fatty acids, palmitic acid content was found to be present at highest levels, with measured mounts of 27.51 g per 100g d.m., whereas stearic acid content was the less quantified, with 27.51 g per 100g d.m. Stevia leaf oil proved to be a rich source of linoleic acid, which may contribute to maintain an ideal fatty acid ratio in the human diet.

Vitamins

Kim, Yang, Lee, and Kang (2011) quantified the amounts of water-soluble vitamins in Stevia leaf (Table 2.8), and determined that the contents of folic acid, vitamin C and vitamin B2 in the leaf extracts were significantly higher than those of callus. In the leaf extract, folic acid was found to be the major compound, followed by vitamin C. In the callus extract, vitamin C was the major compound, followed by vitamin B.

Table 2.8 Amounts of water soluble vitamins detected in S. rebaudiana leaf (mg / 100 g dry base of extract) (Kim et al., 2011).

Vitamin	Leaf
Vitamin C	14.98 ± 0.07
Vitamin B2	0.43 ± 0.02
Vitamin B6	0.00 ± 0.00

Vitamin	Leaf
Folic acid	52.18 ± 0.21
Niacin	0.00 ± 0.00
Thiamin	0.00 ± 0.00

2.3.7 Diterpene (Stevia) Glycosides

The glycosides found mainly in the leaves of the plant, make up to 15% of the content, depending on variety (Giraldo, Marín, & Habeych, 2005). The amount of sweet glycosides in the leaves of Stevia depends on growing conditions (Pól et al., 2007), as well as on the adoption of specific agronomical techniques (Geuns, 2003; Nepovim, Drahosova, Valicek, & Vanek, 1998).

Stevioside traditionally makes up the majority of the sweetener (60–70% of the total glycosides content) and is assessed as being 110–270 times sweeter than sugar. It is also responsible for the bitter aftertaste, sometimes reported as a “licorice” taste. As well as sweetness, Stevioside may have a lingering effect or certain degree of pungency, which is not appreciated by the majority of people, and which reduces its acceptability. Among the components of stevia, Rebaudioside A is of particular interest because it has the most desirable flavour profile (DuBois, 2000). Rebaudioside A is usually present as 30–40% of total sweetener and has the sweetest taste, assessed as 180–400 times sweeter than sugar with no bitter aftertaste (licorice taste or lingering effect).

The content of Rebaudioside B is negligible in comparison to that of Stevioside (Pól et al., 2007). Conversely, purified extracts obtained from Stevia leaves and offered on the market contain mainly Stevioside (>80%) or Rebaudioside A (>90%) (Gardana, Scaglianti, & Simonetti, 2010).

Stevioside has the chemical formula of a diterpene glycoside ($C_{38}H_{60}O_{18}$) and as an active component in Stevia leaves is responsible for the edulcorant properties. Its use has been approved in Brazil, Argentina and Paraguay as well as in China, Korea

and Japan. These molecules are highly stable in aqueous solutions within a broad range of pH and temperature (Abou-Arab et al., 2010; Virendra & Kalpagam, 2008).

Under strong acidic conditions (pH 1.0) forced decomposition of Stevioside was observed which resulted in total decomposition after incubation at a temperature of 80 ºC for 2 h (Abou-Arab, Abou-Arab, & Abu-Salem, 2010).

Similar results were reported by Buckenhuskers and Omran (1997) who showed that the Stevioside possess an excellent heat stability is up to 100 ºC for 1 h at pH range 3–9, but rapid decomposition occurs at pH level greater than 9 under these conditions. All diterpene glycosides isolated from *S. rebaudiana* leaves have the same steviol backbone (Fig. 2.5) and differ mainly in the content of carbohydrate residues (R1 and R2), mono-, di-, and trisaccharides containing glucose and/or rhamnose at positions C13 and C19 (Kochikyan, Markosyan, Abelyan, Balayan, & Abelyan, 2006).

The sweetness of Rebaudiosides increases with increasing amount of sugar units bonded to the steviol aglycone. However, their content in the plant material decreases in parallel (Kovylyaevaet al., 2007). Conversely, the edulcorant properties of those glycosides, differ from one another. Rebaudioside A, for example, which has an extra glucose unit relative to Stevioside, is superior in terms of both sweetness and quality of taste. Pure Stevioside usually produces a significant bitter aftertaste (de Oliveira, Packer, Chimelli, & de Jesus, 2007).

Therefore, there is a need to develop a stevia cultivar that is enriched in Rebaudioside A and has a high steviol glycoside content that can be produced using a relatively low-cost method based on transplants produced from seed.

Brandle (2001) developed the synthetic cultivar AC Black Bird breeding procedure which is characterized by exhibiting a high level of total glycosides (at least 14%), and a high ratio of Rebaudioside A to Stevioside (at least 9.1:1).

As the structure of glycoside molecules plays a key role in determining sweetness or bitterness in Stevia (Hellfritsch et al. 2012), the ratio of Rebaudioside A to Stevioside can be accepted measure of sweetness quality; the more Rebaudioside A the better. Also, the glycoside with higher sweetener potential is the Rebaudioside A, and Stevioside with less sweetness and contributes as a bitterer of taste.

2.3.8 Other phytochemical constituents

The most important bioactive constituents of plants are alkaloids, tannins and polyphenols (Edeoga, Okwu, & Mbaebie, 2005). *S. rebaudiana* is rich in terpenes and flavonoids. The phytochemicals present in *S. rebaudiana* are austroinullin, b-carotene, dulcoside, nilacin, rebaudi oxides, riboflavin, steviol, Stevioside and thiamine (Jayaraman, Manoharan,&Illanchezian, 2008).

2.3.9 Antioxidant activity

Since the antioxidant potential of plant species, regarded as safe and bioactive, in recent years, considerable attention has been directed towards the identification of plants with significant antioxidant potential (Shukla et al., 2011).

Phansawan and Poungbangpho (2007) studied the antioxidant capacities of *S. rebaudiana* and four other medicinal plants; *Pueraria mirifica*, *Bertoni*, *Curcuma longa Linn.*, *Andrographis paniculata* (*Burm.f.*) *Nees*. and *Cassia alata Linn*. Stevia leaf extract exhibits a high degree of antioxidant activity and has been reported to inhibit hydroperoxide formation in sardine oil with a potency greater than that of either DL-a-tocopherol or green tea extract. A study assessing the in vitro potential of ethanolic leaf extract of *S. rebaudiana* indicates that it has a significant potential for use as a natural antioxidant (Shukla, Mehta, Bajpai, & Shukla, 2009).

Ascorbic acid and other phenolic compounds are good for health and prevention of disease (Ames et al., 1993).

2.3.10 Antimicrobial activity

In some studies the antimicrobial activity of various extracts of *S. rebaudiana* have been investigated and its effect on some selected microorganisms such as *Salmonella typhi*, *Aeromonas hydrophila*, *Vibrio cholerae*, *Bacillus subtilis, Staphylococcus aureus* and others have been examined (Debnath, 2008; Ghosh, Subudhi, & Nayak, 2008; Jayaraman et al., 2008; Seema, 2010; Tadhani & Subhash, 2006b). The biological activity for Stevia compounds has been also studied by Tomita et al. (1997) which studied the bactericidal activity of a fermented hot-water extract towards enterhaemorrhagic *Escherichia coli* and other food-borne pathogenic bacteria. Other microorganisms like *Salmonella typhimurium*, *B. subtilis*, and *S. aureus* have also been found to be inhibited by the fermented leaf extract (Debnath, 2008; Ghosh et al., 2008).

Muanda et al. (2011) reported that combination between essential oils and extract of *S. rebaudiana* possesses high antioxidants, anti-inflammatory and antimicrobial properties against *S. aureus, B. subtilis, E. coli, P. aeruginosa, A. niger and C. albicans.*

2.3.11 Health benefits

In addition to the positive effects of replacing sugar, *S. rebaudiana* leaves contain non-toxic (Gardana et al., 2010), non-mutagenic, and non-carcinogenic and non-caloric sweeteners (steviol glycosides) whose consumption could exert beneficial effects on human health (Gardana et al., 2010), and can act as antigingivitis (Blauth de

Slavutzky, 2010). Regular consumption of these compounds decreases the content of sugar, radionuclides, and cholesterol in the blood (Atteh et al., 2008), improves cell regeneration and blood coagulation, suppresses neoplastic growth and strengthens blood vessels (Barriocanal et al., 2008; Jeppesen et al., 2003; Maki et al., 2008; Wingard et al., 1980).

In addition, parts of the Stevia plant and Stevioside have been used in the treatment of cancer and as substitutes for sucrose in the treatment of diabetes (Chen et al., 2006; Jeppesen et al., 2000; Pól, Hohnová, et al., 2007), obesity and hypertension (Chan et al., 2000; Goyal et al., 2010; Hsieh et al., 2003; Lee et al., 2001; Pól, Hohnová, et al., 2007). Nonetheless, no scientific studies clearly associate Stevia consumption with weight loss.

No allergic reaction has been observed when it is used as a sweetener (Abou-Arab et al., 2010). A number of studies have demonstrated that oral intake of Stevioside has no effect on fertility, neither in mice (Akashi & Yokoyama, 1975), nor in rats (Mori, Sakanoue, Takcuchi, Shimpo, & Tanabe, 1981; Xili et al., 1992), nor in hamsters (Yodyingyuad & Bunyawong, 1991). In fact, its commercialisation, in France for example, as a food or a food ingredient has been prohibited based mainly on economic arguments and not on proven adverse health effects (Serio, 2010). However, it is thought that stevia could provoke allergic reactions in people sensitive to plants of the *Asteraceae* family and it is also recommended that pregnant women should avoid consuming Stevia (Serio, 2010).

2.3.12 Stevia Glycosides approval process in EU legislation

On request from the European Commission, Question No EFSA-Q-2007-071; EFSA-Q-2008-387; EFSA-Q-2008-401, adopted on 10 March 2010, European Food Standards Agency (EFSA) Panel on Food Additives and Nutrient Sources added to Food (ANS), gave Scientific Opinion on the safety of steviol glycosides for the proposed uses as a food additive:

“The results of toxicological testing indicated that steviol glycosides are not genotoxic, carcinogenic, nor associated with any reproductive/developmental toxicity. The NOAEL in the 2-year carcinogenicity study in the rat was 2.5% Stevioside (95.6% purity) equal to 967 mg Stevioside/kg bw/day (corresponding to approximately 388 mg steviol equivalents/kg bw/day).”

However, according to EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), “Different studies assessed the bulk stability of dry steviol glycosides under various storage conditions and in food matrices over a range of pH values, processing conditions, at both room temperature and elevated temperatures”.

The photostability of the preparation was examined under dry and aqueous conditions. The Panel notes that, in these experiments, the extent of degradation of the tested steviol glycoside (Rebaudioside A) verified ranged from low levels up to 63% under different storage (pH and temperature) and food production conditions. The Panel notes that, in the presence of high temperatures (e.g. heating, baking), substantial degradation of steviol glycosides might take place (EFSA Panel, 2010).

Consequently, after the publication of this opinion, the European Commission approved a use of steviol glycosides in EU market through COMMISSION REGULATION (EU) No 1131/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council with regard to steviol glycosides (Table 2.9).

Annex II to Regulation (EC) No 1333/2008 is amended as follows: (1) in Part B2 the following entry for E 960 is inserted after the entry for E 959.

(2) in Part E the following entries for E 960 are inserted in numerical order in the food categories referred to: PART E: Authorised food Additives and Conditions of use in food categories.

Table 2.9 Steviol Glycosides maximum rate and restrictions for use as additive in fruit and vegetable preparations excluding compote, as published in REG. (EU) nº 1131/2011, Annex //

Category number	E - Number	Name	Maximum level (mg/kg or mg/l as appropriate)	Footnote	Restrictions/ exceptions
04.2.4.1	Fruit and vegetable preparations excluding compote				
	E 960	Steviol Glycosides	200	(60)	only energy-reduced
	(60): expressed as steviol equivalents				

Only breath-freshening micro-sweets, with no added sugar are allowed to have a maximum 2000 mg of E 960 per 1 kg of micro-sweets.

2.3.13 Industrial applications

Stevia sweeteners, as extracts from the leaves of this herb, are commercially available in Japan, Korea, China, South-East Asia and South America, where they have been used for some decades to sweeten a variety of foods (Koyama et al., 2003). Leading countries substituting sucrose with Stevia are shown in Fig. 2.7.

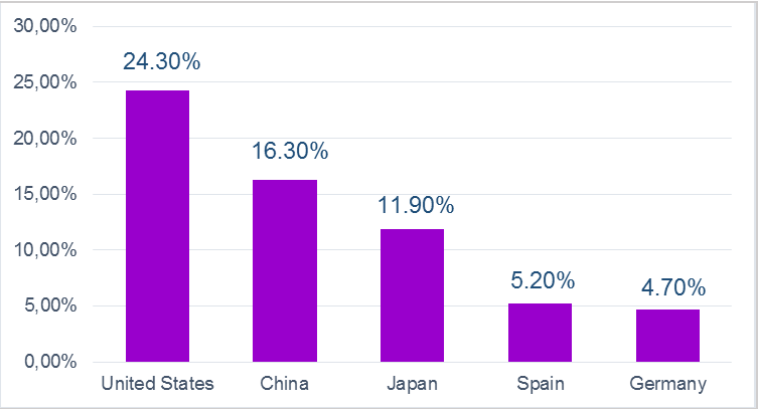


Figure 2.6 Rank of global leaders in using stevia as a sugar replacer for new products (Source: Innova Market Insights, June 2015)

In these countries Stevioside is being used to sweeten foodstuffs and beverages. In the USA powdered Stevia leaves and their extracts are used only as a dietary supplement and a skin care product, but not as a sweetener.

The steviol glycosides are currently in use as a sweetener in a number of industrial foods, with evident increase (Fig. 2.8), such as soft drinks or fruit drinks (Goyal et al., 2010; Jayaraman et al., 2008; Tadhani & Subhash, 2006a; Wallin, 2007), desserts, cold confectionery, sauces, delicacies, sweet corn, breads, biscuits, table-top sweetener. They replace sucrose, for exemple in ready-to-eat cereals (Wallin, 2007), pickles (Koyama et al., 2003), yoghurt (Amzad-Hossain et al., 2010; Tadhani & Subhash, 2006a; Wallin, 2007), candies (Goyal et al., 2010; Koyama et al., 2003), soju, soy sauce (Amzad-Hossain et al., 2010; Tadhani & Subhash, 2006a) and seafoods (Goyal et al., 2010; Koyama et al., 2003).

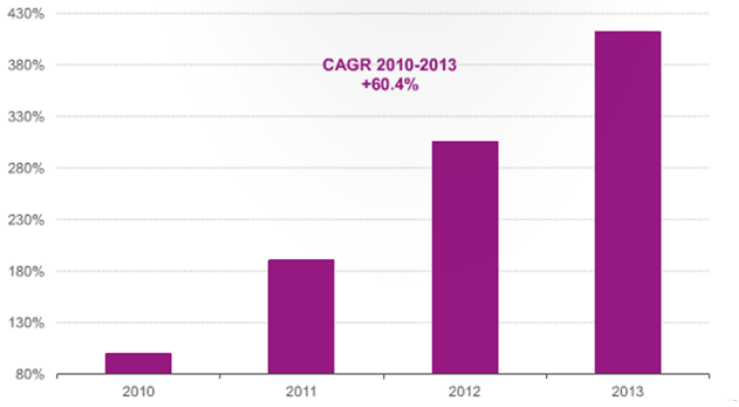
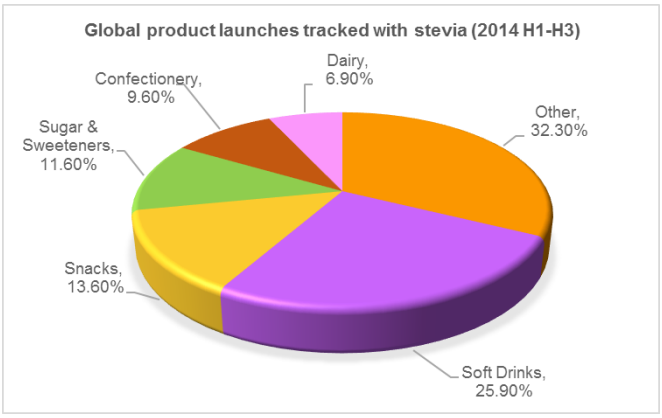


Figure 2.7 Applications of Stevia increased in recent years, with new products launches index 60.4% in 2010-2013 (index: 2010=100)

Stevia was the fastest growing non-nutritive sweetener in terms of number of global food and beverage launches from 2010 to 2014. The soft drinks category represents a quarter of total launches with stevia (Fig. 2.9, Innova Market Insights, June 2015).



Wholesome Sweeteners Organic Stevia (USA, Sep 2014)

Lipton Green Lemon Icetea (Hungary, Sept. 2014)

Starbucks Via Refreshers Strawberry Lemonade (USA, Sep 2014)

Figure 2.8 Soft drinks as a leader in using stevia as sweetener and latest lunches in EU and USA market

In one recent research work (Zahn et al. 2013) the combined use of Rebaudioside A and fibres for partial sucrose replacement in muffins was conducted. To evaluate whether steviol glycosides may be used for partial replacement of sucrose in bakery products, muffins were produced where 30% sucrose of the formulation was exchanged against an iso-sweet amount of Rebaudioside A in combination with several fibres. Baked products were subjected to chemical, colour and texture analysis, and sensory characteristics were assessed by flash profiling. Multivariate analysis of instrumental and sensory data indicates that a combination of inulin or polydextrose with Rebaudioside A results in products with characteristics close to those of a reference. The incorporation of these replacers reduced energy by 6 or 5 kJ/100 kJ, and increases fibre content from 1.30 g/100 g to 4.60 or 7.10 g/100 g, respectively. The use of wheat bran or apple fibre as bulk replacer for sucrose gives products which mainly deviate in crumb colour and are characterised by a wholemeal off-taste, whereas increased crumbliness and reduced elasticity is the consequence of partial sucrose replacement by oat, pea or wheat fibre, cellulose or maltodextrin.

The glycosides from Stevia, namely Stevioside, Rebaudioside A, B and C, and some others, can be easily extracted with water, purified, concentrated and dried (Carakostas, et al. 2008).

The stevia leaves are extracted with hot water or alcohols. In some cases, the leaves are pre-treated with non-polar solvents such as chloroform or hexane to remove the essential oils, lipids, chlorophyll and other non-polar substances. The extract can be clarified by precipitation with salt or alkaline solutions (Midmore and Rank, 2006). The extract is concentrated and redissolved in methanol for crystallization of the glycosides. The crystals are formed almost by pure Stevioside.

2.4 Sugar cane, sugar beet and further sugar plants

Sugar is a carbohydrate found in every fruit and vegetable. All green plants assembly sugar as a reserve product though photosynthesis, but sugar cane (Fig. 2.10 (left), S*accharum officinarum)* and sugar beets (Fig. 2.10 (right), *Beta vulgaris*) have the highest natural accumulation rates, due to high photosynthetic capacity of these plants (Cuba/FAO Conference, 1999) and because biosynthesis and accumulation of sweet compounds is related to density of cells where the synthetisation occurs.

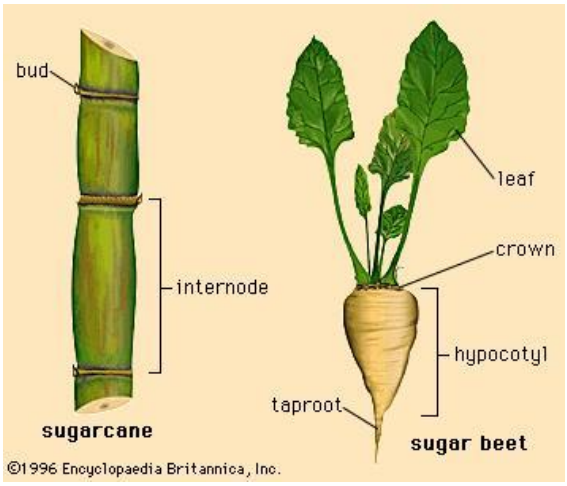


Figure 1.9 Plant structure of the sugar cane and sucrose accumulation in culm tissue (left), Grof and Campbell (2001) and sucrose intake in root discs of mature sugar beet root tissue (right), Wyse R. (1979)

When sugar cane is used worldwide as a sweetener, preservative and in the cosmetics industry**,** sugar is extracted from the sweet, juicy stems. This species is grown in Cuba, the Philippines, Colombia, and other prime areas (Clarke, 2014).

When sugarcane is harvested it is stripped of its leaves and sent to the sugar factory. At the factory the stems are crushed and shredded by rollers in a process called grinding. During grinding hot water is sprayed over the shredded material to extract the remaining sugar. The solid waste that is left after extraction of the sugar is known as pulp or sugarcane bagasse, which is dried and used as a fuel (Harris and Staples, 1998).

The raw juice is then heated and spun in a centrifuge in tubes whose walls are pierced with small holes through which the thick syrup is forced out. This syrup is called

molasses, and is in itself a very valuable product. The molasses, which ironically is a waste product of sugar refining, can be sold as syrup, to flavour rum and other foods, to feed animals, or even as an additive for ethyl alcohol. Molasses is even used in processed tobaccos (Harris and Staples, 1998).

Sugar beet is essentially grown in temperate regions. It is mainly grown at latitudes between 30 and 60ºN, as a summer crop in maritime, prairie, semi continental and some semi-arid and arid climates and as a winter and/or summer crop in Mediterranean and other semi-arid and arid conditions (Draycott, 1972).

The environmental conditions under which the plants grow influence characteristics that impact sugar content. For instance, sugar concentrations have been inversely correlated with root yield (Artschwager, 1930) and positively correlated with the density of cambial rings within the radius of the root (Pack, 1927; 1930). Similarly, inverse correlations between root yield and sugar concentration have been shown between varieties and for crops grown with differential amounts of nitrogen. Crops that are well supplied with nitrogen tend to produce larger yields of beet containing a lower concentration of sugar than crops grown with less nitrogen (Draycott & Christenson, 2003).

Sugar beet has a conical, white, fleshy root and a flat crown. Sugar is formed through photosynthesis in the rosette of leaves, the size of which differs according to the sugar beet variety and growing conditions. The root serves as a reservoir for the sugar, which can represent between 15% and 21% of the sugar beet's total weight (FAO, 2009).

The sugar content in sugar beet can vary from 12% to 20%. It is the sugar that gives value to the sugar beet crop but the by-products of the sugar beet, such as pulp and molasses, give an added value of up to 10%. The sugar extraction rate depends on the sugar content of the sugar beet at the moment of its arrival in the processing plant. With exceptions, European norms define the sugar beet as marketable if it contains 14% sugar or more (in Ukraine, for instance, the average sugar content is only 11.2%). The standard sugar beet should have a sugar content of 16%, which would yield 130 kg of sugar per 1 ton of standard sugar beet processed at a sugar plant (ideal efficiency is 82.5%) (FAO, 2009).

Sugar beet root is cut in strips in order to offer maximum surface area for extraction of raw sugar beet juice. Raw juice is purified in a series of liming and carbonation steps, often with filtration or thickening being conducted between the first and second carbonation, and after concentration and crystallization, sugar is separated from mother liquor in basket centrifuges, and it is dried in either rotary louvered driers or fluidized-bed dryer-coolers (Clarke, 2014).

One ton of sugar beet yields (*Le Sucre*, 2006-2007) 160 kg of sugar, 500 kg of wet pulp and 38 kg of molasses.

Corn syrup, a mixture of glucose and fructose produced from corn, is a relatively recent arrival as a sweetener; obtained from starch (which, like most starches, is a polymer composed of long interlinked chains of glucose molecules) and break it down into isolated glucose molecules using the enzymes amylase and maltase. Commercial amounts of corn syrup were available by the middle of the 20th century (Alexander**,** 1992). The most common form of high fructose corn syrup (HFCS) has 55% fructose and 45% glucose.

Corn syrup was so much cheaper than sucrose, leading to extensive use as a sucrose substitute for thickening foods and to help retain moisture. It wasn't much used solely as a sweetener because it isn't as sweet as sucrose.

Added sugars like HFCS and sucrose are both carbohydrate ingredients that contribute ≈4 kcal/g on a dry solids basis, and both as a caloric ingredient can lead to weight gain if products sweetened with it are consumed to excess (White, 2008).

2.5 DRYING

The control of food moisture is based on the product water removal, which promotes the reduction of water activity and the consequent reduction of microbiological deterioration rates, reducing the chemical changes, and the "collateral" advantage in substantive reduction costs of packaging, storage, transport and distribution.

The water content, which in fruits and vegetables ranges between 80% and 90% , is thereby reduced to 20-30% in fruits and vegetables to 8-12% (Niketic - Aleksic, 1994) to 5 % in mushrooms and 10-15 % in aromatic and medicinal plants .

There are other dehydration techniques (such as salting, smoking, lyophilisation, and other), which differ from dehydration stimulated by the heat, above all in amount of remaining water or the product temperature at which the food is dried.

In regions where the sun was sufficiently hot to permit air-drying, the advantages of this form of processing in extending the storage life of succulent fruit, in an acceptable form, must have been appreciated very early. It had the added advantage that the process also concentrated the material to the stage where its value as a source of energy was quite high, and in dried form, fruits such as dates, grapes and figs were an important element in the food intake and ranked as local staple crops.

Many agricultural products (especially medicinal herbs) have a short supply period and must be preserved and stored for later use. As drying is one of the most common ways for preserving the material, applying the appropriate drying methods,

product quality can be increased and losses reduced (Barbosa-Canovas and Vega-Mercado, 1996).

Water is a significant component of biological materials. The physical and chemical properties of aromatic and medicinal plants are determined by their moisture content. The first step in many postharvest operations is removal of water that is, drying. Drying is basically defined as the decreasing of plant moisture content, aimed at preventing enzymatic and microbial activity, and consequently preserving the product for extend shelf life. For this reason, adequate dryers are needed, using temperature, velocity and humidity values for drying air that provides a rapid reduction in the moisture content without affecting the quality of the active ingredients of medicinal plants. Drying process may also contribute to a regular supply and facilitate the marketing of plants, because drying results in reduction of the weight and volume of the plant with positive consequences for transport and storage (Calixto, 2000).

For various medicinal plant species a maximum value of final moisture content is prescribed in different pharmacopoeias all over the world (Farias, 2003). Most of examples are showing a range of final moisture content between 8 and 12% considerate adequate to preserve the product after dried. There is no obvious correlation between final moisture content and the used part of the plant such as root, herb, flower or seed. Drying of aromatic and medicinal plants must meet the following requirements: (1) Moisture content has to be brought down to be at an equilibrium level that is defined for certain relative air humidity and temperature. This is defined as storage condition by standards; (2) minimum quality reduction in terms of active ingredients, color, flavour and aroma; and (3) microbial count must be below the prescribed limits. No chemical additives may be used (Oztekin and Martinov, 2007).

2.5.1 Drying Methods

The methods used for drying fruits and vegetables are natural sun-drying, drying with heated air (convection), pulverize by hot air (spray-drying), by contact with a hot surface, sublimation and drying foam.

Drying with heated air (Lidon and Silvestre, 2008) is carried out at normal pressure, result in elimination or fixation (by solutes) of moisture from moist material and aims to stabilize food. This system requires temperatures (that may oscillate depending on food and on drying process), and relative humidity which vary depending on the type of food and dehydrated state (consequently requiring ventilation with control of air velocity). In this process, the moisture removal from food material in the form of vapour is evacuated by the air stream. The air humidity is an important factor because it affects the air's ability to carry vapour, determining the level of water activity

in the final product; this factor also determines the velocity at which the moist air is evacuating moist from material.

2.5.2 Solar drying

Economic consideration, environmental concerns and product quality aspects are the three main goals of drying process research in the food industry (Chou and Chua, 2001)

Solar drying of agricultural products in enclosed structures by forced convection is an attractive way of reducing post-harvest losses and low quality of dried products associated with traditional open sun-drying methods (Jain and Tiwari, 2003). In many rural locations in most developing countries, grid-connected electricity and supplies of other non-renewable sources of energy are either unavailable, unreliable or, too expensive. In such conditions, solar dryers appear increasingly to be attractive as commercial propositions (Mekhilefa et al. 2011; Xingxing et al. 2012).In order to assure preservation for long term storage of fruit and vegetables, it is necessary to process them by drying (Jairaj et al., 2009).

According to working principle Solar energy dryers can broadly be classified into direct, indirect and hybrid solar dryers (Ching et al., 2012). The working principle of these dryers mainly depends upon the method of solar energy collection and its conversion to useful thermal energy for drying.

Indirect Solar Drying (ISD) These differ from direct dryers with respect to heat transfer and vapor removal. The crops in these indirect solar dryers are located in trays or shelves inside an opaque drying cabinet and a separate unit termed as solar collector is used for heating of the entering air into the cabinet. The heated air is allowed to flow through/over the wet crop that provides the heat for moisture evaporation by convective heat transfer between the hot air and the wet crop. Drying takes place due to the difference in moisture concentration between the drying air and the air in the vicinity of crop surface (Sharma et al., 2009).

In terms of construction type and air circulation regime, several designs of the mixed-mode natural-circulation solar-energy dryer have been reviewed by *Ekechukwu and Norton (1999) (Fig. 2.11).*

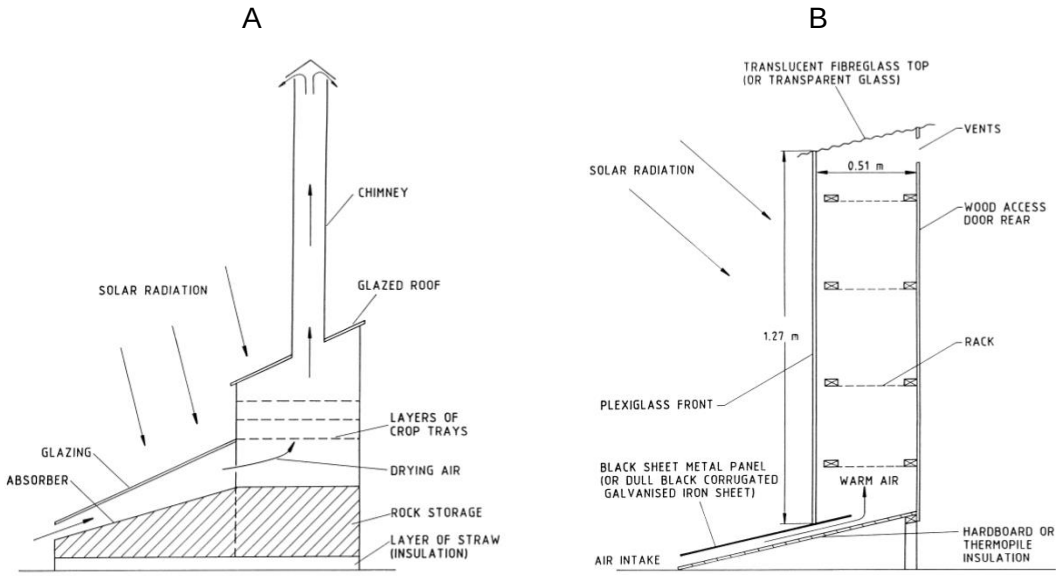


Figure 2.10 Mixed-mode natural-circulation solar dryers (Ekechukwu and Norton, 1999) A) Solar-energy dryer with thermal storage, B) A multi-stacked solar-energy dryer

Sharma (1987), described the design details and performance of two types of low cost solar crop dryers, conventional cabinet dryer with direct heating mode and an integrated solar collector-cum-drying system based on the principle of natural convection. Results obtained with several vegetables viz. cauliflower, green peas and potato showed satisfactory overall efficiency and performance of both dryers. This cabinet type solar dryer model is capable of predicting the instantaneous temperature inside the dryer, the moisture content and drying rates (Sharma et al., 1990).

Banout et al. (2011) recently had studied the design and the performance of a double pass solar dryer for drying of red chilli and Montero et al. (2010) has studied the design and the performance for agro-industrial by-products.

According to *Visavale, 2009,* with the circulation fan that supplies fresh air, powered by 1.5 kW, during experiments the air velocity, temperature and relative humidity in the cabinet were in the range 0.9-1.0 m/s, 40-60°C and 50-65%, respectively*, (Fig.2.12)*

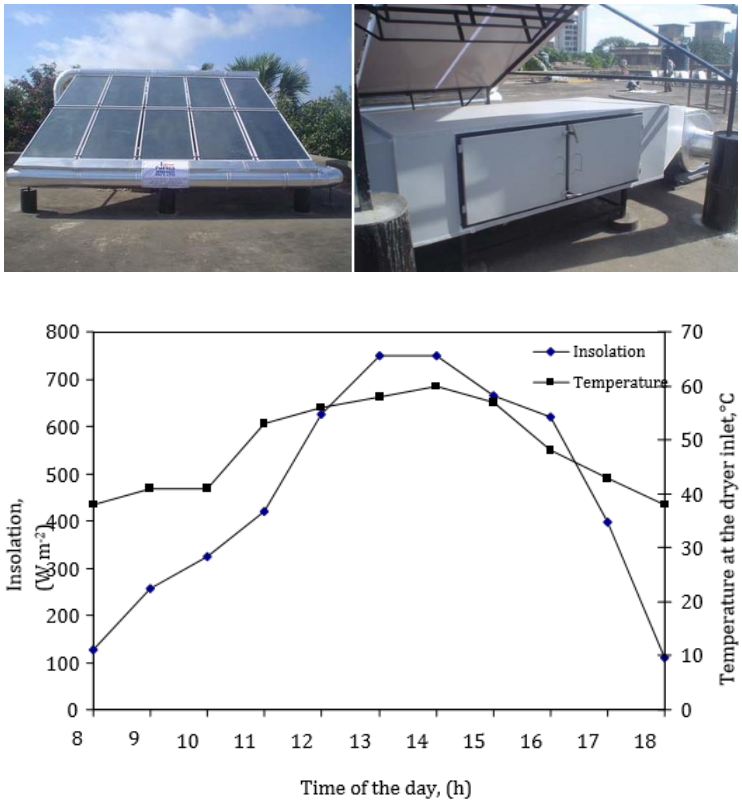


Figure 2.11 Temperature variation during day of drying in function of solar radiation (23rd March, 2008) (Visavale, 2009)

During drying, the weakest bound water is removed first; removing moisture by breaking stronger bonds requires energy. Removal of free water does not change the character of the material in either the dried or rehydrated states.

Significantly higher energy and special procedures are required to remove bound water, i.e., to decompose the higher bonding energies (Ginzburg, 1968; Ginzburg 1976).

During the process of dehydration, there are changes in nutritional quality (Sablani, 2006).

The green colour of most vegetables is due to chlorophyll, which is the most widely distributed plant pigment. The most common change that occurs in green vegetables during thermal processing and storage is the conversion of chlorophyll to pheophytins, causing a colour change from bright green to olive-brown, which is undesirable to the consumer (Schwartz and Elbe, 1983; An-Erl King et al., 2001).

Studies on beta-carotene retention in dried vegetables have shown that maximum retention of beta-carotene is obtained by drying vegetables in a solar drier, compared with open-air sun drying because beta-carotene is highly sensitive to direct sunlight (Mulokozi and Svanberg, 2003).

Spinach, cowpeas, sweet potato and cassava leaves were dried in enclosed solar dryer and maximum retention of ascorbic acid and total carotene was observed in

enclosed solar dryer with shade as compared to sun drying. Sun drying showed minimum retention of this nutrients as reported by Maleda and Salunkhe (1981).

The colour of solar dried (dehumidification assisted) Pegaga leaf did not become darker due to the lower air temperature used (T< 56°C) and the lower RH used (RH < 36%). Pegaga leaf dried at 65°C using warm air became darker (Yahya et al. 2004)

According to Bahloul et al. (2009) the values of L* parameter of the solar dried (Indirect forced convection) olive leaves increase compared to the fresh one. The luminance of the leaves was improved by solar drying but the greenness of the leaves reduced. Dried olive leaves dried at 60°C (at 3.3 m3/min) showed total phenols close to the fresh leaves. The olive leaves dried at 40°C (1.62 m^3/min) exhibited the lowest DPPH radical scavenging activities.

Continued use of the dryer rather than seasonal use will decrease the drying cost and payback (Arinze et al., 1996). Economic analysis on a solar dryer should also incorporate the cost benefits due to improved quality, higher yields, less floor area and quicker drying (Sreekumar, 2010).

In general, solar drying shows several benefits as follow (Esper and Mühlbauer, 1998):

- Significant improvement in product quality (colour, texture and taste)
- No contamination by insects, microorganism and mycotoxin
- Reduction in drying time up to 50%
- Reduction of drying and storage losses
- Considerable increase in shelf life of dried products.

Large-scale commercial active solar dryers employ mostly air-heating solar collectors as supplements to electricity or fossil-fuel fired dehydrators to reduce the overall conventional energy consumption. Practically-realised designs of these ``hybrid systems'' have been reported widely (Bowrey et al., 1980; Bassey, 1985; Huang and Toksoy, 1983; Chakraverty and Das, 1986).

Combining solar energy and heat pump technology is a very attractive concept. Solar assisted heat pump drying systems have been studied and applied since the last decades in order to increase the quality of products where low temperature and well-controlled drying conditions are needed (Daghigh et al. 2010).
For heat sensitive materials improved quality control can be achieved due to low drying temperatures and independency of the outdoor air. In these systems reduced energy consumption is achieved due to the high coefficient of performance of the solar assisted heat pump dryer and the high thermal efficiency of the dryer when properly designed (Daghigh et al., 2010).

A solar assisted heat pump system is composed of a vapour compression cycle unit which is combined with a solar collector and this combined system possesses a high coefficient of performance (Chaturvedi et al., 1984; Morrison, 1994; Kuang, 2003).

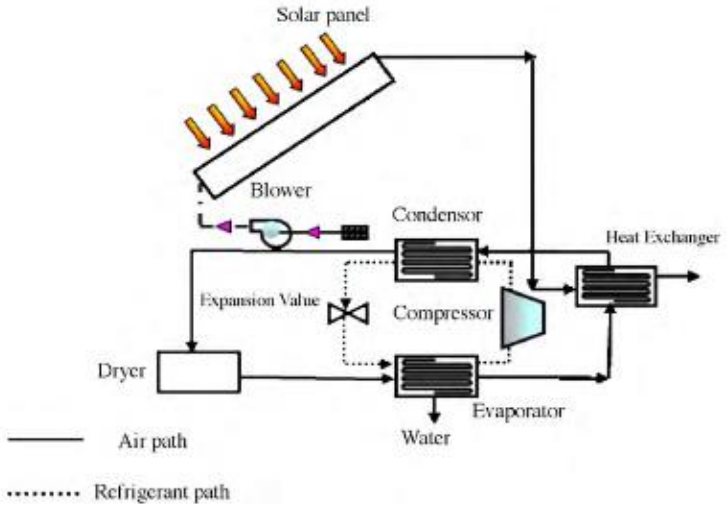


Figure 2.12 Solar assisted heat pump Dryer scheme (Daghigh et al. 2010)

Therefore, further attempts should be conducted in this field and the integration of combined renewable energy technologies into the heat pump drying systems might be much more developed (Daghigh et al. 2010).

3. MATERIALS AND METHODS

3.1 PILOT SOLAR DRYER DESIGN AND DRYING MONITORING

3.1.1 Pilot Solar Dryer Design

A pilot semi-active Solar Dryer, denominated Model “DNB” was designed, developed and constructed under the scope of this thesis and to be used in this work.

Main parts of a dryer are: solar absorber panel with heating “tunnel” and drying chamber (see Fig. 3.1) and in the pilot constructed both parts of the dryer were designed to be exposed to solar irradiation, thus generating a double effect of greenhouse and chimney.

The principal objective of the solar absorber panel is absorbing and irradiating heat in solar absorber area (heating “tunnel”) and heating air provoking natural lifting of hot air (chimney effect) throughout the drying chamber, generating an heated air flow rate.

The difference between the temperatures on the entrance of the solar absorber panel, equals the ambient temperature in area, and out of chamber is producing the difference of pressure between that two spots. This factor leads to conducting the air throughout the chamber, by natural convection.

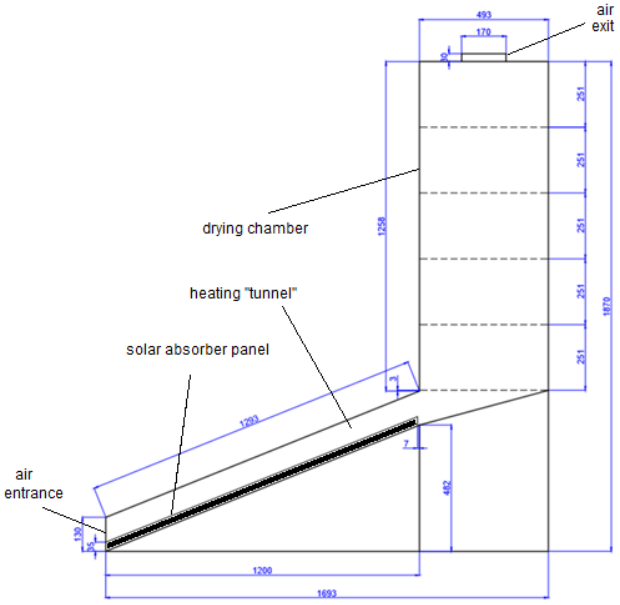


Figure 3.1 Schematic representation of the semi-Active Solar Dryer - Model "DNB"

Materials used in the construction, that took place at *SISELM, Sistemas Inteligentes Electromecânicos, Lda.*, included metallic structures molded and welded into final shapes to obtain the solar absorber panel box and the drying chamber with doors (Fig. 3.2). A solar absorber panel also included a styrofoam layer as isolation material, aluminum corrugated profile painted in black and temperate glass (heat at

600ºC). Principal criteria for choosing those specific materials were to be: non-toxic, non-corrosive, non-absorbent, easy to clean and maintain, fully complying with food safety requirements.

Figure 3.2 Illustrative aspect of the "DNB" solar dryer while under the construction process at SISELM, Lda.

3.1.2 Drying Parameters and measurement procedures

During the process of semi-active solar drying of the leaves of *S. rebaudiana* by solar energy, parameters of both air and leaves were measured.

Moist air parameters determination

The characteristics of air that are relevant for this work are temperature, relative humidity and velocity. Monitoring the temperature of the air was managed with different thermometers placed in different spots: absorber air entrance (ambient air), chamber in and chamber out (air exit). Mini data logger, Model TESTO 174H, serial no. 36695128, and digital probe type, Model DUAL TERM PRO, serial no. C0703030643 were used.

For monitoring relative humidity of ambient air and of the air exiting the chamber, the same digital Mini data logger, Model TESTO 174H was used.

A probe type, Model TESTO 405-V1, Art. No. 0560 4053 was used to monitor the air velocity out of chamber.

Leaves parameters determination

The temperature of the material to be dried was monitored using a digital probe type, non contact infrared for surface temperature and an attached stainless steel probe for internal temperature, Model DUAL TERM PRO, serial no. C0703030643 by placing the top of probe between layers of leaves. Contact temperature range: -55 to 330°C, and resolution of 0.2°C.

3.2 PLANT MATERIAL AND EXPERIMENTAL DESIGN

3.2.1 Stevia plant and sample identification and codification

Stevia rebaudiana variety 'STEVIA/ 2149E15' plants, with origin in Spain were used.

The field experiment was performed at the *CERCICA – Cooperativa para a Educação e Reabilitação de Cidadãos Inadaptados de Cascais* aromatic plant field plots (+38° 42' 44.28" N, -9° 22' 19.92" W) managed under the concept of social horticulture, except for the experimental treatments, when specified.

The experimental design included four crossed treatments: fertilizer frequency × pruning severity. Each treatment had 4 replications of 10 plants each, meaning 16 plots (Fig. 3.4).

Briefly (more details on each treatment are given below, in sub-section **3.2.3**):

- Plants established in the field with only pre-planting incorporation and top-dressing during planting with organic and inorganic fertilizer. This treatment is herein referred to as **non-fertilized**;
- Plants grown with additional foliar fertilization, starting from week 21 ending in week 51 (herein referred to as **fertilized** (suffix F),
- Plants submitted to **light pruning** (suffix LP), meaning that cuts were done at the 3rd node of the stalk counting from the base of plant;
- Plant submitted to **severe pruning** (suffix SP), meaning that cuts were done just at face of the basal node of each plant.

Figure 3.3 Plant grown in plots

- Non-fertilized with light pruning (plots 1, 3, 5, 7), sample code: **LP**
- Non-fertilized with severe pruning (plots 2, 4, 6, 8), sample code: **SP**
- Fertilized with sever pruning (plots 9, 11, 13, 15), sample code: **SPF**
- Fertilized with light pruning (plots 10, 12, 14, 16), sample code: **LPF**

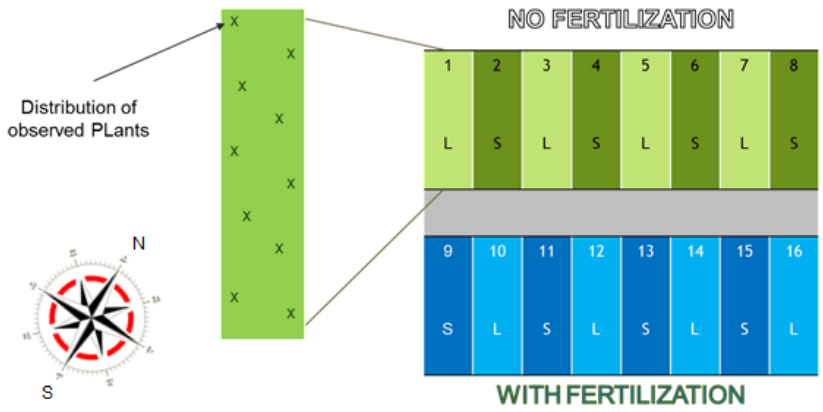


Figure 3.4 – Scheme of the field experimental design including non-fertilized (green) and fertilized (blue) stevia plants and their combination with the two pruning severity types: light (L) and severe (S).

3.2.2 Edafo-climatic conditions

3.2.2.1 Soil characteristics and preparation

The experimental aromatic plants site at CERCICA had a clay soil type and, in 2013, the chemical characteristics were quantified at ISA Laboratory and are presented in Table 3.1

Table 3.1 Chemical characteristics determined from soil samples collected from the experimental field plot at CERCICA

pH (H_2O)	EC (dS m^{-1})	Organic Matter (%)	P_2O_5 (mg kg^{-1})	K_2O (mg kg^{-1})	$CaCO_3$ (mg kg^{-1})	Sodium exchange base	Calcium exchange base
8.2	0.08	2.4	+200	+200	78	0.6	31.5

The total area of the experimental site was 1000 m^2, on fallow land since 2011. The soil was mobilized, ploughed, scarified and cleaned, in weeks 4 and 6 in 2013 (Fig. 3.5).

During week 37-40 (2013) 9 m^3 of horse compost was incorporated in order to improve the soil organic matter (OM) content, soil structure and drainage, and the soil was covered with weed barrier (artificial textile) (Fig. 3.5).

Figure 3.5 – Aspect of the preparatory soil mobilization and weed protection operations in the experimental site A) ploughing B) scarified and cleaned soil and C) artificial textile cover

3.2.2.2 Climate conditions

The average daily temperature from planting to the second harvest at Time 1 (25-09-2014) was 24.3ºC (Source: Estoril - AccuWeather.com).

3.2.3 Cultural practices for crop establishment and growth

3.2.3.1 Propagation

Stevia vegetative propagation was done by stem cuttings and was performed during March 2014 (week 14). The stem rooting occurred in a heated propagation bench, with temperatures ranging from of 22ºC to 25ºC. The stem cuttings were made from young axial ramifications of the mother plants, with 10 cm height and 2-4 leaf buds. The bottom leaves were removed and the upper leaves were chopped to reduce the transpiration leaf area. After rooting in the heating bench, during week 19 the plants were transferred into pots and maintained in a greenhouse (fig. 3.6) until planting in the field.

(a) (b)

Figure 3.6 (a) Stem cuttings preparation before rooting in the heated bench (b) potted plants in the greenhouse, from week 19 until transplanting to the field

3.2.3.2 Planting

Stevia was planted by hand-transplanting from pots to the field, in March 2014 (week 11), in elevated plots of 16 m^2 each (20 m x 0.80 m), to allow water drainage (Fig. 3.7). The distance between plots was set to 0.80 m and 0.40 m between plants. It was not verified any significate mortality of plants in result of transplanting to the field.

Figure 3.7 Aspect of the plants growing in the permanent field, before treatment imposition

3.2.3.3 Crop fertilization

In addition to the manure incorporated during the soil preparation operations (see 4.1.2), at planting, in the "fertilized" treatment plots only, 750 kg ha^{-1} of certified organic fertilizer Guanito (6% N, 15% P_2O_5, 3% K_2O, 10% CaO, 2% MgO, pH 6.5) were additionally applied to the soil.

From week 21 until week 51, a mixture of at least two organic foliar fertilizers (TCOBRA, DUETTO, in rotation with MYR N, MYR Mg, MYR Ca), certified for organic production, with the following composition were applied:

DUETTO:

- 2.7% organic N, 7.1% K_2O, 14% organic C, 5.4% humic acids, 24% organic meter, relation C/N: 4.8%, pH: 5.5, density 1.21 kg/l
- Dose: 10-30 l/ha (used in concentration: 4.5 l per 100 l of water)

TCOBRA (specific fertilizer, reach in amino acids, completely soluble in water):

- 3.2% organic N, 4.6% Cu, 4.4% free amino acids, dry matter: 51%, density: 1.28 g/cm^3, pH in water: 4.5
- used in concentration: 1 l per 100 l of water.

MYR Ca (prevent and cure the deficiency of Calcium and to provide vegetal amino acids): organic N 3%, calcium oxide (CaO) 5%, organic C 18.5%.

MYR Mg (particular composition of the product makes Magnesium easily assimilable by the plant): magnesium oxide (Mg) 5%, organic C 3%.

MYR N (organic liquid nitrogen fertilizer with vegetal amino acids): total N 5%, total organic C 19%, vegetal amino acids and peptides 27%. pH 5-6.

3.2.3.4 Plant protection

During May, attacks by aphids (adults and eggs) were observed. However, at the same time, the presence parasitoids and predators ladybug (all life cycle forms were presented in Stevia plants), syrphids and lacewings (Fig. 3.8), were also observed, which helped to control potential damages caused by the aphids.

(a) (b) (c)

Figure 3.8 – Presence of a) parasitoids, b) ladybugs and c) lacewings in stevia plants during the assay

Every two weeks (in spring and summer) or monthly (in winter and autumn), *Bacillus thurigiensis* (Turex– 100 g per 100 l of water) together with potassic oil (Limbio - 200 ml per 100 l of water) and sugar (1 kg) against caterpillar, moth and aphids, was applied. In September, copper solutions (Ttigre – 1l per 100 l of water) where applied to control soil fungus *Rhizoctonia* spp. and *Phomopsis* spp. It should be noted that the incidence of *Rhizoctonia* was more severe in plots showing more water accumulation. All solutions were applied according to the manufacturer's recommended doses.

3.2.3.5 Irrigation

Irrigation started in May, when the rains become less frequent, with sprinklers irrigation system. The distance between sprinklers was 50 cm, with debits set to 2.3 mm/h. As the summer of 2014 was unusually cooler and rainy, the most frequent irrigation debit was 20 min/day (daily debit 0.8 mm) and, in some days, the crop was not irrigated.

3.2.3.6 Harvest

The plants were harvested in two different times, and the material was used as sample for the yield and quality determination assays. The cutting material was always cleaned and disinfected with a 95% ethylic alcohol solution.

- First harvest - Time 0 (T0), which took place 4th of July 2014 (about 120 days after transplanting to the field). All plants harvested at T0 had been equally managed. Decision for this harvest was made by visual inspection of the plants and took place when plants had an average plant height of 30-35 cm, a very good ramification structure and a healthy aspect (Fig. 3.9).

During the first harvest, two different types of pruning were applied: (L) light pruning and (S) severe pruning; which served as experimental treatment for the assay, with the expected effects in the second harvest

- Second harvest - Time 1 (T1), after plants had been submitted to all four treatments. This harvest took place by 25th of September 2014 (83 days after first harvest).

Figure 3.9 Aspect of the stevia crop four months after transplanting (just before first harvest), with an average plant height of 30-35 cm

The detailed procedure for stevia harvest was:

- 7 days before harvest, all flowers were removed;
- The harvest took place after 2 consecutive days without raining;
- The plants had reached 30-35 cm high in the first harvest and ca. 75 cm in the second harvest. When the harvest was done at the base of the plant in order to produce the "severe pruning" samples, it induced more resistance to *Phomopsis* and stimulated the growth of more branches comparing to plants that were harvested under light pruning.

3.2.4 Plant yield evaluation

For the four stevia crop treatments, each with four independent replications, 10 plants were observed in each replication (40 plants per treatment). The procedure used to evaluate the plants is summarized in table 3.2.

Table 3.2 Summary of sampling protocol for each parameter evaluated of the stevia crop treatments.

	Before harvest	After harvest
Plant height was measured	X	
For leaf area one leaf from the 4th position counting from base, was collected	X	

	Before harvest	After harvest
Fresh weight of total harvested plant material was determined		X
Stem weight was determined		X
Leaf weight was determined		X
Ca. 200 g fresh leaves sample were sent for laboratorial quality analysis. Samples were frozen in liquid nitrogen and stored at – 80ºC until use.		X
± 150 g fresh leaf sample was sent for solar drying		X

3.2.4.1 Fresh leaves

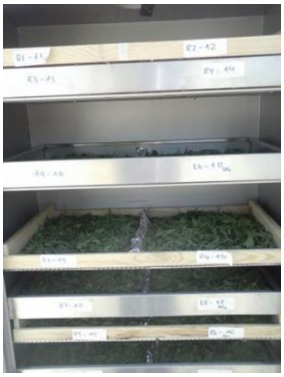
In first harvest a bulked sample from leafs from the total 160 fresh plants was analyzed for Moisture Content, Leaf Area, pH, Total soluble solids, Sum of targeted Glycosides, Rebaudioside A : Stevioside Ratio, Total phenolic compound (Folin), Antioxidant capacity (DPPH and ABTS), (unique reference sample - REF) to provide a characterization of the starting material. The first harvest was done applying pruning techniques: severe (just above basal node), light (from 3rd node counting from base of the plant).

In second harvest, per treatment, 10 plants from each replicate (R) were observed, as described in *3.2. Plant material and experimental design* and for which, ***f*** stands for “fresh leaves”.

3.2.4.2 Dried plants

Dried plant material was chosen after determination of the treatment that led to the highest content of the total observed glycoside compounds (Stevioside, Rebaudioside A and C and Dulcoside A), while simultaneously required lower production costs. This treatment corresponded to the non-fertilized with severe pruning cultivation treatment (fSP) (see section 3.2). The dried leaves (dSP) were obtained after drying fresh leaves samples from Time 1 and for which, ***d*** stands for “dried leaves”

(A)



(B)

(C)

Figure 3.10 Samples for drying A) and B) Samples in trays C) Dried leaves

3.2.4.3 Dried and fresh leaves water infusion

The infusion was performed by adding 14.30 g of fresh (64.80 % water content) sample into 50 ml of distilled H_2O, and in case of dried leaves sample it was weigh 5.60 g (10% water content) and added in 50 ml of distilled H_2O and it was left 1h at 70ºC. After this period the infusion is filtered (Whatman® qualitative filter paper, Grade 1) and distilled water is added to complete 50 ml of infusion.

3.3 PHYSIOLOGICAL DETERMINATIONS - Leaf yield, Plant height and Leaf area

The height of 10 whole plants per replicate was determined using a digital balance, CAS ER PLUS-30CB (LSD). After weighting, leaves and stalks were separated and independently weighted. The weight of plants and leaves was determined as a single biological replicate of 10 plants. Each sample had 4 biological measurements.

Leaf area was determined as mean value (expressed in square millimeters, mm^2) after creating a digital image of 10 leaves from each replicate (one leaf from the 4^{th} position counting from base, was collected from each plant). To accomplish that, 40 individual leaves per sample were scanned into a digital format using a Hewlett & Packard ScanJet 6200C desktop scanner with HP Precision ScanPro (version1.1, imagesscannedat 150 dpi) software. The preliminary image was converted from color to black and white scale 72 pixels (selected from the output type menu). The highlight and shadow levels within the exposure adjustment (selected from the tools menu) were

manipulated to create a black image on a white background. The final version was saved as a TIFF format with 72 pixels. The public domain software (ImageJ 4.0.2 for Windows, National Institutes of Health, Bethesda, MD) was used to measure the surface area of objects in a digital format, using as scale parameters: Distance in pixels: 72; Know Distance = 25.4; and Unit of length: mm.

3.4 ANALITICAL DETERMINATION

3.4.1 Moisture content

The determination of the moisture content of the leaf samples was carried out according to the gravimetric method (AOAC, 1984). On a precision balance (Kern 870), 1 g of sample was weighed into a porcelain crucible. The sample was dried in an oven (Binder) 105 ± 2°C until constant mass. This determination was performed in technical triplicates (fresh leaves) and technical triplicate (dry leaves).

3.4.2 Glycosides quantification

3.4.2.1 Extracts preparation

A preliminary assay was performed to determine the best water extraction method to obtain steviol glycosides from *S. rebaudiana* leaves. Cold and hot extraction (at 70°C) in3 different periods of extraction (10, 30 and 60 minutes) were tested. All methods were done on atmospheric pressure.

The selected procedure was: a starting amount of 4.30 g of fresh leaves or 5.60 g of dried leaves were weighted on a precision balance (Mettler AE 240), 45 ml of distilled water was added, the mixture was heated up until 70ºC and left for 1h and then the infusion was filtered (0.45 microns Nylon filter VWR). The volume was finally completed up to 50 ml. An independent infusion was prepared for each treatment and replicate, meaning 16 measurements.

3.4.2.2 Quantification method

The quantitative content of glycosides in leaf plant material was determined by High-performance liquid chromatography (HPLC), according to Kovylyaeva, et al. (2007) with minor modifications.

HPLC was carried out on a Dionex UltiMate™ 3000 liquid chromatographer with an injector loop and UV spectrophotometer, Dionex UltiMate™ 3000 Diode Array Detector, providing a wavelength detection range from 190 nm to 800 nm.

An XBridge Amide (NH_2) Column (3.5 µm, 4.6 x 250 mm) was used as stationary phase. The mobile phase was 80:20 acetonitrile (CH_3CN) : distilled water, with phosphoric acid (H_3PO_4) pH 3.8, as an acidity regulator in order to adjust the pH to 4.

Injection samples were prepared adding 200 µl infusion to 2 ml CH_3CN:H2O (80:20). Each sample was divided in two, to provide duplicate technical runs.

The flow rate was 1ml min^{-1} with running time of 10 minutes. Detection was at λ = 211 nm.

Calibration curves were prepared with standard compounds of known concentration (Stevioside, Rebaudioside A: 0.01 – 0.5 mg/ml in range; Rebaudioside C, Dulcoside A: 0.005 – 0.35 mg/ml in range). The calibration curves can be seen in Appendice IV.

Necessary corrections were made to obtain results expressed in a weight base (W/W) concentration from volume base (W/V) concentration, using the coefficient: 0.0163427 and 0.0163636 for fresh and dried leaves respectively, taking in consideration sample weight and its moisture content.

3.4.3 Total Soluble Solid content (°Brix)

Total soluble solids were determined from extracted water infusions from fresh and dried leaves using an ATAGO DDR-A1 refractometer in 3 technical measurements per each replicate.

The results obtained were expressed in °Brix (standing as percentage of solid soluble in 100g of dry leaf).

3.4.4 Potential de Hydrogen (pH)

The determination of pH was done through direct reading at room temperature, using a potentiometer (Crison pH 20 Basic) fitted with a liquid electrode (Crison Electrodes 5011). Three technical readings were performed in each replicate extract.

3.4.5 Color

Color was determined instrumentally by direct-readings using a Konica Minolta colorimeter CR- 300. The reflection color by D65 was measured.

The unit determined the L, a*, b* values, and results are expressed in hue angle (°h) and Chroma (C), according to McGuire (1992):

Formula for calculation of Hue Angle (ºh) is:

When a* value is negative = $180 + \frac{\tan^{-1}(\frac{b^*}{a^*})}{6.2832} \times 360$

When a* value is positive = $\frac{\tan^{-1}(\frac{b^*}{a^*})}{6.2832} \times 360$

Chroma (C) was calculated as: $C = \sqrt{(a^{*2} + b^{*2})}$

See Appendice V and Colorimetric circle for Hue Angle, the so called “Color Wheel”.

To translate values into color description, the Konica Minolta Manual (Konica, 1998) was used.

3.4.6 Vitamin C

The determination of the vitamin C content was performed by the HPLC-based method described by Romero Rodrigues et al. (1992). Briefly, 70 mg fresh leafs were weighed on a precision balance (Mettler AE 240) and homogenized in 700 uL of a chilled solution of 3% metaphosphoric acid, (w/v) (3 g metaphosphoric acid added with 8 ml of glacial acetic acid and made up to 100 mL with MilliQ water). The mixture was stirred for 15 minutes in a vortex mixer (BIOSAN V-1 plus) and the volume was completed to 1.75 mL with a cold solution of 3% metaphosphoric acid (w/v) and sonicated (Bandelin Sonorex RK 514H) for 5 minutes with cold water. Then, the sample was centrifuged (Hermle Z 383 K) for 5 minutes at 10,000 g at 4 °C and the supernatant was filtered (0.45 microns Nylon filter VWR), constituting the extract being injected into the HPLC. The sample was injected into the HPLC (Beckman System Gold 126 Solvent Module) with a 20 uL injector, using a C18 column (Waters SunFire) 250 x 4.5 mm, particle size 5 µm at room temperature, for separation. The detector used was the photo-diodes (DAD) (Beckman System Gold 168 Detector) performing readings at a wavelength of 254 nm. The eluent used was MilliQ water acidified with sulfuric acid (pH 2.2) at a flow rate of 0.4 ml min-1 for 15 minutes. These determinations were performed in technical triplicates.

3.4.7 Measurements of total phenolic Compounds and Antioxidant activity

3.4.7.1 Extract preparation

The extracts used for the quantification of phenol compounds and antioxidant activity were obtained following an adaptation of the method described by Swain & Hillis (1959). Briefly, 1g of sample was weighed on a precision balance (Mettler AE 240) and dissolved in 4 mL of absolute methanol.

To obtain the alcoholic extract from water infusions (obtained as in section 4.1.4.2), 10 volumes of absolute ethanol were added.

Samples were centrifuged (Hermle Z 383 K) for 1 min at 11200 g at 4 °C and then incubated at 4°C for 24 hours protected from light, to promote a complete extraction of all extractable compounds. After that incubation period, another centrifugation (4°C, Hermle Z 383 K) for 8 minutes at 4°C 11200 rpm followed and the supernatants were collected and stored at 4°C protected from light, until use.

This procedure was triplicated.

3.4.7.2 Phenolic compounds

The method used for the determination of total phenolic compounds was adapted from Swain & Hillis (1959) and is based on the colorimetric reaction caused by the Folin-Ciocalteu reagent.

In 5 ml tubes, 150 µl of extracts were added with 50 µl of 0.25N Folin-Ciocalteu reagent. The mixture was stirred and, after 3 minutes incubation at room temperature, it was added with 300 µl of a 10% sodium carbonate solution, stirred again and left to stand for 2 hours, protected from light, at room temperature.

After this time, absorbance measurements were performed (ATI Unicam UV/VIS Spectrometer UV2) at a wavelength of λ=725 nm.

The results are based on interpolation of absorbance values on calibration curve obtained with Gallic acid (0.045 - 0.35 mg/ml), and expressed in mg equivalent (Eq) to Gallic acid per gram of dry matter. Calibration curve with solutions with known concentration of Gallic acid in 100% methanol, conducted by to the same treatment as samples, is showed in Appendice IV.

For each replicate, 2 technical measurements were performed.

3.4.7.3 Antioxidant activity - DPPH method

The method used to determine the antioxidant capacity based on the free radical capture method provided by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was adapted from Brand-Williams et al. (1995). In summary, 150 µl of extracts obtained in sub-section 4.1.4.2 were mixed in 2850 µl of a fresh (daily prepared) solution of DPPH diluted in methanol (100%) to a 1 absorbance value at a wavelength of 517 nm. The mixture was stirred and left to stand for 40 minutes protected from light at room temperature. After this time, an absorbance reading at 517 nm was recorded. The results are expressed in mg of Trolox eq/g of dry product. A calibration curve was prepared with solutions of pattern concentration (in a 0.05 – 0.16 mg/ml range) of Trolox solution in 100% methanol, conducted by to the same treatment as samples and can be found in Appendice IV. For each biological replicate, 2 technical measurements were performed

3.4.7.4 Antioxidant activity - ABTS free radical capture method

The 2,2'-Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) - (ABTS) free radical capture method adapted from Miller et al. (1993) was also used to determine the antioxidant activity. In brief, 66.6 µl of extract solution obtained in sub-section 4.1.4.2 was mixed with 3330 µl of a fresh (daily prepared) ABTS solution diluted in MilliQ water containing 9.8 mg of potassium persulfate to an absorbance of 0.7, measured under a wavelength of 734 nm. The mixtures were then stirred and allowed to stand at room temperature for 6 minutes, protected from light. After this time, absorbance readings at 734 nm were performed using (Unicam UV/VIS spectrometer UV2) spectrophotometer. The results are expressed in mg of ascorbic acid eq/g of dry product. The standard curve provided in Appendice IV, was prepared with solutions of pattern concentration (range of 0.01 – 0.2mg/ml) of ascorbic acid in MilliQ water, conducted by to the same

treatment as samples. For each biological replicate, 2 technical measurements were done.

3.5 Statistical analysis of data

All quantified data, except drying parameters, were subjected to analysis of variance (ANOVA) using the STATISTICA 8 software (StatSoft, Inc. 2007).

For correct choosing the post-hoc test it was taken in consideration type of data, how a data organization, number of samples and interconnection of data.

Additional exploration of the significance of the differences among means detected by Variance Analysis (ANOVA) was undertaken by the Scheffe's test.
Means were separated on the basis of least significant difference (LSD) only when the ANOVA F test showed significance at 0.05 or 0.01 probability level, and when groups where small and of similar dimension.

A Two-way completely randomized ANOVA was carried out separately for each treatment to estimate the variance components of cultivation treatments (T), their replications (R) and their interaction (T*R).

In the Table 3.3 it is shown the measurements methodology and type of statistical analyses for analyses included in this work.

Table 3.3 Measurement methodology applied in work

Analyze	Sample	Measurements	N	ANOVA
HPLC Glycosides	1 treatment = 4 replicates	1 replicate = 10 plants = 1 infusion = 2 x 2 technical measurements 1 sample = 16 technical measurements = 4 biological measurements	4	One-way
Color	1 treatment = 4 replicates	1 replicate = 10 plants = 10 biological measurements 1 sample = 40 biological measurements	40	Factorial
		1 replicate = 1 infusion = 10 technical measurements 1 sample = 4 biological measurements	4	One-way
Height	1 treatment = 4 replicates	1 replicate = 10 plants 1 sample = 40 biological measurements	40	Factorial

Leaf yield	1 treatment = 4 replicates	1 replicate = 10 plants = 1 biological measurements 1 sample = 4 biological measurements	4	One-way
Leaf area	1 treatment = 4 replicates	1 replicate = 10 plants 1 sample = 40 biological measurements	40	Factorial
TSS	1 treatment = 4 replicates	1 replicate = 1 infusion/extract = 3 technical measurements 1 sample = 4 biological measurements	4	One-way
pH	1 treatment = 4 replicates	1 replicate = 1 infusion/extract = 3 technical measurement 1 sample = 4 biological measurements	4	One-way
ABTS **DPPH** **Folin**	1 treatment = 4 replicates	1 replicate = 1 infusion/extract = 2 technical measurements 1 sample = 4 biological measurements	4	One-way

4. RESULTS AND DISCUSSION

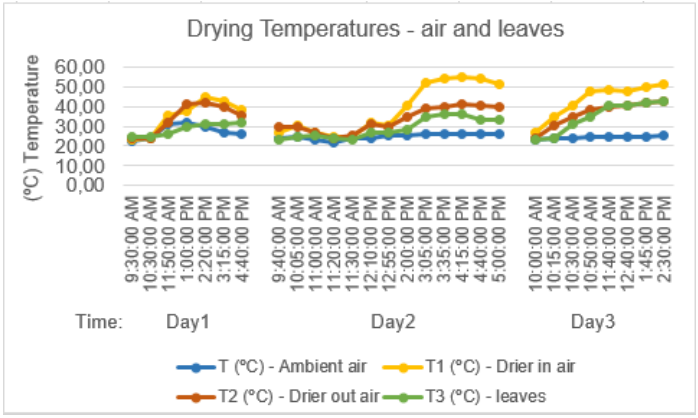
4.1 Drying parameters

With the solar dryer designed and construction in this work, it was possible to achieve drying with an average air temperature of 39.19 ±10.62 ºC and 37.13 ±6.55ºC, inside and outside of chamber respectively, and with a corresponding temperature in the leaves surface during drying of 30.45 ±6.03ºC in average (Fig. 4.1, A). Ambient average temperature for the same period was 25.33 ±2.30ºC. Also, the average air velocity in the chamber was 0.69 ±0.18 m/s. In the same time, during continuous, active period of drying, an average relative humidity of air exiting from the chamber was 40.52 ±12.24%, while ambient air average relative humidity was 41.74 ±10.73% (Fig. 4.1, B).

The drying process were completed effectively after 18h24m, during which, the leaves water content (humidity) decreased from 78.36 ±1.21 % to 10.00 ±0.11 %.

All measurements recorded during drying are presented in Appendice VI.

(A)



(B)

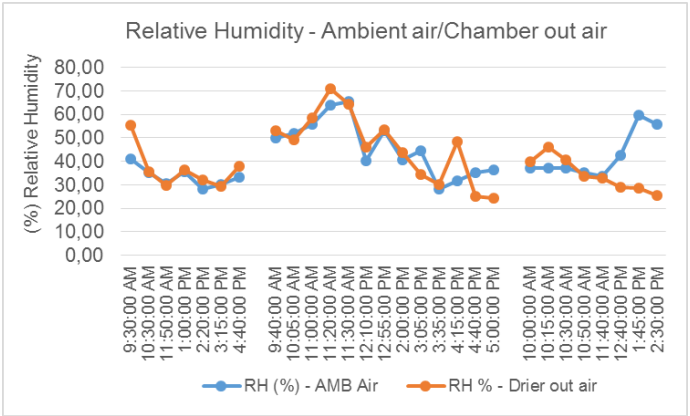


Figure 4.1 Drying parameters evolution along the process. A) Temperatures – air and leaf material B) Relative Humidity and air velocity

Taking in consideration the known impact of moderate heat stress on accumulation of secondary metabolites in leaves (Jochum et al., 2007; Tanveer et al., 2012), a heating incubation period of the fresh leaves for ca. 2 hours inside the dryer was applied prior to the drying period itself.

The proposed model of dryer was demonstrated to be adequate for achieving desirable drying conditions, with average air temperature exiting the chamber at 37.13 ±6.55ºC, as recommended for both preserving nutrient and functional compounds in leaf tissues and avoiding mold growth. The air velocity recorded can be considered adequate as Martins et al. (2002) observed that drying lemon grass leaves with air velocities of 0.5 and 1.0 m/s had no statistically significant effect on the final product quality. On the other hand, among different drying treatments oven drying at 40 °C for 24 h produced higher mean total steviol glycoside and found statistically at par with oven drying at 40 °C for 48 h, oven drying at 70 °C for 24 h and shade drying. Total mean steviol glycoside was statistically lowest in oven drying at 70 °C for 48 h and direct sun drying (Saurabh Sharma et al., 2014).

4.2 Impact of fertilization frequency and pruning severity

4.2.1 Characterization of the reference material, before treatment imposition

Stevia rebaudiana plants obtained in the first harvest were investigated in terms of their quality parameters, as a single sample.

This sample showed relatively low moisture content, 66.56 ±3.81%, with leaves with the average area of 1523.18 mm^2 ±400.53, a slightly acidic pH of 5.51 ±0.03, and total soluble solids of 2.70 ±0.12 in °Brix.

As expected, before treatments, the most abundant steviol glycosides in leaves were Rebaudioside A and Stevioside, with a content of 1.85 ±0.27% and 1.62 ±0.19%, respectively (Table 4.1). A quality indicator of this composition, expressed as Rebaudioside A : Stevioside ratio, was 1.15 ±0.15, indicating that sweetening effect is marginalizing bitter aftertaste.

The antioxidant activity measured using DPPH and ABTS methods, both evidencing high ability of antioxidants to neutralize free radicals, and expressed as equivalent quantity of Trolox (DPPH) or Ascorbic acid (ABTS), in fresh leaves and in infusions is shown on Table 4.1. A significant ca. 50% decrease of antioxidant activity in leaves infusion was observed, when compared to fresh leaves. Phenolic compounds content expressed as Gallic Acid equivalent, also showed a high concentration of phenolic compounds in fresh leaves and in infusion from fresh leaves.

Table 4.1 Characteristics of S. rebaudiana fresh leaves at first harvest, growing before treatment imposition

Parameters	Values
Moisture Content (%)	66.56 ±3.81
Leaf Area (mm^2)	1523.18 ±400.53
pH	5.51 ±0.03
º Brix (total soluble solids)	2.70 ±0.12
Sum of targeted Glycosides (mg/g dry leaf)	38.06 ±4.42
• **Stevioside (mg/g dry leaf)**	16.16 ±1.90
• **Rebaudioside A (mg/g dry leaf)**	18.49 ±2.66
• **Rebaudioside C (mg/g dry leaf)**	2.94 ±0.49
• **Dulcoside A (mg/g dry leaf)**	0.47 ±0.04
Rebaudioside A : Stevioside Ratio	1.15 ±0.15
Phenolic compound - mg Gallic Acid/g dry leaf	
• **Fresh leaves**	8.33 ±0.77
• **Fresh leaves infusion**	5.63 ±0.06
Antioxidant capacity (DPPH) - mg Trolox/g dry leaf	
• **Fresh leaves**	10.34 ±2.48
• **Fresh leaves infusion**	5.65 ±0.78
Antioxidant capacity (ABTS) - mg Ascorbic Acid/g dry leaf	
• **Fresh leaves**	4.42 ±1.33
• **Fresh leaves infusion**	2.72 ±0.59

4.2.2 Morphological parameters of plant

By the second harvest and sampling, conducted at 25th of September 2014, the plants developed under the effect of each of the 4 treatments applied.

(A) (B)

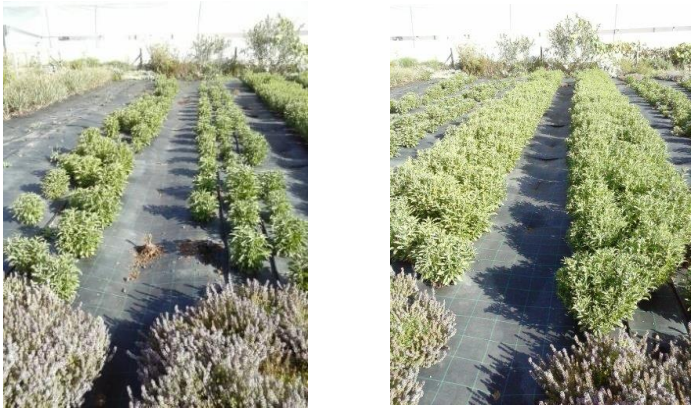


Figure 4.2 Plant growth in non-fertilization regime after (A) light and (B) sever pruning in end of July 2014

Plants were then examined according to the protocol described in sub-section 3.2.

The amount of dry matter produced by *S. rebaudiana* per unit area of production, determines the yield of the available land area. Production measured as leaf yield per hectare, indicated ca.407 kg fresh leaves per ha, or ca. 117.20 kg of dry leaves kg /ha. This result is considered very low and it is influenced hugely by the conditions designed by this work with low plant density (2100 plants per 1 ha).

In Appendice I are presented results from all morphological measurements.

4.2.2.1 Plant height

It was evidenced that, when grown under the “no fertilization” treatment, the plants were higher, independently of the pruning severity. Plants with additional fertilization were always shorter, particularly those that had been light pruned (Fig. 4.3 (A)).

The increased plant height when subjected only to fertilization during implantation, reaching 80.38 ±6.88cm, is not in compliance with evidences in earlier publications of Maheswar (2005), which reported that the increasing dosage of nitrogen from 60 kg N ha^{-1} to 105 kg N ha^{-1} could increase height of stevia for about 16.01% (from 48.96 into 56.8 cm). Aladakatti et al. (2012) also reported that the increasing dosage of nitrogen from 200 to 400 kg N ha-1 could increase height of stevia for about 14.94% (from 49.59 into 57 cm). From other reports, plant height was significantly higher in unfertilized treatments which could be attributed to reduced leaching and movement of nutrients and more availability to the crop. For instance, Ossom and Matsenjwa (2007), reported similar results in field bean (*Phaseolus vulgaris L.*).

4.2.2.2 Leaf yield

In terms of leaf yield no differences were detected due to the fertilization regime and the only effect was, that under fertilization treatment, plants that had been light pruned produced more leaves that those submitted to a more severe pruning (Fig. 4.3 (B)).

The higher leaf yield ratio measured in w_{leaf} / w_{stem} (0.43 ±0.03) observed in samples under the fertilization foliar reinforcement with light pruning is similar to previous research reported from Italy conditions (Andolfi et al., 2006) where the high values obtained in both years (1999 and 2000) resulted from the first cutting (0.6 and 0.7 respectively), when two cuttings were performed. This authors suggest that leaf yield ratio goes from a minimum in the year of planting (0.4) to a maximum 3 years latter (0.7), taking in consideration high survival rates during the winter, with temperatures above 0 ºC, as stevia suffers from the cold and cannot tolerate long periods under 0 ºC and under snow (Angelinia & Tavarinia, 2014). Higher leaf yield can

be obtained in the first year if the plants are early harvested (82 days after the vegetative regrowth rather than 127 days after planting), as suggested by Angelini. Also, long vegetative growth periods before the onset of flowering are known to increase leaf yield (Angelini and Tavarini, 2014).

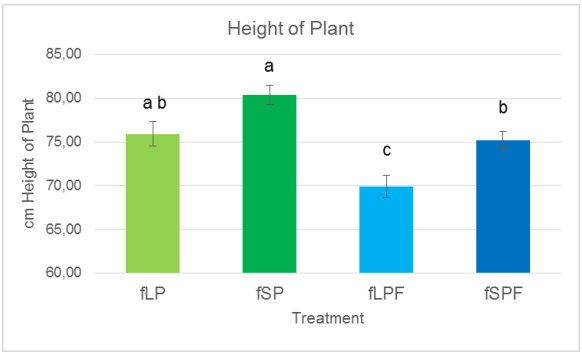
No significant difference in the present study was observed in leaf yield when foliar fertilizer was applied. This result does not agree with results obtained with the application of 60 kg N per ha, 30 kg P per ha and 45 kg K per ha, in which stevia plants achieved higher dry leaf yield and simultaneously higher nutrient uptake (Chalapathi et al. 1997, 1999). Sood and Kumar (1994) also reported that green and dry foliage yield increased with increasing levels of N and P. No effect on plants treated with comfort might be attributed to increased availability of micronutrients in soil through greater microbial and enzymes activity in organic manures, and uptake of major nutrients by the stevia plant is significantly influenced by the organic manures (Kumar, 2012) and this may indicate that no additional foliar fertilization is necessary in the first year of production, when basal fertilization is sufficient source of nutrient uptake.

4.2.2.3 Leaf area

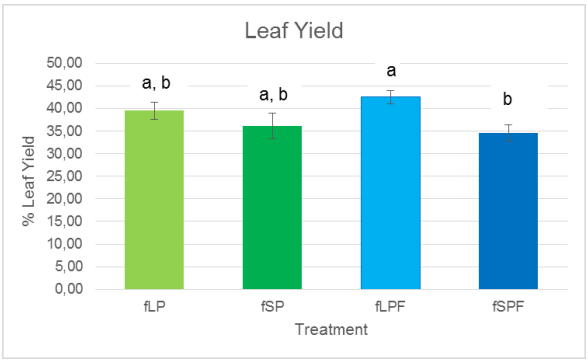
Regarding leaf area, the pruning severity had no effect in each individual fertilization regime. Considering the 4 treatments as independent, leaves with increased area were produced by plants grown with additional fertilization and with severe pruning, contrasting leaves from plants light pruned just with fertilization incorporated at culture establishment as a source or nutrients (Fig. 4.3 (C)).

The higher values of leaf area (17.63 ±5.24 cm^2) were observed under combination of severe pruning and foliar fertilization reinforcement when compared individually with other treatments, and it is in accordance with previous reports, in which Shaikh and Hulmani (1997) observed the increase in leaf area (43.29 cm^2) due to severe pruning compared to mild pruning (39.62 cm^2) of Guava (*Psidium guajava L.*) leaves.

(A)



(B)



(C)

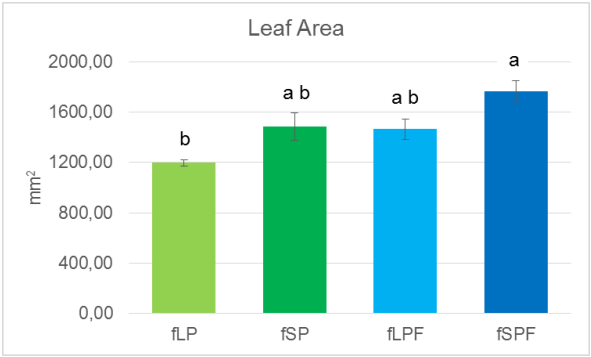


Figure 4.3: Effect of pruning severity and regime of fertilization on A) Plants Height B) Leaves Yield C) Leaf Area, measured on the S. rebaudiana plants assayed. Bars represent means ± standard error of at least 10 biological replicates in the experimental design with 4 randomized blocks. Different letters indicate statistically significant difference for $p < 0.05$. A) Scheffé test, B) LSD test, C) Scheffé test. Legend: fLP – fresh plant, light pruning; fSP - fresh plant, severe pruning; fLPF - fresh plant, light pruning with additional fertilization; fSPF - fresh plant, light pruning with additional fertilization

4.2.3 Glycosides concentration in fresh leaf material

Under the conditions of the assay, the total amount of quantified steviol glycosides -changed when different type of pruning was applied, and showed to be unaffected by the amount of nutrients delivered to the plants.

The higher content of measured glycosides was detected in leaves from plants with the severe type of pruning, when compared with light type of pruning. Average values of 36.82 ±2.63 mg and 36.05 ±4.93 mg glycosides per g of dry leaves were quantified in fSP and fSPF treatments, respectively. The concentration of glycosides when plants were cut in less stressful light pruning was observed significant decrease ($P<0.05$) to 28.45 ± 3.59 mg per g of dry leaf, and 25.31 ± 3.41 mg per g of dry leaf, fLP and fLPF treatments respectively.

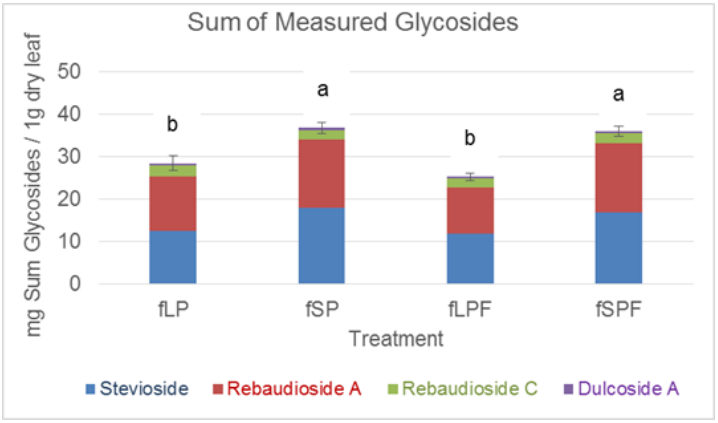


Figure 4.4 Effect of type of pruning and regime of fertilization on the accumulation of the 4 more representative Stevia glycosides in S. rebaudiana, Bars are representing means ± standard error of 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05, according to the Scheffé test. Legend: fLP – fresh plant, light pruning; fSP - fresh plant, severe pruning; fLPF - fresh plant, light pruning with additional fertilization ; fSPF - fresh plant, light pruning with additional fertilization

The higher content of Stevia glycosides obtained from severe type of pruning, independently on fertilization regime is in accordance to relevant reports indicating that secondary metabolites synthesis and accumulation occurred as a plant's response to induced wound-stress and previous works has shown that wounding and pathogens induced the transcription of genes that encode glucosyltransferases that can glycosylate secondary metabolites and hormones (O'Donnell et al., 1998). The synthesis of secondary metabolites, such as phenylpropanoids, terpenoids, and alkaloids according to Wink (1999) is to create antimicrobial properties or antioxidant activities. However, due to their cellular toxicity, many of these compounds are converted to their glyco-conjugates and then accumulated in vacuoles or specialized plastids. Thus, the glycosylation reaction is one of the mechanisms that modulates the biological activity of secondary metabolites and allows the metabolic homeostasis of plants to be maintained (Jones and Vogt, 2001).

The relatively lower concentration of total observed glycosides (3.68 ±0.27 % in fSP), compared to some previous reports can be explained by the effect of decreased

glycosides content when the plant is harvested after flowering like in this study, as demonstrated by Kang and Lee (1981) that report that the maximal content of Stevioside in leaves is achieved during the formation of flower buds and it then gradually declines, and as suggested by Angelini and Tavarini (2014) that acknowledge the long vegetative growth period before the onset of flowering as responsible to increased Steviol glycosides yield. Another sources are reporting that a steady increase from the summer period to the beginning of autumn, Steviol glycosides content decreased by 20 % during late autumn, coinciding with the onset of flowering (Mitsuhashi et al., 1975).

This may indicate that the steviol glycosides are transported to generative organs. Similar results were obtained for ecdisteroids in *Rhaponticum carthamoides*, *Ajuga reptans* and *Serratula coronata* (Vereskovskii et al. 1983; Revina et al. 1986; Tomas et al. 1993; Anufrieva et al. 1998). Even larger decreases, ranging from 35 to 50 %, were observed by Bian et al. (1981), Yoshida (1986), Truong and Valíček (1999) and Vanidze et al. (2009).

On the other hand, no significant differences in total observed glycosides between plants with additional foliar fertilization and unfertilized plants is not in conformity with the results from Inugraha et al. (2014) reporting that increasing application of nitrogen from 100 into 250 kg N ha-1 had significantly increased Stevioside level in stevia's leaf. Also, research conducted in Egypt described that the increasing dosage of nitrogen from 10 into 30 kg N has significantly increased Stevioside for about 1.99 % (Allam et al., 2001). This result can be explained by the fact that the soils from both fertilization regimes were already enough reach in nutrients from basal fertilization and that in the first year of cultivation, nutrient reinforcement may not have any difference in a level of glycosides concentration.

When each individual glycoside was quantified separately, with the exception of Rebaudioside C whose accumulation was not affected by the treatment imposed, it was evidenced that, in general, conditions that promote its accumulation are associated to severe pruning, regardless of the regime of fertilization. In fact, both Stevioside (Fig. 4.5 (A)) and Rebaudioside A (Fig. 4.5 (B)) content were higher in leaves from plants with severe type of pruning, 17.95 ±1.15 mg and 16.00 ±1.61 mg per 1 g of dry leaf, in leaves from plants with only basal fertilization, respectively, and 16.87 ±2.35 mg and 16.23 ±2.49 mg per 1 g of dry leaf, in leaves from plants with more availability of nutrients, in case of additional fertilization. Regarding the specific case of Dulcoside A, the trend for a higher accumulation in response to severe pruning was also verified, but no fertilization condition led to higher accumulation of this glycoside (Fig 4.5 D).

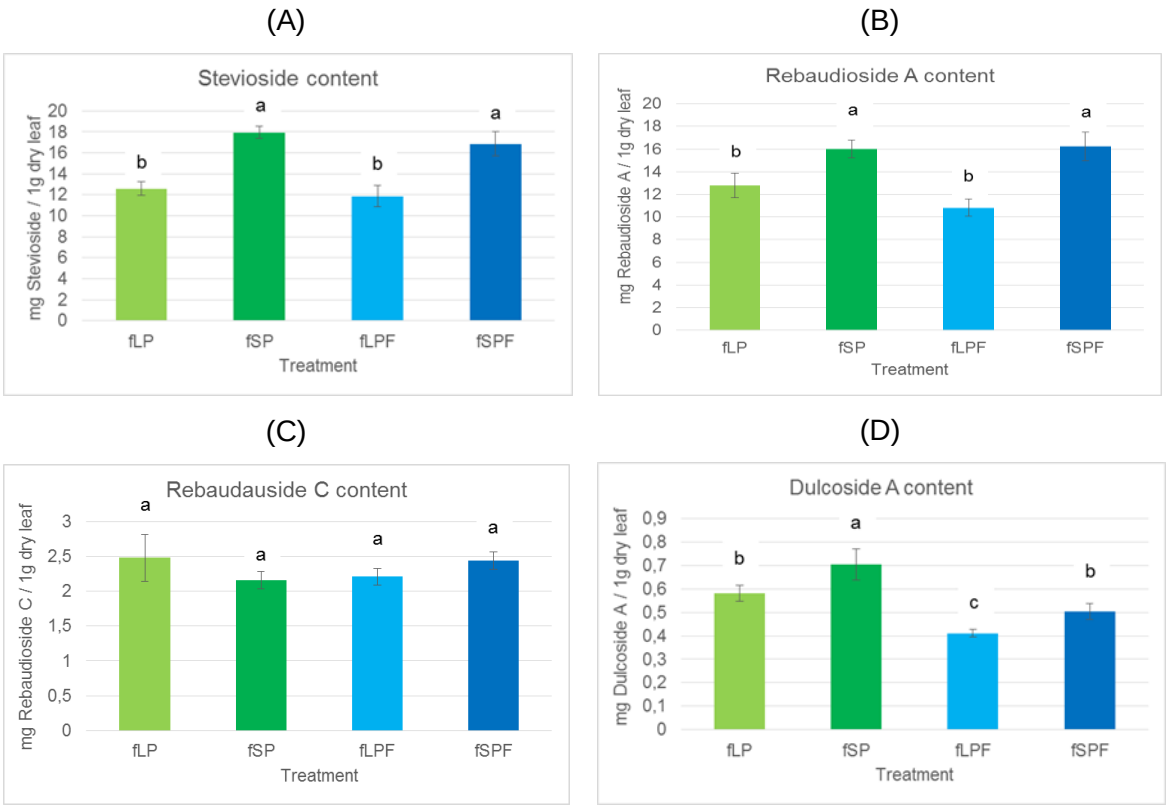


Figure 4.5 Effect of type of pruning and regime of fertilization on the accumulation of A) Stevioside B) Rebaudioside A C) Rebaudioside C D) Dulcoside A in S. rebaudiana. Bars represent means ± standard error of 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05 (Scheffé test). Legend: fLP – fresh plant, light pruning; fSP - fresh plant, severe pruning; fLPF - fresh plant, light pruning with additional fertilization ; fSPF - fresh plant, light pruning with additional fertilization

However, in terms of the level of concentration of individual glycosides the results from this work are more similar to the previous study from Mandala et al. (2013) where concentration of Stevioside ranged from 2.10 to 2.90 % and those of Reb-A from 2.16 to 2.30 %, in two different treatments, two concentrations of phosphorous, 0 and 50 mg P kg^{-1} as K_2HPO_4 in the soil (basal fertilization), assay conducted in the Botanical Garden, Department of Botany at the University of Delhi.

From Gardana et al. (2010) was reported from southern Italy, that level of Steviol Glycosides found in samples of Stevia was for Stevioside (5.8±1.3%), Rebaudioside A (1.8±1.2%) and Rebaudioside C (1.3±1.4%) as those three were the most abundant steviol-glycosides.

When the ratio between the two most significant glycosides, Rebaudioside A and Stevioside, was compared, and how it was altered in response to the different conditions of pruning and different regimes of fertilization, it could be observed that, although that ratio is unchanged in response to fertilization (fLPF = 0.92 ±0.07 and fSPF = 0.96 ±0.08), leaves from plants growing under conditions of “no fertilization”,

showed statistically significant differences (P<0.05) according to the type of pruning, being higher when leaves were under light pruning (1.02 ±0.16) and lower under severe pruning (0.89 ±0.08), as showed on Fig. 4.6. The same ratio (1.02) was obtained by Mandal et al. (2013) in non-nutrition (0 mg P kg^{-1} as K_2HPO_4) treatment, and in nutrition (50 mg P kg^{-1} as K_2HPO_4) one the ratio was 0.79.

This result indicates that leaves from plants grown after light pruning, just with basal fertilization are more prone to be less bitter aftertaste.

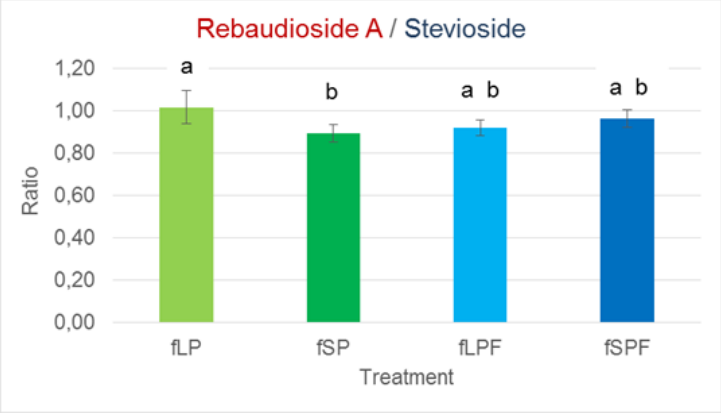


Figure 4.6 Effect of type of pruning and regime of fertilization on bitter aftertaste, as addressed by the quantification of the ratio between Rebaudioside A : Stevioside. Bars are representing means ± standard error of 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05, (Scheffé test). Legend: fLP – fresh plant, light pruning; fSP - fresh plant, severe pruning; fLPF - fresh plant, light pruning with additional fertilization ; fSPF - fresh plant, light pruning with additional fertilization

Higher ratio observed in conditions of reduced availability of nutrients and light pruning (1.02 ±0.16) indicate that wounding promotes the Stevioside synthesis rather than Rebaudioside A synthesis as pathway terminates more with Stevioside, and less with the glycosylation of Stevioside of which forms Rebaudioside A.

This can be explained as a natural adaptation of the plant on physical stress provoked by severe pruning, responding with higher accumulation of bitter substances, as defence compounds in response to attack from herbivorous.

4.3 Impact of drying on leaf quality

Based on evaluation of the highest sweetener (steviol glycosides) concentration per g of dry leaves (Fig. 4.4) and lower production inputs, among four observed treatments of fresh plants the chosen sample was composed by leaves from plants grown in “no fertilized” treatment and severe type of pruning, codified as “fSP”, so the impact and influence of solar semi-active drying was assessed over this sample.

According to results from biomass yield of fSP sample, based on 40 observed plants, when projected on 2100 plants per hectare, the yield of fresh leaves of *S.*

rebaudiana planted in this experimental field, as referred in section 4.2.2, can be considered ca. 407kg, and after drying in semi-active solar dryer until leaf's moisture of 10.00 ± 0.11% (Tab. 4.2), it would be 117.20 kg of dry leaves kg /ha per harvest.

4.3.1 Moisture content

Moisture content was determinate for unique samples per treatment, from both fresh (fSP) and dried (dSP) leaves. *S. rebaudiana* leaves, as shown in Table 4.2, it was observed a after drying 10.00 ± 0.11% moisture content in dried leaves, contrasting with the initial value of 78.36 ± 1.21% in fresh leaves. It should be notated that, in addition to this usefulness to biologically characterize the material assayed, these values were also taken as a reference moisture content of *S. rebaudiana* leaves and used in all subsequent calculations of W/W concentrations.

Table 4.2 Moisture content determined in fresh and dried leaves of S. rebaudiana

Samples	Moisture (% H_2O)
fSP - Fresh plants – T1	78.36 ± 1.21
dSP – Dried plants – T1	10.0 ± 0.11

4.3.2 Glycoside concentration after drying

The sum of measured glycosides showed to be different (P<0.0001) between fresh and dried leaves in favor of dried leaves. According to the results obtained, fSP sample (fresh leaves) showed 36.82 ± 2.63 while dSP sample (dried leaves) showed an increased content of 43.06 ± 4.65 mg of total glycosides per gram of dry leaf (Fig. 4.7). This is a significant increase of ca. 17% (calculated on means bases).

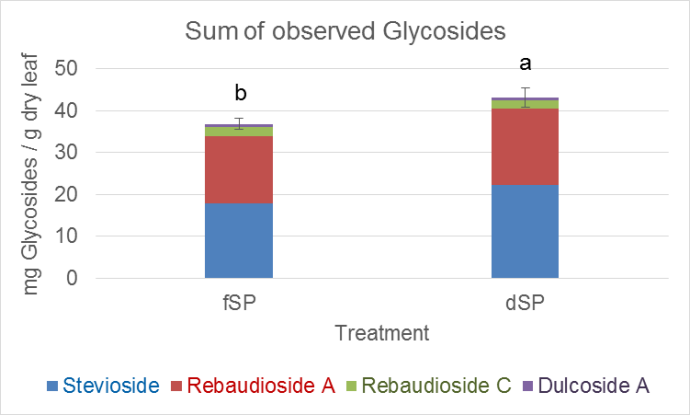


Figure 4.7 Effect of drying on accumulation of the 4 most representative Stevia glycosides in S. rebaudiana dried leaves. Bars are representing means ± standard error of 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05, following the Scheffé test. Legend: fSP – fresh leaves, severe pruning; dSP – dried leaves, severe pruning

Increased concentration of total observed glycosides after drying of ca. 17% (4.31 ±0.47 %), is similar to previous reports as can be attributed to pre-imposed moderate heat stress incubation, as expected. As a response of leaves, as described in the literature, plants overproduce secondary metabolites under heat stress as one of the adaptive strategies (Jochum et al., 2007; Tanveer et al., 2012).

When the accumulated individual glycosides content was addressed, we could observe significant differences (P<0.05) in Stevioside, Rebaudioside A and Dulcoside A levels While Stevioside and Rebaudioside A contents increased with drying from 17.95 ±1.15 to 22.20 ±2.47mg/g representing about 24% and from 16.00 ±1.61 to 18.35 ±2.39mg/g representing about 14% respectively, the opposite effect was perceived for Dulcoside A, that decreased in ca. 23% (Fig. 4.8). Rebaudoside C was not affected by drying of the leaves.

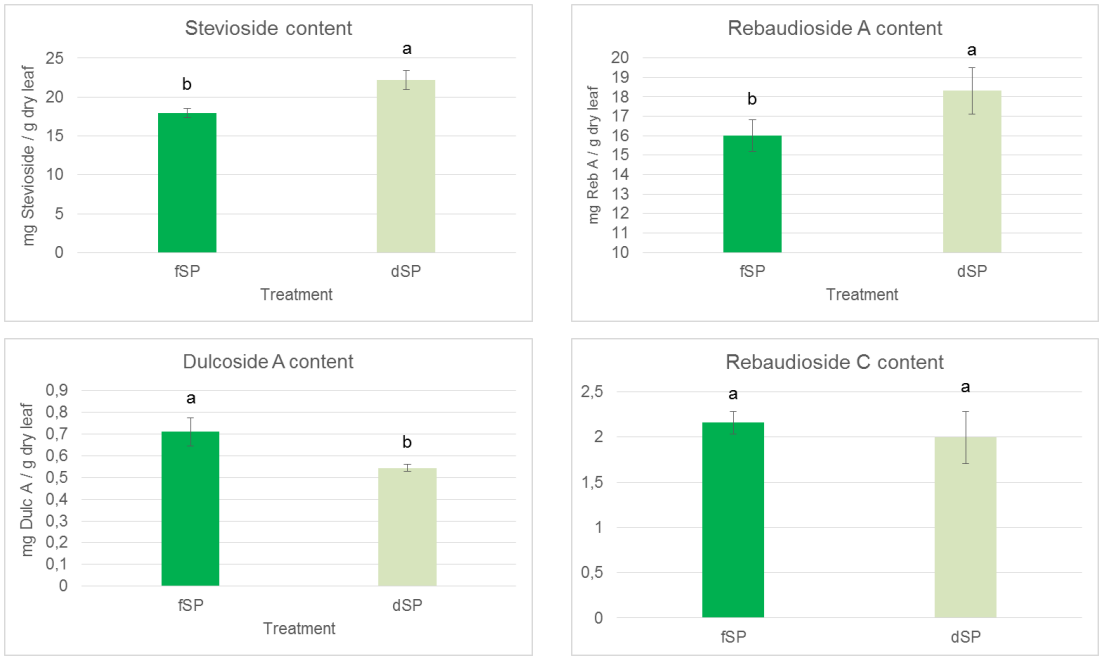


Figure 4.8 Effect of drying on accumulation of A) Stevioside B) Rebaudioside A C) Rebaudioside C D) Dulcoside A in S. rebaudiana. Bars are representing means ± standard error of 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05, (Scheffé test). Legend: fSP – fresh leaves, severe pruning; dSP – dried leaves, severe pruning

To understand if drying produced an effect on potential perceivable taste, the Rebaudioside A: Stevioside ratio was also compared for this condition. The results showed a significant change (Fig. 4.9) after drying, being altered in response to the specific conditions of the process, including the pre-imposed heat stress incubation and we could observe that the ratio in fact decreased ca. 7%, statistically significantly, changing from 0.89 ±0.08 to 0.83 ±0.08, indicating that additional accumulation of two

most abundant sweeteners after drying, was not proportional and that could result in slight increase of bitter aftertaste.

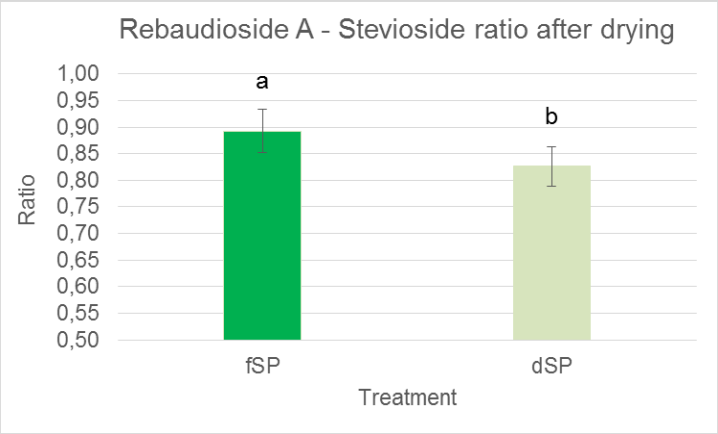


Figure 4.9 Effect of drying B) ratio of Rebaudioside A : Stevioside in S. rebaudiana. Bars represent means ± standard error of 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05, (Scheffé test). Legend: fSP – fresh leaves, severe pruning; dSP – dried leaves, severe pruning

N. Kolb et al. (2001) reported that when 30 samples of *S. rebaudiana* dried leaves obtained from different places in Misiones (Northeastern Argentina) were analyzed, a wide variability was found in the ratio Rebaudioside A: Stevioside which ranges from 0.28 to 1.2. The samples were harvested at different places and at different times throughout the year, dried through exposure to sunlight. The authors suggest that this may be related to the time of harvesting through the year as well as to the stage of development of the plant.

4.3.3 Total Solid Soluble (TSS)

The measured total soluble solid (TSS, ºBrix) showed a significant increase from 2.05 ± 0.11 in fSP to 2.43 ± 0.11 in dSP, with a P value = 0.006, after LSD test, indicating statistically significant differences. The difference can be observed on Fig. 4.10

It seems that drying promoted accumulation of total soluble solid, with an increment of ca. 18 %

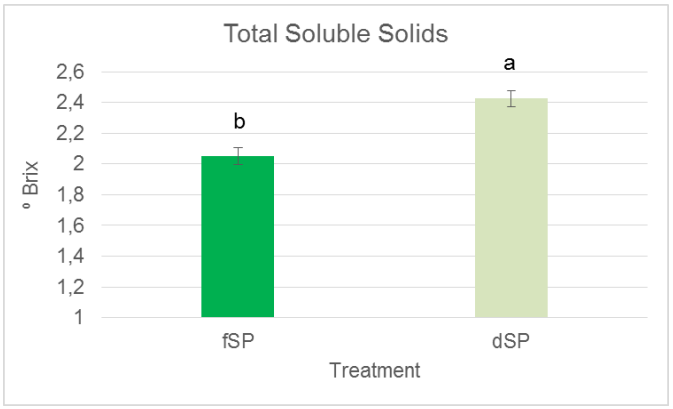


Figure 4.10 Effect of drying on total solid soluble content Bars are representing means ± standard error of at 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05, (LSD test). Legend: fSP – fresh leaves, severe pruning; dSP – dried leaves, severe pruning

The significant increase in ca. 18 % of total soluble solids is in accordance with previous results obtained by Samsudin (2013) where about 1.6% of soluble sugars were detected in the fresh leaves and the amount increased to 5.5 – 6.9% in the dried leaves. Likewise, as reported by Abou-Arab et al. (2010), total soluble sugars of Stevia leaves grown in Egypt recorded to be around 4.13%. As Kaplan et al., (2004) suggests, drying can promote accumulation of total soluble solid. Besides sugar, other soluble materials include organic and amino acids, soluble pectins, and this accumulation can be the result of plant's adaptation to adverse, stress conditions, under which plants appear to re-organize their metabolic network in order to adapt to different adverse conditions.

4.3.4 pH

Regarding pH, it was showed that drying led to more acidic extracts (in ca. 4%). In fact, fSP showed 5.90 ± 0.07 and dSP showed 5.66 ± 0.04, with a P value = 0.002, after LSD test, indicating significant difference, that can be attributed to a slight accumulation of amino and phenolic acids. The difference can be verified on Fig. 4.11

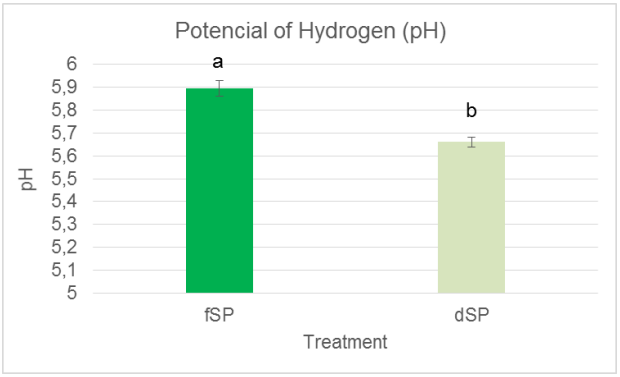


Figure 4.11 Effect of drying on potential of hydrogen (pH). Bars are representing means ± standard error of 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05, (LSD test). Legend: fSP – fresh leaves, severe pruning; dSP – dried leaves, severe pruning

The pH value observed is similar to results obtained by Mishra et al. (2010) that observed pH of 5.95. Similar pH readings were also obtained from the analysis of Stevia grown in Egypt (Abou-Arab et al. 2010). Mehmet Ozgur et al (2011), which found similar pH of fresh and dried leaves Leeks (*Allium porrum L.*) of 6.02 ±0.01 and 5.02 ±1.71, respectively. It can be related to accumulation of some soluble solid like amino acids and some phenolic compounds like phenolic acids. Alternatively, as suggested by Samsudin (2013), the slight acidity increase might be due to delay in drying, where the leaves started to ferment.

4.3.5 Leaf Color

Color parameters expressed in terms of the hue (color tone), lightness (brightness), and chroma or saturation (vividness), were determined in fresh leaves and dry leaves, and their infusions.

4.3.5.1 Fresh and dried leaves color

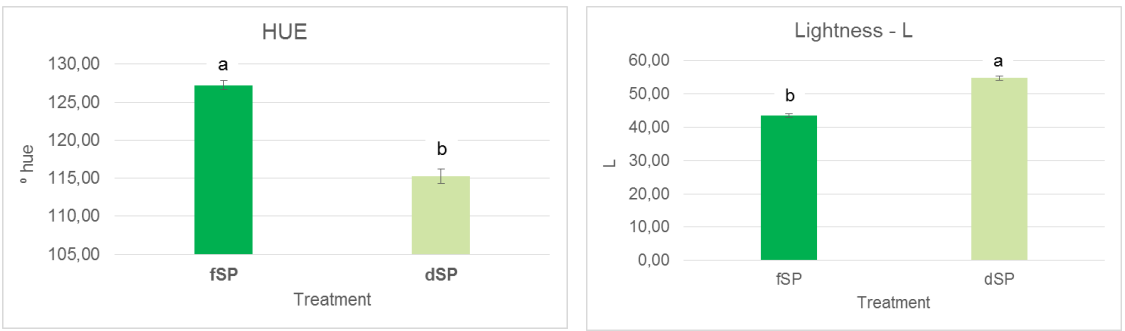
Even though both types of leaves, fresh and dried, appeared visually green, their objective colors showed to be different. In terms of hue, fresh leaves (fSP) showed to be green at 127.23 ±1.22 and dried leaves (dSP) showed to be green-yellow at 115.25 ±1.90, indicating significant difference ($P<0.05$).

The color of the dried leaves was brighter, with Lightness (L) values of 43.51 ±1.28 for fresh leaves and 54.79 ±1.32 after solar drying, indicating significant difference.

Moreover, the color of the dried leaves appeared more vivid, as expressed by the increased chroma value (C) that reached C=21.48 ±1.41 for dried leaves in contrast with C=18.16 ±2.52 for fresh ones, indicating significant difference ($P<0.05$).

All reported differences can be verified on Fig. 4.12

(A) **(B)**



(C)

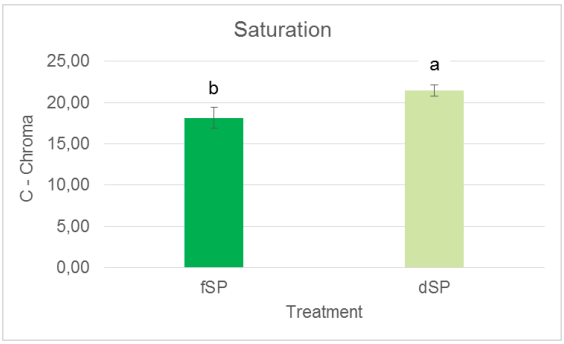


Figure 4.12 Effect of drying on color change expressed as A) HUE - Tonality B) L – Lightness C) C - Saturation on leaves of S. rebaudiana. Bars are representing means ± standard error of at 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05, (Scheffé test). Legend: fSP – fresh leaves, severe pruning; dSP – dried leaves, severe pruning

The significant decrease in the hue value h (a* and b* values), after drying observed in the dried leaves, used as a color tonality indicator that showed changes from green to yellowish green, is in accordance to previous reports from Arabhoseseini A. et al. (2006) that observed tarragon leaves after drying at 45ºC, air velocity 0.6 m/s, with obtained h dried leaves = 105 from h fresh leaves = 124.5, (as threshold for dried tarragon was established to be 95 (h_o), indicating that results with h> 95 are with leaves of acceptable quality). This can be explained as a reduction in the pigments content (chlorophyll A and B, carotenoids and total pigments) as reported by Abou-Arab (2010).

The increase in ca. 26% the lightness of the color of dried leaves, turning from darker green into yellow-green, promoted green color bleaching instead of much darkening or browning, which is in accordance with reports from Shaw M. et al. (2006) in which oven drying was performed on coriander leaves, with drying air temperature set to 50ºC, and RH below 5% and air velocity 1.1 m/s. Under these conditions, the observed lightness increased from ca. 12, 18 and 24%. The fact that the color didn't turn from green into brown suggests that thermal stress was responsible for inhibiting the enzymatic activity, preventing enzymatic browning, as suggested by Murata et al. (2004) and that the pre-heating (moderate heat stress) could even reinforced that inhibition. However, this type of drying could not avoid certain blanching of original green color.

The significant increase of vividness (saturation, C value) for ca. 18% in dried leaves, is an important change and is different from the one reported by Arabhosseini et al. (2007) which observed tarragon leaves (fresh tarragon: C* = 9.65) after drying, and reported that saturation (C*) of the dried tarragon leaves dropped for ca. 9% after drying.

It was also reported that exposure to mild heat at 40 - 50 ºC reduced browning and improved the quality of lettuce and spinach (Delaquis, Stewart, Toivonen, & Moyls, 1999; Gomez et al., 2008; Murata, Tanaka, Minoura, & Homma, 2004; Roura, Valle, & Pereyra, 2008).

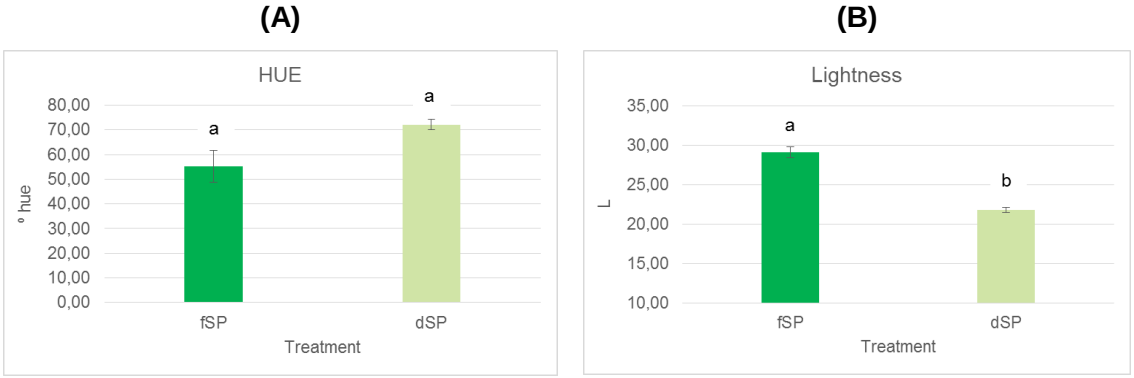
4.3.5.2 Leaves water infusion color

Regarding the color of plant infusions, when two leaves infusions were compared, both infusions appeared dark green, with no significant differences measured in terms of color tonality. However, infusions from fresh leaves (fSP) showed to be dark orange-yellow, with a measured hue angle of 55.12 ±12.96, while infusions from dried leaves (dSP) were more dark yellow-orange, as revealed by hue angles of 72.13 ±4.16, and after LSD test with P value 0.074.

The color of the dried leaves infusion was darker, as revealed by Lightness (L) that decreased from 29.15 ±1.41 for fSP to 21.82 ±0.58 for dSP, with a P<0.001, after LSD test, indicating extremely significant difference in terms of brightness.

The color of the dried leaves infusion appeared significantly more vivid, as expressed by chroma values, which in case of fresh leaves (fSP) was 2.76 ±0.75 and in case of dried leaves (dSP) was 7.29 ±0.54, with P<0.001, after LSD test, indicating extremely significant difference in terms of color saturation.

All reported differences can be verified on Fig. 4.13



(C)

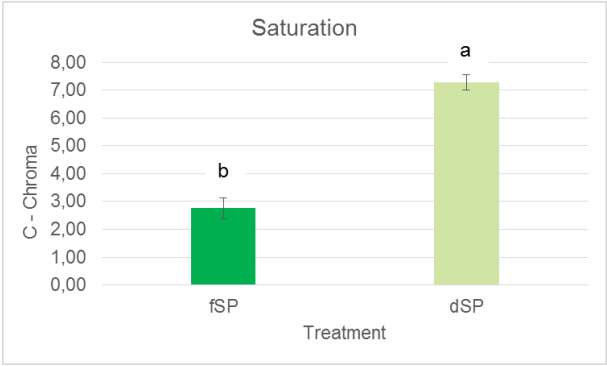


Figure 4.13 Effect of drying on color change expressed as A) HUE - Tonality B) Lightness C) Saturation on leaves infusion of S. rebaudiana. Bars are representing means ± standard error of 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05, (LSD test). Legend: fSP – infusion from fresh leaves, severe pruning; dSP – infusion from dried leaves, severe pruning

The significantly lower hue value h (a* and b* values), of dark yellow-orange dried leaves infusion, when compared to dark orange-yellow fresh leaves infusion, shows a maintenance of the same level of color difference (Δh) from two starting materials before water infusion, and is in accordance with Abou-Arab (2010) that pointed a reduction in the pigments content (chlorophyll A and B, carotenoids and total pigments) as a principle cause of color change.

The lightness of dried leaf infusion dropped for ca. 25%, turning from dark orange-yellow to even darker yellow-orange. The significant increase of vividness (saturation, C value) for ca. 2.65 fold in dried leaves infusion, is an important change.

4.3.6 Phenolic Compounds and Antioxidant Activity

Fresh and dried leaves extracted antioxidant capacities, measured based on inhibition in absorption of both ABTS and DPPH techniques, showed significant differences (P<0.05), disclosing increased antioxidant capacity in dried leaves, expressed in mg of EAA (Equivalent of Ascorbic Acid) per g of dried leaves was 4.4 fold higher (ABTS fresh leaves = 2.46 ±0.31, ABTS dried leaves = 10.87 ±0.51) with the same tendency when expressed in mg of ET (Equivalent of Trolox) per g of dried leaves triplicated (DPPH fresh leaves = 4.25 ±0.61, DPPH dried leaves = 13.03±0.45). Tadhani et al., (2007) have reported that a water-based extract from stevia leaves have an antioxidant activity of 9.66 to 38.24 mg EGA, EAA, EBHA (Equivalent of Beta-hydroxy Acid) and Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carJboxyl;'c acid) per g on a dry weight basis and increased antioxidant activity may be related to increase of Stevioside content after drying as furthermore, Xi et al., (1998) have proposed that Stevioside could have an anti-oxidant activity. In Appendice III are presented all measurements.

Folin–Ciocalteu method, used to determine phenolic compounds, also showed significant difference (P<0.05), with increase of phenolic compound content in dried leaves, expressed in 10.39 ±0.26 mg EGA per g dry leaves, compared to fresh leaves from the same treatment that evidenced 5.21 ±0.31 mg EGA per g dry leaves.

In Fig. 4.14 can be evidenced reported differences.

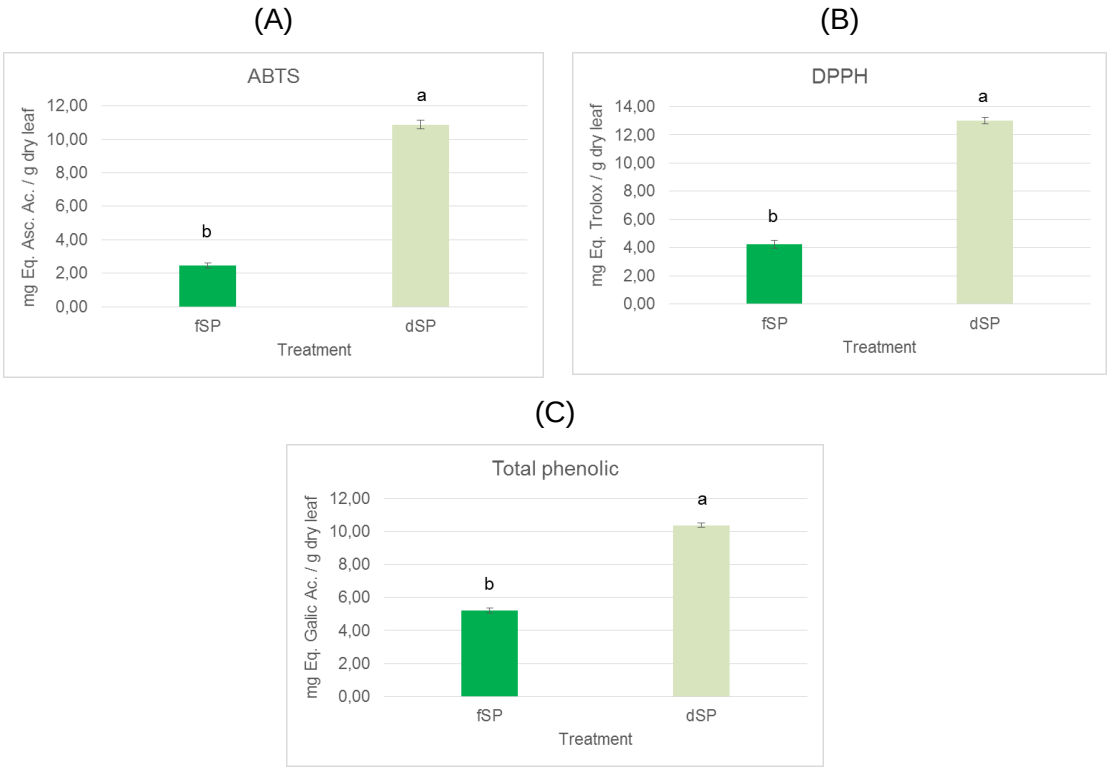


Figure 4.14 Effect of drying on antioxidant activity A) ABTS B) DPPH and on accumulation of phenolic compounds C) Total Phenolic Content in S. rebaudiana leaves. Bars are representing means ± standard error of at 4 biological replicates. Different letters indicate statistically significant difference for p < 0.05, (LSD test). Legend: fSP – fresh leaves, severe pruning; dSP – dried leaves, severe pruning

Likewise, observed increase of ca. 100% in phenolic compounds in dried leaves compared to fresh leaves indicates that phenolic compounds accumulation occurred during drying. As previously reported, thermal stress induces the production of phenolic compounds, such as flavonoids and phenylpropanoids (Nozolillo et al. 1990, Christie et al 1994, Dixon and Paiva 1995, Bharti and Khurana 1997).

The level of phenolic compounds found in dried leaves (10.39 ±0.26 mg GAE/g) is similar to ones reported by Katarzyna Gaweł-Bęben et al. (2015) in which content of total phenols in water (aqueous extract), 96% ethanol (ethanolic extract) and 4:1 propylene glycol to water mixture (glycol-aqueous extract) dried stevia leaves extracts, expressed as mg of EGA per gram of extracted stevia leaves, was noted respectively 3.85, 7.65 and 15.50. Work with lettuce has shown that tissue with low initial levels of phenolic compounds had a higher phenolic accumulation in response to heat shock stress (Saltveit 2000). The accumulation of phenolic compounds as previously reported

(Wahid et al., 2008; Fardus et al., 2014) may be related with better protection against oxidative damage, screening of harmful radiations, stabilization of sub-cellular structures and improvement in cell water balance. Moreover, it can be attributed to the phenomenon known as phenolic induction and syntheses of phenylalanine ammonia-lyase (PAL), as defense related enzyme, known as PAM activity. Increases in PAL activity are associated with induced defence reactions such as hypersensitive response, systemic acquired resistance and wounding in several host pathosystems (Kiba et al. 2003).

The same trend was observed when the infusions were investigated. The antioxidant capacity was leveraged from 4.16 ±0.69 mg EAA per g dry leaves for infusion obtained from fresh leaves to 7.46 ±0.84 mg EAA per g dry leaves for infusion obtained from dried leaves according to ABTS method (Fig. 4.2.12, A), representing an increase for ca. 80%.

Based on DPPH method (Fig. 4.2.12, B) the antioxidant activity almost doubled and from 7.55 ±0.05mg of Eq. Trolox per g dry leaves for infusion obtained from fresh leaves to 15.23 ±1.94 mg Eq. Trolox per g dry leaves for infusion obtained from dried leaves.

These results are similar to reports from Monika Sabolová et al. (2012) who suggested that disruption of plant tissues during drying process could have caused a greater release of active substances during their extraction and therefore higher antioxidant activity of dried herbs aqueous extract than extracts from fresh herbs.

Likewise, phenolic compounds, showed significant differences with drying when Folin–Ciocalteu method was used, with an increase of phenolic compound in dried leaves infusion, measured at 10.01 ±0.43 mg EGA per g dry leaves, compared to fresh leaves infusion from the same treatment in which 6.88 ±0.82 mg EGA per g dry leaves were recorded. In Fig. 4.15 C) can be evidenced reported differences.

The higher content of total phenolic compounds for ca. 46% in dried leaves infusion compered to fresh leaves infusion, is expected, suggesting that heat treatment during infusion (1h/70ºC) just changed the ratio from total phenolic compounds in initial material that was ca. 1:2 for fresh and dried samples, becoming ca. 1:1.5 for two infusions, being in both cases in favour of dried leaves material. This can suggest that in fresh leaves infusion could occurred phenolic compounds accumulation due to heat stress from infusion heat treatment (1h/70ºC).

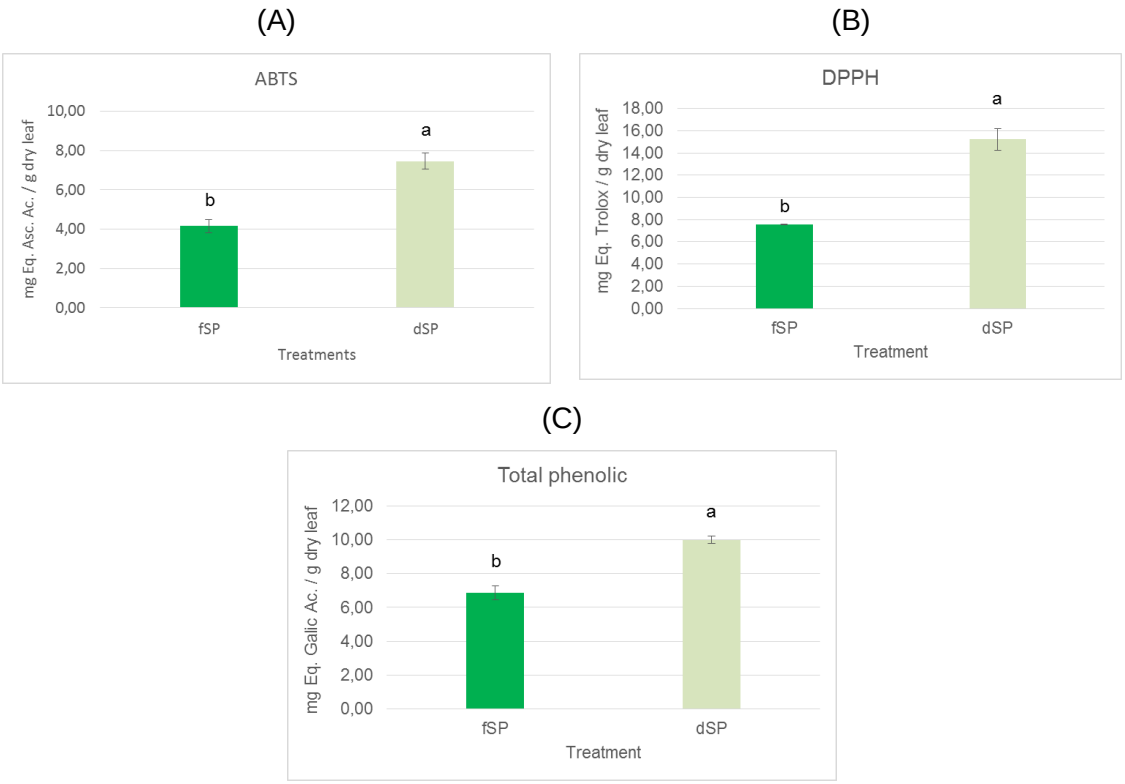


Figure 4.15 Effect of drying on antioxidant activity A) ABTS B) DPPH and on accumulation of phenolic compounds C) Total Phenolic Content in S. rebaudiana leaves infusion. Bars are representing means ± standard error of at 4 biological replicates. Different letters indicate statistically significant difference for $p < 0.05$, (LSD test). Legend: fSP – infusion from fresh leaves, severe pruning; dSP – infusion from dried leaves, severe pruning

As in previous report from Periche et al. (2014), for even higher phenolic compounds content the temperature has more influence (minimum values at 50 °C and maximum at 90 °C during 1 minute) than time in the case of antioxidants, when dry Stevia leaves solid-liquid extracts were observed under different temperatures (from 50 to 100 °C), times (from 1 to 40 min) and microwave powers (1.98 and 3.30 W/g extract).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 CONCLUSIONS

The present work contributed to assist optimization of agronomic practices for production of Stevia in Portugal and, through validating suitable processing practices, to obtain value added final products that have potential as crude Stevia to be commercialized as a dietary supplement (Business-to-Consumer concept) or as raw material for further production (Business-to-Business concept) of high purity Stevia extracts, Stevioside and Rebaudioside A, as natural sweetener. The investigation was focused to deliver a quantitative determination of effects of two types of pruning severity and two regimes of fertilization on biomass yield and concentration of compounds of interest in Stevia plants, cultivated under the context of the social institute CERCICA, Council of Estoril in Lisbon District, Portugal, during the first year of production, as well as the effects on the targeted compounds and other relevant qualitative parameters in Stevia leaves after solar drying.

This study provided evidence for:

- A particular positive aspect that must be considered from this assay is that in this region Stevia can be grown successfully as plurennial species because the crop survival observed over the winter was high.
- Among the agronomical practices assayed, those producing the most beneficial effects were with sever pruning, regardless on type of fertilization.
- Biomass yield evaluation indicated that higher growth doesn't necessary bring higher leaf yield and bigger leaf area. No significant effect were found in leaf area and leaf yield when additional nutritional intake was promoted, most probably because basal fertilization had sufficient and enough effect on full morphological development of plants during the first year of production. Among plants with lower growing rate (additionally fertilized), those with light pruning promoted higher leaf yield, while those with severe pruning promoted bigger leaf area. Higher plants didn't need additional fertilization.
- As for the concentration of compounds of interest due to the main commercial value, namely Stevioside, Rebaudioside A and C, and Dulcoside A, it was observed that the conditions that promote higher accumulation of secondary metabolites are severe pruning, regardless of the regime of fertilization. Relatively low concentration of sum of observed glycosides in fresh leaves can be related to very stable and mild climate conditions in the Estoril area, central coastal zone of Portugal. The results suggest that production of fresh Stevia may be more rentable in regions of interior and/or with higher altitudes where

oscillations of temperature have a higher magnitude and extreme episodes are more frequent, stimulating temperature stress during the growth period, favourable when compared to the suitable natural climate of semi-humid subtropical with temperature extremes from 21 to 43°C and average 24°C.

- Regarding to desirable prevalence of sweeter Rebaudioside A over Stevioside content with bitter aftertaste, this assay confirmed some previous investigations that Stevioside is significantly prevailing when severe pruning is applied in lower nutrients intake conditions, probably as response to wound stress that program the plant to be turned bitterer and more undesirable for herbivores.
- The glycosides increase in ca. 17% after solar drying at relatively lower temperatures (35-40ºC), coupled with a pre-heating stress period demonstrates that this type of processing is appropriate and can leverage the accumulation of higher sweeteners concentration in leaves.
- Rebaudioside A – Stevioside ratio decrease after drying in ca. 6.7%, it can confirm that plants reacts on heat stress accumulating compounds that are with more bitter aftertaste. Accumulation rate for Stevioside was ca. 24%, being 1.7 fold higher from Rebaudioside A that accumulated in ca. 14%. From this perspective it's highly recommendable a use of varieties and techniques that hugely prevail in Rebaudioside A in fresh leaves.
- Color change after drying under low temperatures was used as external quality criteria. As green color of fresh leaves after drying slightly turn in to more yellow tonality and got a bit lighter, the drying process used confirmed inexistence of degradation processes, avoiding browning, and better preservation of nutrients. Getting lighter proves some degradation of heat-sensitive colour compounds and pigment reduction.
- Fresh and dried leaf samples showed high antioxidant activity. The increase of total phenolic to double values in the dried leaf samples proves that thermal stress positively affects phenolic compound accumulation as natural response of plant against oxidative damage, and among them, tannins and flavonoids with bitter aftertaste are likely to accumulate as well. One can speculate that this is the reason why it is expected an increased bitterer aftertaste of stevia leaves after drying.
- Replicability of best practices in production for CERCICA and other similar institutions can be a value added solution as low input crop production and Stevia leaf post-harvest processing by semi-active solar drying can contribute to make the whole process self-sufficient. Nonetheless, some additional costs have to be considered in labor for harvest and leaves separation.

This assay further demonstrates that engaged agricultural practices have to take in consideration correlation and necessary balance between obtained biomass yield and the composition of observed compounds.

5.2 SUGGESTIONS FOR FURTHER WORK

Based on the results obtained after the research developed to produce the present dissertation, we suggest future research in the following areas:

- As foliar fertilization didn't influence the higher leaf yield and leaf area, with opposite impact on plant height, further investigation should provide evidence if higher foliar penetration rates can be achieved with positive impacts on leaf yield and leaf area. Likewise, the effect of foliar maintenance fertilization after depletion of nutrients available due to the basal fertilization applied during crop installation must be monitored.
- As total phenolic compounds doubled after drying it should be studied the impact on antioxidant activity when leaves are dried in different regimes of temperature (38, 45 and 60ºC) using new innovative drying combined technique that includes pre-drying by convection, drying by convection in vacuum (all free water content removal), and microwave drying in vacuum (partial captured water removal) compared to also hybrid drying using combination of heat bomb and solar energy.
- Also due to high antioxidant potential of Stevia leaves water infusions, both fresh and dry, it is important to carry on further studies on crude Stevia in order to evaluate if it meets the requirements for approval of commercialization and use for human consumption, especially dried Stevia leaves and powder.
- As significant accumulation of secondary metabolites after drying including phenolic compounds, among them tannins and flavonoids with bitter aftertaste it is very important to investigate if their accumulation is determinant for change into bitterer aftertaste.

Finally, all assays must be replicated in different years and in experimental fields established in other geographical regions.

REFERENCES

(AOAC) Association of Official Analytical Chemists (1984). Official Gravimetric Method.

Abdullateef RA, Osman M. Effects of stem cutting types, position and hormonal factors on rooting in Stevia rebaudiana Bertoni. Journal of Agricultural Science. 2012;4(1).

Abou-Arab, A., Abou-Arab, A., & Abu-Salem, M. F. (2010). Physico-chemical assessment of natural sweeteners steviosides produced from Stevia rebaudiana Bertoni plant. African Journal of Food Science, 4, 269–281.

Akashi, H., & Yokoyama, Y., (1975). *Security of dried-leaf extracts of Stevia: Toxicological tests.* Food Industry, 18, 34–43.

Aladakatti, Y. R., Y. B. Palled., M. B. Chetti., S. I. Halikatti., S. C. Alagundagi., P. L. Patil., V. C. Patil. and A. D. Janawade, (2012). *Effect of nitrogen, phosphorus and potassium levels on growth and yield of stevia (Stevia rebaudiana Bertoni)*. Karnataka. J. Agric. Sci., 25(1), 25-29.

Alexander, R.J., (1992). *Maltodextrins: production, properties and applications. In: Starch Hydrolysis Products*. Worldwide Technology, Production, and Applications (F.W. Schenck and R.E. Hebeda, eds.), pp.233-276. VCH Publishers, New York.

Alicja Mortensen (2006). *Sweeteners permitted in the European Union: safety aspects*. Department of Toxicology and Risk Assessment, Danish Institute for Food and Veterinary Research, Søborg, Denmark - Scandinavian Journal of Food and Nutrition; 50 (3): 104-11 - Taylor & Francis ISSN 1748-2976

Allam, A.I., Nassar, A.M. and Besheit, S.Y., (2001). (Online) Available: Nile.enal.sci.eg/ArcJournal/uga.html.

Ames, B.N., Shigenaga, M.K., Hagen, T.M., (1993). *Oxidants, antioxidants and degenerative diseases of aging.* Proc Natl Acad Sci., 90:7915–22.

Amzad-Hossain, M., A. Siddique, S. Mizanur-Rahman, M. Amzad-Hossain, (2005). *Chemical composition of the essential oils of Stevia rebaudiana Bertoni leaves.* Asian Journal of Traditional Medicines, 5 (2010), pp. 56–61.

Andolfi, L., Macchia, M. & Ceccarini, L. (2006). *Agronomic-productive Characteristics of Two Genotype of Stevia Rebaudiana in Central Italy.* Ital. J. Agron. Volume 2. No. 2. pp. 257-262.

Andrade, A. E., (2012). *Análisis de la incidencia y relación de los factores ambientales en el desarrollo vegetativo de la Stevia rebaudiana B. en condiciones de invernadero en la Sabana de Bogotá .* Dissertação de Mestrado. Universidad de la Sabana. pp. 24-50.

An-Erl King, V., Liu, C.-F., Liu, Y.-J., (2001). *Chlorophyll stability in spinach dehydrated by freeze-drying and controlled low-temperature vacuum dehydration.* Food Research International, 34 (2-3), pp. 167-175.

Angelini, L.G., Tavarini, S., (2014). *Crop Productivity, Steviol Glycoside Yield, Nutrient Concentration and Uptake of Stevia rebaudiana Bert. under Mediterranean Field Conditions. Communications in Soil Science and Plant Analysis, 45:2577–2592*

Anufrieva, E. N., Volodin, V. V., Nosov, A. M., Garcia, M. and Lafont, R., (1998). *The content and composition of ecdysteroids in plants and tissue cultures of Serratula caranata*. Russ. J. Plant Physiol. 45: 326-332.

Arabhosseini, A., Huisman, W., van Boxtel, A., Müller, J. (2007). *Long-term effects of drying conditions on the essential oil and color of tarragon leaves during storage.* Journal of Food Engineering 79, pp. 561–566.

Arakawa, O., (1991). *Effect of temperature on anthocyanin accumulation in apple fruit as affected by cultivar, stage of fruit ripening and bagging.* J Hort Sci 66(6): p 763–768.

Arinze, E.A., Sokhansanj S., Schoenau, G.J., Trauttmansdorff, F.G., (1996). *Experimental evaluation, simulation and optimization of a commercial heated-air batch hay drier.* J. Agr. Eng. Res., 63, pp. 301-314.

Artschwager, Ernst, (1930). *A study of the structure of sugar beets in relation to sugar content and type.* J. Agr. Res. 40: pp 867-915.

Asrul Afandi, Shazani Sarijan and Ranajit Kumar Shaha, (2013). *Optimization of Rebaudioside extraction from Stevia rebaudiana (Bertoni) and quantification by High Performance Liquid Chromatography Analysis*. Journal of Tropical Resources and Sustainable Science. Volume 1, Number 1: pp 62-70.

Bagley, (2013). Article from the Food Ingredients First, January 2013

Bahloul, N., Boudhrioua, N., Kouhila, M., Kechaou, B., (2009). *Effect of Convective Drying on Colour, Total Phenols and Radical Scavenging Activity of Olive Oil Leaves (Olea europea L.).* Int. J. Food Sci. and Techn., 44, pp. 2561-2567.

Barbosa-Cánovas Gustavo V. and Humberto Vega-Mercado, (1996). *Dehydration of food.* New York: Chapman & Hall.

Barriocanal, L., M. Palacios, G. Benitez, S. Benitez, J. Jimenez, N. Jimenez, et al. (2008) - Apparent lack of pharmacological effect of steviol glycosides used as sweeteners in humans, a pilot study of repeated exposures in some normatensive and hypotensive individuals and in type 1 and type 2 diabetics. Regulatory Toxicology and Pharmacology, 51, pp. 37–41.

Bassey, M.W., (1985). *Design and performance of hybrid crop dryer using solar-energy and sawdust.* In: Proc ISES Cong INTERSOL 85. Montreal, Canada, Pergamon Press, Oxford, p. 1039-1042.

Bharti A.K., Khurana J.P. (1997). *Mutant of Arabidopsis astools to understand the regulation of phenylpropanoids pathway and UV-B protection mechanism.* J Photochem Photobiol, 65: pp. 765–776

Bian, Y.M., (1981). *Studies on Stevia rebaudiana a new sweettasting plant: refining stevioside and determination of its concentration.* Plant Physiol. Commun. 3: 15-17. (in Chinese, English abstract.).

Blauth de Slavutzky, S. (2010). *Stevia and sucrose effect on plaque formation.* Journal für Verbraucherschutz und Lebensmittelsicherheit, 5, 213–216.

Blumenthal, M., (1996). *Perspectives of FDA'S new Stevia Policy, After four years, the agency lifts its ban-but only partially.* Whole Foods Magazine, pp. 1–5.

Bowrey, R.G., Buckle, K.A., Hamey, I., Pavenayotin, P., (1980). *Use of solar-energy for banana drying*. In: Food Technol. Aust., 32(6):290-291.

Brandle J.E., Telmer P.G. - Steviol glycoside biosynthesis – Phytochemistry, 2007 Jul; 68(14):1855-63. Epub 2007 Mar 29.

Brandle, J., (2001). *Stevia rebaudiana with altered steviol glycoside composition.* U.S. Patent no. US 6255557 B1.

Brandle, J., P. Telmer, (2007). *Steviol glycoside biosynthesis Phytochemistry,* 68, pp. 1855–1863.

Brand-Williams, W., Cuvelier, M.E., Berset, C., (1995). Use of free radical method to evaluate antioxidant activity. Lebensm Wiss Technology; 28:25-30.

Braz de Oliveira, A. J., Correia Gonçalves, R. A., Cantuaria Chierrito, T. P., Müller dos Santos, M., Merade Souza, L., Gorin, P.A.J.,et al., (2011). *Structure and degree of polymerisation of fructooligosaccharides present in roots and leaves of Stevia rebaudiana (Bert.) Bertoni.* Food Chemistry, 129, 305–311.

Bright. Graham (1999). *Low-Calorie Sweeteners.* IUFoST World Conference. Barcelona. April 1999". Brussels: Corti A.

Buckenhuskers, J., Omran, H., (1997). *Stevia rebaudiana Bertoni* and stevioside, sugar and its substitutes. Food Proc. Nutr.; pp. 157-178.

Burton. W.G. (1982). *Post-Harvest Physiology of Food Crops*. New York: Longman Inc.. ISBN 0-582-46038-7

Calatayud A., Roca D., Gorbe E., Martynez P.F., (2007). *Light acclimation in rose (Rosa hybrida cv. Grand Gala) leaves after pruning: effects on chlorophyll a fluorescence, nitrate reductase, ammonium and carbohydrates*. Scientia Horticulture 111:152– 159.

Calixto, J.B., (2000*). Efficacy, safety, quality control, market and regulatory guidelines for herbal medicines (phytotherapeutic agents)*. Brazilian Journal of Medical and Biological Research. 33:179-189.

Carakostas, L. Curry, A. Boileau, D. Brusick, (2008). *Overview: The history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages,* Food and Chemical Toxicology, 46, pp. S1–S10.

Carakostas, M., Curry, L., Boileau, A., & Brusick, D., (2008). *Overview: The history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages.* Food and Chemical Toxicology, 46, S1–S10.

Carneiro, J., A. Muniz, T. Guedes, (1997). *Greenhouse bedding plant production of Stevia rebaudiana (Bert) bertoni.* Canadian Journal of Plant Science, 77, pp. 473–474.

Cassacia, J. & Alvarez, E., (2006) – *Recomendaciones tecnicas para una producción sustentable del ka'a he'e (Stevia rebaudiana (bertoni) bertoni) en el Paraguay*. Ministerio de Agricultura y Ganadería. Instituto Agronómico Nacional. Programa de investigación de Ka´á He´é, Manual técnico No. 8. Paraguay.

Cedus - Le Sucre - Statistics Memo 2006–2007. Link visited in 7th of May of 2015, at http://www.lesucre.com/

Chakraverty A., Das S.K., (1986). *Design and testing of an integrated solar-cum-husk ®red paddy dryer of one tonne per day capacity.* Int Drying Symp, Drying 86, Cambridge, Mass, U.S.A., 692-702.

Chalapathi, M.V. and S. Thimmegowda, (1997). *Natural non-calorie sweetener stevia (stevia rebaudiana Bertoni).* A future crop of India. Crop Research Hisar, 14 (2): 347-350.

Chan, P., Linson, B., Chen, Y., Liu, J., Hsieh, M., & Cheng, J., (2000). *A double blind placebo-controlled study of the effectiveness and tolerability of oral stevioside in human hypertension.* British Journal of Clinical Pharmacology, 50, 215–220.

Chatsudthipong, V., Muanprasat, C., (2009). *Stevioside and related compounds: Therapeutic benefits beyond sweetness.* Pharmacology & Therapeutics, 121 pp. 41–54

Chaturvedi, S.K., Shen, J.Y., (1984). *Thermal performance of a direct expansion solar-assisted heat pump.* Solar Energy. 33(2):155–62.

Chen, J., Jeppesen, P.,Abudula, R., Dyrskog, S., Colombo, M., & Hermansen, K., (2006). *Stevioside does not cause increased basal insulin secretion or b-cell desensitization as does the sulphonylurea, glibenclamide: Studies in vitro.* Life Science, 78, 1748–1753.

Chou S.K., Chua K.J., (2001). *New hybrid drying technologies for heat sensitive foodstuffs.* Trends Food Sci Tech. 12(10):359–369. http://dx.doi. org/10.1016/S0924-2244(01)00102-9

Chou S.K., Chua K.J., Mujumdar A.S., Tan M., Tan S.L. (2001). *Study on the osmotic pretreatment and infrared radiation on drying kinetics and colour changes during drying of agricultural products*. ASEAN J Sci Technol Dev. 18(1):11–23.

Choudhary, K., & Bandyopadhyay, N. (1999). *Preliminary studies on the inorganic constituents of some indigenous hyperglycaemic herbs on oral glucose tolerance test.* Journal of Ethnopharmacology, 64, 179–184.

Christie, P.J., Alfenito, M.R., Walbot, V., (1994). *Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings.* Planta, 194: pp. 541-549.

Commission Regulation (EU) No 1131/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council with regard to steviol glycosides.

Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council.

Cortés, C.J. (2012). *Análisis de crecimiento del cultivo de stevia (Stevia rebaudiana) con proyección agroindustrial en el valle del cauca.* Universidad de san buenaventura cali. 84 p.

Crammer B. & Ikan R., (1986). *Sweet glycosides from the Stevia plant.* Chemistry in Britain, 22, 915–917.

Crammer, B., & Ikan, R. (1987*). Progress in the chemistry and progress of the rebaudiosides.* In T. Grenby (Ed.), Developments in Sweeteners (pp. 45–64). London, UK: Elsevier Applied Science.

Crane, E., (1975). *Honey: A comprehensive Survey*. London: Heinemann.

Cuba/FAO International Sugar Conference in Havana (1999). *Sugar: Sustainability in the 21st Century – Outlook to 2005 and Inter-Regional Competitiveness*.

Curry, E., (1997). *Temperatures for optimum anthocyanin accumulation in apple tissue.* J. Hortic. Sci. 72(5): p 723–729.

Daghigh, R., Ruslan, M.H., Yusof Sulaiman, Kamaruzzaman Bin Sopian, (2010) Article: *Review of solar assisted heat pump drying systems for agricultural and marine products*. Renewable and Sustainable Energy Reviews (Impact Factor: 5.9). 14(9):2564-2579. DOI: 10.1016/j.rser.2010.04.004.

De Oliveira, B., Packer, J., Chimelli, M., & de Jesus, D., (2007). *Enzymatic modification of stevioside by cell-free extract of Gibberella fujikuroi.* Journal of Biotechnology, 131, 92–96.

Debnath, M., (2008). *Clonal propagation and antimicrobial activity of an endemic medicinal plant Stevia rebaudiana.* Journal of Medicinal Plants Research, 2, 45–51.

Delaquis, P.J., Stewart, S., Toivonen, P. M. A., & Moyls, A. L. (1999). *Effect of warm, chlorinated water on the microbial flora of shredded iceberg lettuce.* Food Research International (Ottawa, Ont.) Food Research International, 32(1), 7-14.

Dicosmo F., Misawa M., (1985). *Eliciting secondary metabolism in plant cell cultures.* Trends Biotechnol; 3:318-22.

Dixon, R.A., and Paiva N.L., (1995). *Stressed induced phenyl propanoid metabolism.* Plant Cell, 7:1085-97.

Draycott, (1972). *Sugar-beet nutrition*. New York : Wiley. ISBN 0470221607.

Draycott, A.P & Christenson, D.R., (2003). *Nutrients for Sugar Beet Production: Soil–Plant Relationships.* CABI Publishing, Wallingford. ISBN 0-85199-623-X.

Drew, M. (1998). *Stress physiology.* In: Taiz L, Zeiger E, editors. Plant physiology. Sunderland, Mass.: The Sinauer Associates, Inc. p 725–757.

DuBois, G.E., (2000). *Sweeteners: non-nutritive.* Pages 2245-2265 in F. J. Francis, ed. Encyclopedia of food science and technology Vol.4. 2nd ed. John Wiley and Sons, Inc., New York, NY.

Dwivedi, R.S., (1999). *Unuttered and untapped super sweet nonsaccharіferous plant species in India.* Current Sci. (Bangalore) 76, pp. 1454–1461.

Edeoga, H., Okwu, D., & Mbaebie, B., (2005). *Phytochemical constituents of some Nigerian medicinal plants.* African Journal of Biotechnology, 4, 685–688.

(EFSA) European Food Safety Authority Panel on Food Additives and Nutrient Sources added to Food (ANS), (2010). Scientific Opinion on the safety of steviol glycosides for the proposed uses as a food additive. EFSA J., 8 (4), 1537.

Ekechukwu, O.V., Norton, B., (1999). *Review of Solar-Energy Drying Systems II: An Overview of Solar Drying Technology.* Energy Conversion and Management, 40, pp. 615–655.

Esper, A., Mühlbauer, W., (1998). *Solar Drying – An Effective Means of Food Preservation.* Renewable Energy, 15, pp. 95-100.

FAO, 2009 – "Sugar Beet - White Sugar" - Agribusiness Handbook.

FAO, 2014 – Food and Nutrition, ISBN 978-92-5-108617-9

Faragher, J., Brohier, R., (1984). *Anthocyanin accumulation in apple skin during ripening: regulation by ethylene and phenylalanine ammonia-lyase.* Sci Hortic 22: p 89–96.

Fardus, S., Wahid A., Javed F. and Sadia, B., (2014). *Changes in leaf phenolics concentrations determine the survival of evening primrose (Oenothera biensis) in various seasons.* Int. J. Agric. Biol., 16: 819– 824.

Farias, M.R., (2003). *Assessment of quality of raw vegetables.* In: Simões, C.M.O. et al. Pharmacognosy: the plant drug. 5th ed. Porto Alegre / Florianópolis: UFRGS Publisher / Editor of UFSC.

Fernando Lidon and Maria Manuela Silvestre, (2008). *Conservação de Alimentos – Princípios e Metodologias.* Escolar Editora, Lisboa. pp 96.

(FMI) Future Market Insights (2014). Global Stevia Market Analysis & Opportunity Assessment. 2014 – 2020. Report

Folin, O., Ciocalteu, V., (1927). *On tyrosine and tryptophan determination in proteins.* Journal of Biology and Chemistry; 27:627-650.

Free, J.B., (1982). *Bees and Mankind*. Boston, MA: George Allen and Unwin.

Gardana, C., Scaglianti, M., & Simonetti, P. (2010). *Evaluation of steviol and its glycosidesin Stevia rebaudiana leavesand commercialsweetener byultra-highperformance liquid chromatography–mass spectrometry.* Journal of Chromatography A, 1217, 1463–1470.

Geuns, J., (2003). *Stevioside.* Phytochemistry, 64, 913–921.

Ghosh & Sudha, M.L., (2012). *A review on polyols: new frontiers for health-based bakery products,* SOMA

Ghosh, S., Subudhi, E., & Nayak, S., (2008). *Antimicrobial assay of Stevia rebaudiana Bertoni leaf extracts against 10 pathogens.* International Journal of Integrative Biology, 2, 27–31.

Ginzburg, A.Sz., (1968). *Drying in Food Industry.* Budapest, Hungary, Muszaki Konyvkiado, pp. 39-47.

Ginzburg, A.Sz., (1976). *Principles of Drying Theory and Techniques of Foods.* Budapest, Hungary, Mezogazdasagi Kiado, pp. 28-51.

Giraldo, C., Marín, L., & Habeych, D. (2005). *Obtención de Edulcorantes de Stevia rebaudiana Bertoni.* Revista CENIC Ciencias Biológicas, 36, 3–10.

Given, N.K., M.A. Venis, D. Gierson, *(1988). Hormonal regulation of ripening in the strawberry, a non-climacteric fruit.* Article at Planta, 174, Issue 3, pp 402-406.

Global Stevia Institute. Link visited in 9th December 2014 at http://globalsteviainstitute.com/consumers/stevia-story/what-is-stevia/.

Gomez, F., Chaves, A., Bartoli, C. G., Guiamet, J. J., Fernandez, L., & Gergoff, G. (2008). *Heat shock increases mitochondrial H_2O_2 production and extends postharvest life of spinach leaves.* Postharvest Biology and Technology, 49, 229-234.

Gordana Niketic – Aleksic, (1994). *Tehnologija Voca i Povrca,* Poljoprivredni Fakultet, Beograd – Zemun.

Goyal S. K., Samsher, Goyal R. K. (2010). *Stevia (Stevia rebaudiana) a bio-sweetener: a review*. International Journal of Food Sciences and Nutrition. Vol. 61. No. 1: Pages 1-10

Grof, C.P.L. and Campbell, J.A., (2001). *Sugarcane sucrose metabolism: scope for molecular manipulation.* Australian Journal of Plant Physiology 28: 1-12.

Handro, W., Ferreira, C.M. and Floh, E.I.S., (1993). *Chromosomal variability and growth rate in cell suspension cultures of Stevia rebaudiana.* Plant Sci. 93:1

Harris, B., Jr., and Staples, C. R., (1998). *Sugarcane Bagasse*. Studies by the University of Florida.

Hellfritsch C, Brockhoff A, Staehler F, Meyerhof W, Hofmann T. Human, (2012). *Psychometric and Taste Receptor Responses to Steviol Glycosides.* Journal of Agricultural and Food Chemistry, 60(27):6782–6793.

Hossain A.B.M.S., Amru N.B., Normaniza O., (2007). *Postharvest Quality, Vase Life and Photosynthetic Yield (Chlorophyll Fluorescence) of Bougainvillea Flower by Applying Ethanol.* Australian Journal of Basic and Applied Sciences, 1: 733-740.

Hossain, A.B.M.S., Fusao, M., (2008). *Determination of abscisic acid hormone (ABA), mineral content, and distribution pattern of 13C photoassimilates in bark-ringed young peach trees.* Moj Int J Sci Tech 2: 274-284.

Hsieh, M., Chan, P., Sue, Y., Liu, J., Liang, T., Huang, T., et al., (2003). *Efficacy and tolerability of oral stevioside in patients with mild essential hypertension: A two-year, randomized, placebo-controlled study.* Clinical Therapeutics, 25, 2797–2808.

Huang, B.K., Toksoy, M., (1983). *Design and analysis of greenhouse solar systems in agricultural production.* Energy in Agric, 2: 115-136.

(IMS) Innova Market Insights, (2015). Link consulted in 5th of February 2015, at http://www.foodingredientsfirst.com/IngredientFocus/Stevias-Continued-Surge.html

Inugraha, Maghfoer, M.D., and Widaryanto, E., (2014). *Response of Stevia (Stevia rebaudiana Bertoni M) To Nitrogen and Potassium Fertilization.* IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS). e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 7, Issue 10 Ver. I, pp 47-55.

Ishwar Singh, Sapna Langyan, Pranjal Yadava, (2014). *Sweet Corn and Corn-Based Sweeteners*. Sugar Tech, Journal - June 2014, Volume 16, Issue 2, pp 144-149

J.Banout, P. Ehl, J. Havlik, B. Lojka, Z. Polesny, and V. Verner, (2011). *Design and performance evaluation of a Double-pass solar drier for drying of red chilli (Capsicum annum L.).* Solar Energy, vol. 85, nº. 3: 506–515.

Jain, D., Tiwari, G. N., (2003). *Thermal aspects of open sun drying of various crops.* Energy 28, pp. 37–54.

Jairaj, K.S., Singh, S.P., Srikant, K., (2009). *A Review of Solar Dryers Developed for Grape Drying.* Solar Energy, 83, pp. 1698–1712.

Jayaraman, S., Manoharan, M., & Illanchezian, S. (2008). *In-vitro antimicrobial and antitumor activities of Stevia rebaudiana (Asteraceae) leaf extracts.* Tropical Journal of Pharmaceutical Research, 7: 1143–1149.

Jayaraman, S., Manoharan, M., & Illanchezian, S., (2008). *In-vitro antimicrobial and antitumor activities of Stevia rebaudiana (Asteraceae) leaf extracts.* Tropical Journal of Pharmaceutical Research, 7, 1143–1149.

Jeppesen, P., Gregersen, S., Poulsen, C., & Hermansen, K., (2000). *Stevioside acts directly on pancreatic a cells to secrete insulin: Actions independent of cyclic adenosine monophosphate and adenosine triphosphate-sensitive K+ channel activity*. Metabolism, 49, 208–214.

Jeppesen, P., Gregersen, S., Rolfsen, S., Jepsen, M., Colombo, M., Agger, A., et al. (2003). Antihyperglycemic and blood pressure-reducing effects of stevioside in the diabetic Goto-Kakizaki rat. Metabolism, 52, 372–378.

Jimoh, F.O. and Oladiji, A.T., (2005). *Preliminary studies on Piliostigma thonningii seeds: Preliminary analysis, mineral composition and phytochemical screening*. Afr. J. Biotechnol. 4: 1439-1442.

Jochum, G.M., K.W. Mudge and R.B. Thomas, (2007). *Elevated temperatures increase leaf senescence and root secondary metabolite concentrations in the understory herb Panax quinquefolius (Araliaceae).* Amer. J. Bot., 94: 819–826.

Kader AA (1992) Postharvest biology and technology: an overview. In: Kader AA (ed) Postharvest technology of horticultural crops. UC Publication No. 3311. University of California, Division of Agriculture and Natural Resources, Oakland, pp 15–20.

Kalt W, Howell A, Duy JC, Forney CF, McDonald JE. 2001. *Horticultural factors affecting antioxidant capacity of blueberries and other small fruit.* Hort. Technol. 11(4): 523–528.

Kang, K.H., Lee, E.W. (1981). *Physio-ecological studies on stevia (Stevia rebaudiana Bertoni).* Korean J. Crop Sci. 1981, 26(1), pp. 69-89.

Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N, Sung DY, Guy CL (2004). *Exploring the temperature-stress metabolome of Arabidopsis.* Plant Physiol 136: 4159–4168.

Katarzyna Gaweł-Bęben, Tomasz Bujak, Zofia Nizioł-Łukaszewska, Beata Antosiewicz, Anna Jakubczyk, Monika Karaś and Kamila Rybczyńska, (2014). *Stevia Rebaudiana Bert. Leaf Extracts as a Multifunctional Source of Natural Antioxidants.* Molecules, 20: 5468-5486.

Katayama, O., T. Sumida, H. Hayashi, H. Mitsuhashi, (1976). *The practical application of Stevia and research and development data* (pp. 747) I.S.U. Company, Japan.

Kaushik, R., Pradeep, N., Vamshi, V., Geetha, M., Usha, A. (2010). *Nutrient composition of cultivated Stevia leaves and the influence of polyphenols and plant pigments on sensory and antioxidant properties of leaf extracts.* J. Food Sci. Tech., 47, 27–33.

Kays, S., (1991). *Postharvest physiology of perishable plant products.* In: Kays S, editor. An Avi book. New York: Van Nostrand Reinhold. p 127, 128, 207.

Kennelly, E. (2002). *Sweet and non-sweet constituents of Stevia rebaudiana (Bertoni) Bertoni. In A. Kinghorn (Ed.). Stevia, the Genus Stevia.* Medicinal and aromatic plants–industrial profiles (vol. 19, pp. 68–85). London, NY: Taylor and Francis.

Kiba A., Tomiyama H., Takahashi H., Hamada H., Ohnishi K., Okuno T., Hikichi Y., (2003). *Induction of Resistance and expression of defence related genes in tobacco leaves infiltrated with Ralstonia solanacearum.* Plant & Cell Physiology 44, 287–295.

Kim K.K., Sawa Y. and Shibata H., (1996). *Hydroxylation of entkaurenoic acid to steviol in Stevia rebaudiana Bertoni: purification and partial characterization of the enzyme.* Arch. Biochem. Biophys. 332: 223-230.

Kim, I., Yang M., Lee, O. & Kang, S. (2011). *The antioxidant activity and the bioactive compound content of Stevia rebaudiana water extracts*. LWT – Food Science and Technology, 44, 1328–1332.

Kinghorn, A., Soejarto, D., (1985). *Current status of stevioside as a sweetening agent for human use.* in: H. Wagner, H. Hikino, N. Farnsworth (Eds.), Economics and medicinal plant research, vol. 1, Academic Press, London, UK (1985), pp. 1–52.

Kochikyan, V., Markosyan, A., Abelyan, L., Balayan, A., & Abelyan, V., (2006). *Combined enzymatic modification of stevioside and rebaudioside A*. Applied Biochemistry and Microbiology, 42, 31–37.

Kolb, N., J.L. Herrera, D.J. Ferreyra, and R.F. Uliana, (2001). *Analysis of Sweet Diterpene Glycosides from Stevia rebaudiana: Improved HPLC Method.* J. Agric. Food Chem. 2001, 49, 4538−4541.

Konica Minolta (1998). *Precise Color Communication Manual*. SENSING, INC.

Kovylyaeva, G., Bakaleinik, G., Strobykina, I., Gubskaya, V., Sharipova, R., Al¢fonsov, V., et al., (2007). *Glycosides from Stevia rebaudiana*. Chemistry of Natural Compounds, 43, 81–85.

Kovylyaeva, G.I., Bakaleinik, G.A., Strobykina, I.Y., Gubskaya, V.I., Sharipova, R.R., Al'Fonsov, V.A., Kataev, V.E., Tolstikov, A.G., (2007). *Glycosides from Stevia rebaudiana.* Chem Nat Compd 43(1):81–85.

Koyama, E., Kitazawa, K., Ohori, Y., Izawa, O., Kakegawa, K., Fujino, A., et al. (2003). In vitro metabolism of the glycosidic sweeteners, Stevia mixture and enzymatically modified Stevia in human intestinal microflora. Food and Chemical Toxicology, 41, 359–374.

Kuang, Y.H., Wang R.Z., Yu L.Q., (2003). *Experimental study on solar assisted heat pump system for heat supply.* Energy Conversion and Management. 44(7):1089–98.

Landázuri, P. & Tigrero, J. (2009). *Stevia rebaudiana Bertoni, una planta medicinal.* Sangolquí, Ecuador. Escuela Politécnica del Ejército. 34 p.

Latham, M., (2002*). Macronutrientes: Carbohidratos, proteínas y grasas. In Nutrición humana en el mundo en desarrollo.* vol. 29 (pp. 99–107). Roma: FAO.

Lee, C. N., Wong, K., Liu, J., Chen, Y., & Chan, P., (2001*). Inhibitory effect of stevioside on calcium influx to produce antihypertension.* Planta Medica, 67: 796–799.

Leeper, GW & Uren, NC 1993 Soil Science. 5th edn. Melbourne University Press.

Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L. & Ah-Hen K. (2012). *Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: a comprehensive review on the biochemical, nutritional and functional aspects*. Food Chemistry 132, Pages 1121–1132.

Li K.T., Lakso A.N. Piccioni R., Robinson T., (2009). *Summer pruning reduces whole-canopy carbon fixation and transpiration in apple trees.* J. Hort. Sci. Biotech. 78: 749–754.

Lindsey Bagley (2013). Article *Making the Sweetest Choice*. J. of The World of Food Ingredients, Feb. 2013, pp. 13.

Maeda, E.E., and Salunkhe, D. K., (1981). *Retention of Ascorbic Acid and Total Carotene in Solar Dried Vegetables.* Journal of Food Sci., Vol. 46, issue 4, pp. 1288-1290.

Magnuson, Bernadene A., G.A. Burdock, J. Doull. R.M. Kroes, G.M. Marsh, M.W. Pariza, P.S. Spencer, W.J. Waddell, R. Walker, G.M. Williams, 2007. "Aspartame: A Safety Evaluation Based on Current Use Levels, Regulations, and Toxicological and Epidemiological Studies" - Critical Reviews in Toxicology, 37:629–727, 2007 Copyright Informa Healthcare USA, Inc. ISSN: 1040-8444 print / 1547-6898 online DOI: 10.1080/10408440701516184.

Maheshwar, H.M. (2005). *Effect of different levels of nitrogen and dates of planting on growth and yield of Stevia (Stevia rebaudiana Bert.).* MSc Thesis. Department of Horticulture College of agriculture, Dharwad University of Agricultural Sciences, Dharwad, 66 pp.

Maki, K., Curry, L., Reeves, M., Toth, P., Mckenney, J., Farmer, M., et al., (2008). *Chronic consumption of rebaudioside A, a steviol glycoside, in men and women with type 2 diabetes mellitu*s. Food and Chemical Toxicology, 46, 47–53.

Manish, B., Tadhani and Rema, S., (2006). *In Vitro Antimicrobial Activity of Stevia Rebaudiana Bertoni Leaves.* Tropical Journal of Pharmaceutical Research, 5: pp. 557-560.

Margaret A. Clarke, (2014). Link visited in 5th of January 2015 at http://www.britannica.com/topic/sugar-chemical-compound

Martins, P.M., Melo, E.C., Barbosa, L.C.A., (2002). *Influence of temperature and air drying velocity on the content and chemical composition of essential oil of lemongrass.* Acta Horticulturae, 569, pp. 155-160.

Mcguire, R.G., (1992). Reporting of Objective Color Measurements. Hortscience 27, 1254-1255.

Megeji N.W., Kumar J.K., Virendra Singh, Kaul V.K., Ahuja P.S. (2005). *Introducing Stevia rebaudiana, a natural zero-calorie sweetener*. Current Science, 88,5:801-804.

Megeji, N. W., Kumar, J. K., Virendra, S., Kaul, V. K. and Ahuja, P. S. 2005. *Introducing Stevia rebaudiana, a natural zero-calorie sweetener.* Current Sci. 88: 801-804.

Mehmet Ozgur, Arzu Akpinar-Bayazit, Tulay Ozcan, Lutfiye Yilmaz-Ersan, (2011). *Effect of Dehydration on Several Physico-Chemical Properties and the Antioxidant Activity of Leeks (Allium porrum L.).* Not. Bot. Hort. Agrobot. Cluj., 39(1):144-151.

Mekhilefa, S., Saidurb, R., Safari, A., (2011). *A review on solar energy use in industries.* Renewable and Sustainable Energy Reviews, 15, pp. 1777–1790.

Metivier J., and Viana A.M., (1979). *The effect of long and short day length upon the growth of whole plants and the level of soluble proteins, sugars and stevioside in leaves of Stevia rebaudiana Bert*. J. Exp. Bot. 30: 1211-1222.

Midmore, D.J. & Rank, A.H. (2002). *A new rural industry – Stevia – to replace imported chemical sweeteners.* Rural Industries Research and Development Corporation. 50 p.

Miller N.J., Rice-Evans C.A., Davies M.J., Gopinathan V., Milner A., (1993). *A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates.* Clin. Sci., 84, 407-412.

Mishra, P., Singh, R., Kumar, U., Prakash, V., (2010). *Stevia rebaudiana* – A magical sweetener. Global Journal of Biotecnology & Biochemistry, 5, pp. 62–74.

Mitsuhashi, H., Ueno, J., Sumita, T., (1975). *Studies on the cultivation of Stevia rebaudiana Bertoni. Determination of stevioside*. J. Pharm. Soc. Jpn., 95(1), 127-130.

Mohammad, M., Mohammad, U., Sher, M., Habib, A., & Iqbal, A., (2007). *In vitro clonal propagation and biochemical analysis of field established Stevia rebaudiana Bertoni.* Pakistan Journal of Botany, 39, 2467–2474.

Mohammed Abdalbasit A. Gasmalla, Ruijin Yang , Xiao Hua, (2014). - *Review Article: Stevia rebaudiana Bertoni: An alternative Sugar Replacer and Its Application in Food Industry* - Food Engineering Reviews, Volume 6, Issue 4, pp 150-162.

Mohammed Saifuddin, A.B.B.S. Hossain, N. Osman, M.A. Sattar, K.M. Moneruzzaman, and M.I. Jahirul, (2010). *Pruning impacts on shoot-root-growth, biochemical and physiological changes of Bougainvillea glabra.* Australian J. of Crop Sci. 4(7): 530-537.

Monika Sabolová, Lenka Kourimská, Jan Pánek (2012). *Antioxidant Activity of Lamiaceae Herbs Grown under organic conditions.* International Conference of Chem. Reactions in Foods (CRF 2012), 17. Prague, Czech Republic.

Montero, I., Blanco, J., Miranda, T., Rojas, S., Celma, A.R., (2010). *Design, construction and performance testing of a solar dryer for agro-industrial by-products.* Energy Conversion and Management, 51, pp. 1510-1521.

Mori, N., Sakanoue, M., Takcuchi, M., Shimpo, K., & Tanabe, T., (1981). *Effect of stevioside on fertility in rats.* Journal of the Food Hygienic Society of Japan, 22, 409–414.

Morrison, G.L. (1994). *Simulation of packaged solar heat-pump water heaters. Solar Energy.* 53(3):249–57.

Muanda, F., Soulimani, R., Diop, B., & Dicko, A., (2010). *Study on chemical composition and biological activities of essential oil and extracts from Stevia rebaudiana Bertoni leaves.* LWT – Food Science and Technology. doi:10.1016/ j.lwt.2010.12.002.

Mulokozi, G., Svenberg, U., (2003). *Effect of Traditional Open-Sun Drying and Solar Cabinet Drying on Carotene Content and Vitamin A Activity of Green Leafy Vegetables.* Plant Foods for Human Nutrition, 58, pp. 1-15.

Murata, M., Tanaka, E., Minoura, E., Homma, S., (2004). *Quality of cut lettuce treated by heat shock: prevention of enzymatic browning, repression of phenylalanine ammonia-lyase activity, and improvement on sensory evaluation during storage.* Biosci. Biotech. Biochem. 68, pp. 501–507.

NEDFI, (2007). *Stevia.* Handbook on Medicinal & Aromatic Plants, pp. 79-83.

Nepovim, A., Drahosova, H., Valicek, P., & Vanek, T., (1998). *The effect of cultivation conditions on the content of stevioside in Stevia rebaudiana Bertoni plants cultivated in the Czech Republic.* Pharmaceutical and Pharmacological Letters, 8, 19–21.

Nozolillo P., Isabelle G.D., (1990). Seasonal changes in phenolics constituents of jack pine seedlings (Pinus banksiana) in relation to the purpling phenomenon. Canadian J. of Botany, 68:2010–2017.

O'Donnell, K., (1998). *Molecular genetic approaches for measuring microbial diversity: Examples from filamentous fungi.* National Institute of Agrobiological Resources, Tsukuba, Ibaraki, Japan. pp. 7-23.

Ossom, E.M., Matsenjwa, V.N., 2007. Influence of mulch on agronomic characteristics, soil properties, disease and insect pest infestation of dry bean (Phaseolus vulgaris L.) in Swaziland. World J. Agric. Sci. 3, 696-703.

Pack, D. A., (1927). *Ring density of sugar beets as a character for selection*. Am. J. Bot. 14: 23 8-245.

Pack, D.A., (1930). *Selection characters as correlated with percentage of sucrose, weight and sucrose content of sugar beets.* J. Agr. Res. 40:5 23 -546.

Pande, S. S., & Gupta, P., (2013). *Plant tissue culture of Stevia rebaudiana (Bertoni): A review.* Journal of Pharmacognosy and Phytotherapy. Volume 5. No. 1. pp. 26-33.

Patrik Jones and Thomas Vogt, (2001). *Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers.* Planta, Vol. 213, No. 2 , pp. 164-174

Periche, A., Castelló M.L., Heredia, A., Escriche, I. (2015). *Influence of Extraction Methods on the Yield of Steviol Glycosides and Antioxidants in Stevia rebaudiana Extracts.* Plant Foods Human Nutrition, 70(2): 119-127.

Phansawan, B., & Poungbangpho, S., (2007). *Antioxidant capacities of Pueraria mirifica, Stevia rebaudiana Bertoni, Curcuma longa Linn., Andrographis paniculata (Burm.f.) Nees and Cassia alata Linn for the development of dietary supplement.* Kasetsart Journal: Natural Science, 41, 548–554.

Pól, J., Hohnová, B., & Hyötyläinen, T., (2007). *Characterization of Stevia rebaudiana bycomprehensive two-dimensional liquidchromatography time-of-flight mass spectrometry.* Journal of Chromatography A, 1150, 85–92.

Pól, J., Ostra, E. V., Karasek, P., Roth, M., Karolinka, B., Kotlarikova, P., et al., (2007). *Comparison of two different solvents employed for pressurised fluid extraction of stevioside from Stevia rebaudiana: Methanol versus water.* Analytical and Bioanalytical Chemistry, 388, 1847–1857.

Prakash, I., Dubois, G., Clos, J., Wilkens, K., & Fosdick, L., (2008). *Development of rebiana, a natural, non-caloric sweetener.* Food and Chemical Toxicology, 46, S75–S82.

R. Kumara, S. Sooda, S. Sharmaa, R.C. Kasanab, V.L. Pathaniaa, B. Singha, R.D. Singhc, (2014). *Effect of plant spacing and organic mulch on growth, yield and quality of natural sweetener plant Stevia and soil fertility in western Himalayas.* International Journal of Plant Production 8(3): 311-334.

Rahmesh K., V. Singh, N. Megeji (2006). *Cultivation of Stevia (Stevia rebaudiana (Bert) Bertoni): A comprehensive review,* Advances in Agronomy, 89, pp. 137–177.

Rahmesh, K., V. Singh, N. Megeji, (2006*). Cultivation of Stevia (Stevia rebaudiana (Bert) Bertoni): A comprehensive review*. Advances in Agronomy, 89 (2006), pp. 137-177.

Randi, A.M., (1980). *Germinação de Stevia rebaudiana Bert.* M.Sc. thesis. Universidade Estadual de Campinas, Campinas, Brazil. [English summary.]

Rank, A., & Midmore, J., (2006). *Stevia – An intense natural sweetener: Laying the groundwork for a new rural industry.* RIRDC Publication No. 06/020 RIRDC Project No UCQ-17A.

Ravichandran R., (2004). *The impact of pruning and time from pruning on quality and aroma constituents of black tea.* Food Chem., 84:7-11.

Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives.

René Borstel / KNAUER, 2009 - Separation of steviol glycosides, KNAUER leaflet

Renwick A.G. and Lazarus NR (1998). Human variability and noncancer risk assessment-An analysis of the default uncertainty fac; tor. Regul. Toxicol. Pharmacol. 27: 3-20.

Renwick A.G. et al., (2003). *Risk Characterisation of chemicals in food and diet*. Food Chem Toxicol 41: 1211–1271 [FOSIE].

Revina, T.A., Karnachuk, R.A. and Tailashova, P.Ya., (1986*). The dynamics of contents of ecdisterone in the above ground part of Serratula coronata L. and the effect of light of various spectral composition on it*. Rast. Resursy. 22: 70-72.

Richman AS, Gijzen M, Starratt AN, Yang Z, Brandle JE (1999). *Diterpene synthesis in Stevia rebaudiana: recruitment and up-regulation of key enzymes from the gibberellin biosynthetic pathway.* Plant J.19(4):411-21.

Roger Wyse, (1979). *Sucrose Uptake by Sugar Beet Tap Root Tissue.* Plant Physiol. 64(5): 837–841. PMCID: PMC543374

Romero-Rodrigues, A., L.V. Oderiz, J.L. Hernandez, J.S. Gandara, (1992). *Comparaison de deux máthodes de dosage par CLHP de l'acide ascorbique dans Carica pentagona*. Sci. Aliments, 12, pp 593-600.

Ruta C., De Mastro G., Morone Fortunato I., Marzi V.. 1999. Modalità di propagazione e tecniche di coltivazione di Stevia rebaudiana Bertoni. Acts XXXIII SIA congress 1999: 310-311.

Sablani, S.S., (2006). *Drying fruits and vegetables: retention of nutritional/functional quality.* Drying Technology, 24 (2), pp 123-135.

Saltveit, M.E., (1997). *Physical and physiological changes in minimally processed fruits and vegetables.* In: Tom´as-Barber´an, F.A., Robins, R.J. (Eds.), Phytochemistry of Fruit and Vegetables. Oxford University Press, New York, pp. 205–220.

Samsudin, A., and Aziz, I.Ab., (2013). *Drying of Stevia leaves using laboratory and pilot scale dryers.* J. Trop. Agric. and Fd. Sc. 41(1)(2013): 137–147.

Sanjay Sharma, V.K. Sharma, Ranjana Jha, R.A. Ray, (1990). *Evaluation of the performance of a cabinet type solar dryer.* Article at Energy Conversion and Management. Volume 30, Issue 2, 1990, Pages 75-80.

Sapers, G., Hicks, K., Burgher, A., Hargrave, D., Sondey, S., Bilyk, A., (1986). *Anthocyanin patterns in ripening Thornless blackberries*. J. Am. Soc. Hortic. Sci. 111(6): p 945–950.

Sara Roura, del Valle C.E., Luciana Pereyra (2008). *Phenylalanine ammonia lyase activity in fresh cut lettuce subjected to the combined action of heat mild shocks and chemical additives. Article at* Lebensmittel-Wissenschaft und-Technologie (Impact Factor: 2.42); 41(5):919-924.

Saurabh Sharma, Rakesh Kumar, Bikram Singh, (2014). *Post Harvest drying affects Steviol Glycosides in Stevia (Stevia Rebaudiana Bertoni).* Conference: National Symposium on "Agriculture Diversification, for Sustainable Livelihood and Environmental Security", At Punjab Agricultural University, Ludhiana from November 18–20, 2014., Volume: "Agriculture Diversification, for Sustainable Livelihood and Environmental Security" November 18–20, 2014, Page no. 852-853.

Schwartz, S.J., von-Elbe, J.H., (1983). *Kinetics of Chlorophyll Degradation to Pyropheophytin in Vegetables.* Journal of Food Science, 48, pp. 1303 – 1306.

Seema, T. (2010). *Stevia rebaudiana: A medicinal and nutraceutical plant and sweet gold for diabetic patients.* International Journal of Pharmacy & Life Sciences, 1, 451–457.

Seigler, D.S., (1998). *Plant Secondary Metabolism.* Boston MA: Chapman and Hall (Kluwer Academic Publishers):711.

Sekaran, T., Giridhar, P. and Ravishankar, G.A. (2007). *Production of Stevioside in ex vitro and in vitro grown Stevia rebaudiana Bertoni.* J. Sci. Foof Agric. 87: 420-424.

Serdar *Öztekin* and Milan Martinov, (2007). *Medicinal and Aromatic Crops, Harvesting, Drying and Processing.* Haworth Food & Agricultural Products Press TM , An Imprint of the Haworth Press, Inc., 10 Alice Street, Binghamton, New York 13904-1580 USA

Serio, L. (2010). *La Stevia rebaudiana, une alternative au sucre.* Phytothérapie, 8, 26–32.

Shaikh M.K. and Hulmani N.C., (1997). *Effect of pruning on Shoot Growth, Leaf Area and Yield in Guava. Karnataka J. Agric. Sci., 10 (1), pp 93-97.*

Shantanu Mandala, Heikham Evelina, Bhoopander Girib, Ved Pal Singha, Rupam Kapoora, (2013). *Arbuscular mycorrhiza enhances the production of stevioside and rebaudioside-A in Stevia rebaudiana via nutritional andnon-nutritional mechanisms.* Applied Soil Ecology, 72, pp. 187– 194.

Sharma, A., Chen, C.R., Lan, N. V., (2009), *Solar-energy drying systems: A review, Renewable and Sustainable Energy Reviews*, 13, pp. 1185-1210.

Sharma, S., Ray, R. A., Sharma, K., (1987). *Comparative study of solar dryers for crop drying.* Invention Intelligence, 22, pp. 105-113.

Shaw, M., V. Meda, L. Tabil Jr.and A. Opoku, (2006). *Drying and color characteristics of Coriander foliage using convective thinlayer and microwave drying.* Journal of Microwave Power & Electromagnetic Energy, 41(2), pp. 56-64.

Shibata, H., Sawa, Y., Oka, T., Sonoke, S., Kim, K. K. and Yoshioka, M., (1995). *Steviol and steviol-glucoside: glucosyltransferase activities in Stevia rebaudiana Bertoni: purification and partial characterization.* Arch. Biochem. Biophys. 321: 390-396.

Shibata, H., Sonoke, S., Ochiai, H., Nishihashi, H. and Yamada, M., (1991). *Glucosylation of steviol and steviolglucosides in extracts from Stevia rebaudiana Bertoni.* Plant Physiol. 95: 152-156.

Shock, C. (1982). *Experimental cultivation of Rebaudi's stevia in California.* University of California – Davis, Agronomy Progress Report, April pp. 122.

Shukla, S., Mehta, A., Bajpai, V., & Shukla, S., (2009). *In vitro antioxidant activity and total phenolic content of ethanolic leaf extract of Stevia rebaudiana Bert.* Food and Chemical Toxicology, 47, 2338–2343.

Shukla, S., Mehta, A., Mehta, P., & Bajpai, V., (2011). *Antioxidant ability and total phenolic content of aqueous leaf extract of Stevia rebaudiana Bert.* Experimental and Toxicologic Pathology. doi:10.1016/j.etp.2011.02.002.

Singh, S., Rao, G., (2005). *Stevia: The herbal sugar of 21st Century*. Sugar Tech, 71, pp. 17–24.

Smith, J. and Van-Stadin, H. (1992). *Subcellular pathway of glycoside synthesis.* S. Afr. J. Sci. 88: 206.

Soejarto, D., (2002). *Botany of Stevia and Stevia rebaudiana*. A. Kinghorn (Ed.), Stevia: The genus Stevia, Taylor and Francis, London, New York, pp. 18–39.

Sood B.K., Kumar N. (1994). *Effect of nitrogen and phosphorus on forage yield and nutrient uptake of oat-berseem mixture.* Crop Res. 8:239–244.

Sreekumar, A., (2010). Techno-economic analysis of roof-integrated solar air heating system for drying fruit and vegetables. Energy Conversion and Management, 51, pp. 2230-2238.

Srijani Ghanta, Anindita Banerjee, Avijit Poddar and Sharmila Chattopadhyay, 2007 - Oxidative DNA Damage Preventive Activity and Antioxidant Potential of *Stevia rebaudiana* (Bertoni) Bertoni, a Natural Sweetener - Journal of Agricultural and Food Chemistry, 2007, 55 (26), pp 10962–10967.

Starrat, A.N., Kirby, C.W., Pocs, R. and Brandle, J.E., (2002). *Rebaudioside F a diterpene glycoside from Stevia rebaudiana.* Phytochemistry 59: 367-370.

Stitt, M., Schaewen, A., Willmitzer L., (1990). *Sink regulation of photosynthetic metabolism in transgenic tobacco plants expressing yeast invertase in their cell wall involves a decrease of the Calvin-cycle enzymes and an increase of glycolytic enzymes.* Planta, 183:40–50.

Strauss, S., (1995) *The perfect sweetener?* Technol. Rev. 98: 1820.

Sudesh Kumar Yadav & Praveen Guleria (2012). *Steviol Glycosides from Stevia: Biosynthesis Pathway Review and their Application in Foods and Medicine,* Critical Reviews in Food Science and Nutrition, 52:11, 988-998, DOI: 10.1080/10408398.2010.519447.

Susann Zahn, Anne Forker, Lydia Krugel, Harald Rohm, (2013). *Combine use of Rebaudioside A and fibres for partial sucrose replacement in muffins.* Food Science and Technology 50, pp 695-701.

Swain, T., Hillis, W. E., (1959). *Phenolic constituents of Prunus domestica i quantitative analysis of phenolic constituents.* Journal of the Science of Food and Agriculture, 10, 63–68.

Tadhani, M., & Subhash, R. (2006a). *Preliminary studies on Stevia rebaudiana leaves: Proximal composition, mineral analysis and phytochemical screening*. Journal of Medical Sciences, 6, 321–326.

Tadhani, M., & Subhash, R., (2006b). *In vitro antimicrobial activity of Stevia rebaudiana Bertoni leaves.* Tropical Journal of Pharmaceutical Research, 5, 557–560.

Tadhani, M.B.; Patel, V.H.; Subhash, R. (2007). *In vitro antioxidant activities of Stevia rebaudiana leaves and callus*. J. Food Compos. Anal., 20, pp. 323–329.

Tamura, Y., Nakamura, S., Fukui, H., & Tabata, M., (1984). *Clonal propagation of Stevia rebaudiana Bertoni by stem-tip culture.* Plant Cell Reports, 3, 183–185.

Tannahill, R. (1975). *Food in History*. St Albans, U.K.: Paladin.

Tanveer, H., S. Ali and M.R. Asi, (2012). *Appraisal of an important flavonoid, quercetin in callus cultures of Citrullus colocynthis.* Int. J. Agric. Biol., 14: 528–532.

Te Morenga L, Mallard S, Mann J., (2012). *Dietary sugars and body weight: systematic review and meta-analyses of randomised controlled trials and cohort studies*. B.M.J. (Clinical Reaserch ed.) - 2012 Jan 15; 346:e7492. doi: 10.1136/bmj.e7492.

Teixeira, S., Gonçalves, J. & Vieira, E., (2011) - *Edulcorantes: uso e aplicação na alimentação, com especial incidência na dos diabéticos.* Revista SPCNA - Alimentação Humana. Volume 17. No. 1/2/3. p. 47-54

Tomas, J., Campus, F., Coll, J., Mele, E. and Messeguer, J, (1993). *Phytoecdysteroid production by Ajuga reptans tissue cultures.* Phyotchemistry 32: 317-324.

Tomita, T., Sato, N., Arai, T., Shiraishi, H., Sato, M., Takeuchi, M., et al., (1997). *Bactericidal activity of a fermented hot-water extracts from Stevia rebaudiana Bertoni and other food-borne pathogenic bacteria.* Microbiology and Immunology, 41, 1005–1009.

Truong, T. T., Valicek, P., Nepovim, A. and Vamel, T., (1999). *Correlation between stevioside content in leaves, their surface and the number of roots in the plant.* Sci. Agric. Bohemica. 30: 249-258.

US National Library of Medicine. Environmental Health Information Programme

Van Veen, J.A., L.S. van Overbeek, and J.D. van Elsas (1997). *Fate and activity of microorganisms introduced into soil*. Microbiol Mol Biol Rev. 1997 Jun; 61(2): 121–135.

Vanidze, M.R.; Kalandiya, A.G.; Chanukvadze, K.R., (2009). *Identification and quantitative determination of the content of diterpene glycosides in Stevia (Stevia rebaudiana Bertoni).* Khimiya Rastitel'nogo Syr'ya, 4, 155-158.

Vereskovskii, V. V., Chekalinskaya, I. I. and Pashina, G. V., (1983). *Dynamics of contents of ecdisterone in species of the genus Rhaponticum Ludw.* Rast. Resursy. 19: 60-65.

Virendra, V., & Kalpagam, P. (2008). *Assessment of Stevia (Stevia rebaudiana) natural sweetener: A review.* Journal of Food Science and Technology, 45, 467–473.

Visavale, G.L., (2009). *Design and Characteristics of Industrial Drying Systems*. Ph.D. thesis. Institute of Chemical Technology. Mumbai, India.

Wahid, A., Farooq, M., Basra, S.M.A. and Rasul, E., (2008). *Metabolites accumulation in plants under high temperature stress.* In: Proceedings of the International Workshop on Carbon and Water Exchange in Plants under Changing Climatic Conditions, pp: 103– 115. Nov. 5–6, 2007, Pir Mehr Ali Shah Arid. Agriculture University, Rawalpindi, Pakistan.

Wallin, H. (2007). *Steviol glycosides.* 63rd Joint FAO/WHO Expert Committee on Food Additives (JECFA) – Chemical and Technical Assessment (CTA) (pp. 1–8).

(WHO) World Health Organization (2007). Report of a Joint WHO/FAO/UNU Expert Consultation (2002: Geneva, Switzerland). WHO Technical Report Series; No. 935. ISBN: 92 4 120935 6.

(WHO) World Health Organization, (2004). The world health report 2004 - changing history. http://www.who.int/whr/2004/en/White, John S. (2008). *Straight talk about high-fructose corn syrup: what it is and what it ain't*[1–4]*.* (ASCN) American Society for Clinical Nutrition.

Wingard, R., Brown, J., Enderlin, F., Dale, J., Hale, R., & Seitz, C., (1980). *Intestinal degradation and absorption of the glycosidic sweeteners stevioside and rebaudioside A*. Cellular and Molecular Life Sciences, 36, 519–520.

Wink, M., (1999). *Functions of plant secondary metabolites and their exploitation in biotechnology.* Annual plant reviews, Volume 3, pp. 370.

Wu, S., Ng, L., Huang, Y., Lin, D., Wang, S., Huang, S., et al., (2005). *Antioxidant activities of Physalis peruviana.* Biological & Pharmaceutical Bulletin, 28, 963–966.

Xi, Y., Yamaguchi, T., Sato, M., and Takeuchi, M., (1998). *Antioxidant activity of Stevia rebaudiana.* Journal of Japanese Society of Food Science and Technology (Nippon Shokuhin Kagaku Kogaku Kaishi) 45, 310–316.

Xiaotong, Y., 2012, Review of R&D progress and practical application of the solar photovoltaic/the

Xili, L., Chengjiany, B., Eryi, X., Reiming, S., Yuengming, W., & Haodong, S. (1992). *Chronic oral toxicity and carcinogenicity study of stevioside in rats*. Food and Chemical Toxicology, 30, 957–965.

Xingxing, Z., Xudong, Z., Stefan, S., Jihuan, X., Xiaotong, Y., (2012). *Review of R&D progress and practical application of the solar photovoltaic/thermal (PV/T) technologies.* Renewable and Sustainable Energy Reviews, 16, pp. 599-617.

Yahya M., M.Y. Othman, K. Sopian, W. R. W. Daud and B. Yatim, (2004). *Quality of pegaga leaf dried in solar assisted Dehumidification drying system.* Drying 2004 – Proceedings of the 14the International Drying Symposium (IDS 2004), São Paolo, Brazil, August 22-25, B, 1049-1054.

Yilmaz, G., Kandemir, N., Kinalioglu, K., (2004). *Effects of different pruning intervals on fresh shoot yield and some quality properties of tea (Camellia sinensis L. Kuntze) in Turkey*. Pak. J. Bio. Sci. 7: 1208-1212.

Yodyingyuad, V., & Bunyawong, S. (1991). *Effect of stevioside on growth and reproduction.* Human Reproduction, 6, 158–165.

Yoshida, S., (1986). *Studies on the production of sweet substances in Stevia rebaudiana.* Jpn. J. Crop Sci., 55(2), 189-195.

Zahn, S., A. Forker, L. Krügel, and H. Rohm, (2013). *Combined use of Rebaudioside A and fibres for partial sucrose replacement in muffins.* Food Science and Technology, 50(2): 695-701.

Zieslin, N., Mor, Y., (1981). *Plant Management of greenhouse roses. The pruning.* Sci Hort 14:285–293.

APPENDICES

APENDICE I: Fresh biomass and leaves yield of Stevia in different treatments:

Treatment	Repetition	Plant height Means (cm)	Plant weight (g)	Leaves weight (g)	Leaves Yield
1	1	68.4	4035	1800	44.61%
2	2	73.1	3680	1680	45.65%
1	3	73.5	3535	1455	41.16%
2	4	82.4	5785	2040	35.26%
1	5	80.5	5145	1790	34.79%
2	6	80.8	6385	2105	32.97%
1	7	81.3	4320	1620	37.50%
2	8	85.2	6250	1920	30.72%
3	9	81.5	4965	1680	33.84%
4	10	81.2	5540	2190	39.53%
3	11	78.3	5000	1440	28.80%
4	12	66.1	3560	1425	40.03%
3	13	71.1	3820	1470	38.48%
4	14	63.9	2650	1155	43.58%
3	15	69.9	4930	1830	37.12%
4	16	68.5	2525	1185	46.93%

Stevia plant biomass yield - 4 treatments (summary table, means and SD):

	TREATMENTS			
	Without fertilization		With fertilization	
Parameters	light pruning fLP sample	severe pruning fSP sample	light pruning fLPF sample	severe pruning fSPF sample
Plant Height (cm)	**75.93 ±** 8.86	**80.38 ±** 6.88	**69.93 ±** 8.15	**75.20 ±** 6.08
Leaf Yield (%)	**39.52 ±** 3.71	**36.15 ±** 5.71	**42.52 ±** 2.99	**34.56 ±** 3.73
Leaf Area (mm^2)	1197.86 ± 429.44	1486.07 ± 482.03	1462.61 ± 528.01	1762.83 ± 523.72

APPENDICE II: Glycosides

STEVIOL GLYCOSIDES IN FRESH LEAVES

1) Individual Glycosides concentration in *S. rebaudiana* fresh leaves – 4 treatments:

Treatment	**Measured Glycosides** (mg / 1g dry leaf)				Stand Error	Sum Means
	Stevioside	Rebaudioside A	Rebaudioside C	Dulcoside A		
fLP	12.61 ±1.32	12.78 ±2.20	2.48 ±0.67	0.58 ±0.07	0.85	28.45 ±3.59
fSP	17.95 ±1.15	16.00 ±1.61	2.16 ±0.25	0.70 ±0.13	0.66	36.81 ±2.63
fLPF	11.87 ±2.01	10.82 ±1.48	2.21 ±0.24	0.41 ±0.03	0.85	25.31 ±3.41
fSPF	16.87 ±2.35	16.23 ±2.49	2.44 ±0.24	0.50 ±0.07	1.23	36.05 ±4.93

2) Sum of Observed Glycosides concentration in *S. rebaudiana* fresh leaves – 4 treatments:

Treatment	mg Sum Measured Glycosides / g dry leaf
fLP	28.45 ± 3.59
fSP	36.81 ± 2.63
fLPF	25.31 ± 3.41
fSPF	36.05 ± 4.93

3) Rebaudioside A : Stevioside Ratio in fresh leaves for 4 imposed treatments:

Treatment	Reb-A/Stev	Groups
fLP	1.02 ±0.16	a
fSP	0.89 ±0.08	b
fLPF	0.92 ±0.07	a, b
fSPF	0.96 ±0.08	a, b

STEVIOL GLYCOSIDES IN DRIED LEAVES

4) Sum of Observed Glycosides after drying

Treatment	Sum Glyc	Standard error
fSP	36.82 ±2.72	1.32
dSP	43.06 ±4.65	2.32

5) Individual Glycosides after drying:

mg of Stevioside per g of dry leaves

mg of Rebaudioside A per g of dry leaves

- mg of Rebaudioside C per g of dry leaves

Treatment	Rebaudioside C	Standard error
fSP	2.16 ±0.25	0.12
dSP	2.00 ±0.58	0.29

- mg of Dulcoside A per g of dry leaves

Treatment	Dulcoside A	Standard error
fSP	0.71 ±0.13	0.06
dSP	0.54 ±0.03	0.02

6) Rebaudioside A : Stevioside – Ratio after drying

Treatment	Ratio	Standard error
fSP	0.89 ±0.08	0.04
dSP	0.83 ±0.08	0.04

APPENDICE III: Phenolic Compounds and Antioxidant activity

FRESH AND DRY LEAVES:

a) Measurements

Repetition	ABTS - mg Ascb. Ac./g dry leaves	DPPH - mg Trolox /g dry leaves	FOLIN - mg Gallic Ac./g dry leaves	Treatment
1	2.35	3.79	5.30	T1 - FRESH LEAVES
2	2.91	4.52	5.26	
3	2.06	3.81	4.72	
4	2.51	4.88	5.56	
1	11.62	13.60	10.69	T1 - DRIED LEAVES
2	10.97	12.60	10.13	
3	10.19	13.36	10.59	
4	10.70	12.58	10.14	

b) Mean values and standard error

Treatment	ABTS - mg Ascb. Ac./g dry Stevia	Standard Error
fSP	2.46 ±0.31	0.15
dSP	10.87 ±0.51	0.26

Treatment	DPPH mg Trolox/g dry leaf	Standard Error
fSP	4.25 ±0.61	0.30
dSP	13.03 ±0.45	0.23

Treatment	FOLIN - mg Gallic Ac./g dry leaf	Standard Error
fSP	5.21 ±0.31	0.15
dSP	10.39 ±0.26	0.13

LEAVES INFUSIONS:

a) Measurements

Repetition	ABTS - mg Ascb. Ac./g dry leaves	DPPH - mg Trolox/g dry leaves	FOLIN - mg Gallic Ac./g dry leaves	Treatment
1	5.35	7.55	8.20	T1 - FRESH LEAF INFUSION
2	3.64	7.64	6.89	
3	3.87	7.52	6.38	
4	3.77	7.51	6.05	
1	7.28	17.66	10.53	T1 - DRIED LEAF INFUSION
2	8.81	16.21	10.34	
3	6.47	12.47	9.61	
4	7.28	14.56	9.55	

b) Mean values with standard error:

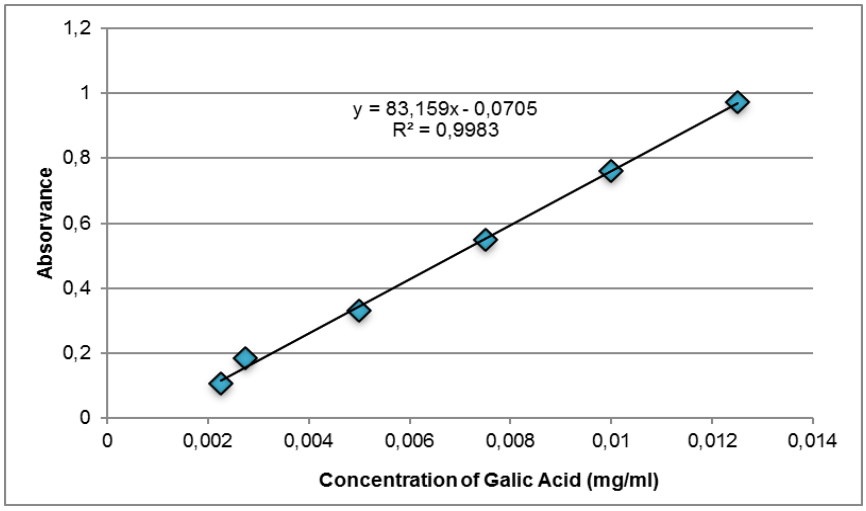
Treatment	ABTS - mg Ascb. Ac./g dry leaves	Standard error
fSP	4.16 ±0.69	0.35
dSP	7.46 ±0.84	0.42

Treatment	DPPH - mg Trolox /g dry leaf	Standard error
fSP	7.55 ±0.05	0.03
dSP	15.23 ±1.94	0.97

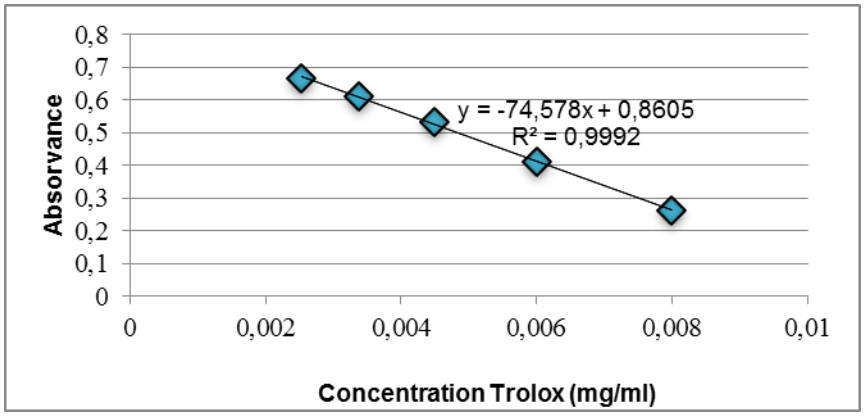
Treatment	FOLIN - mg Gallic Ac./g dry leaf	Standard error
fSP	6.88 ±0.82	0.41
dSP	10.01 ±0.43	0.22

APPENDICE IV: Calibration curves in present work

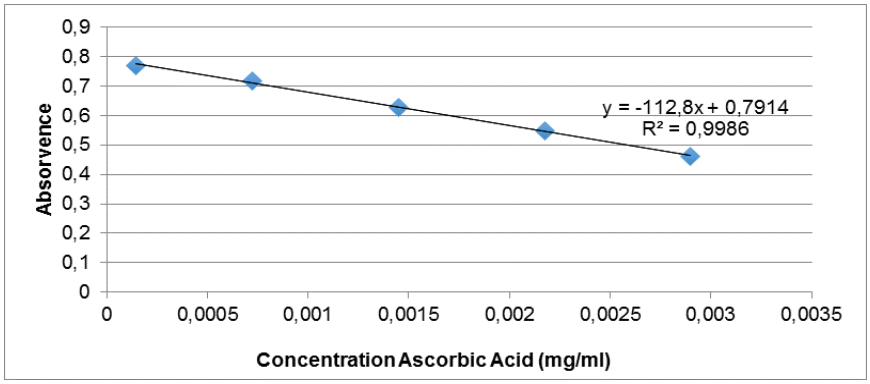
Phenolic Compounds and Antioxidant Activity



Calibration curve for Phenolic Compound determination

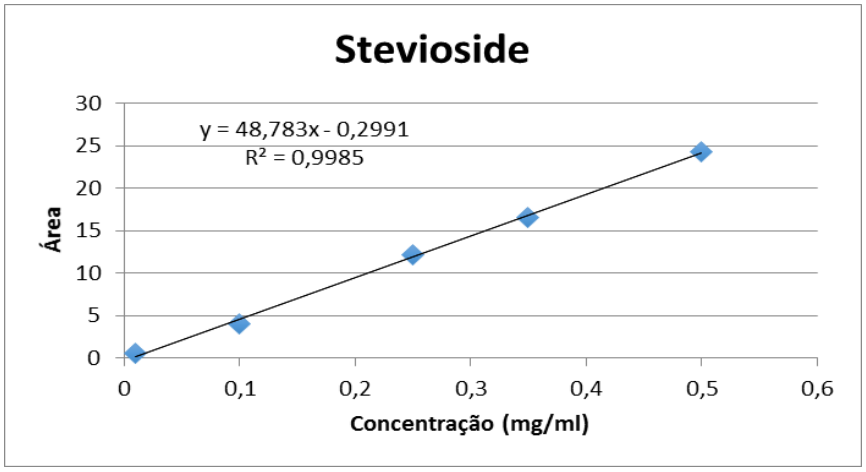


Calibration curve for determination of Antioxidant Capacity (DPPH)



Calibration curve for determination of Antioxidant Capacity (ABTS)

Stevioside:



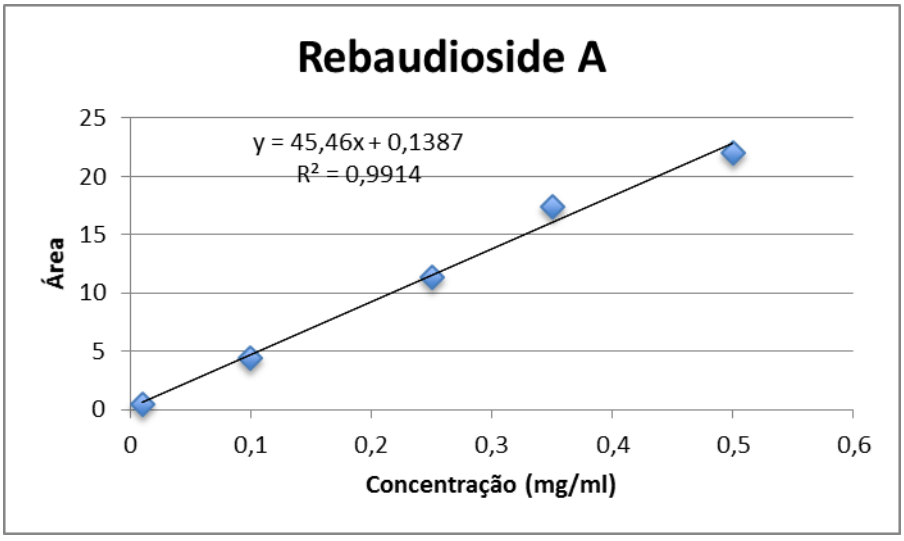
Calibration curve for determination of Stevioside

Stevioside:

Calibration curve function: y=48.783*x – 0.2991 (R^2=0.9985)

Where *y* represents observed retention area. and *x* represents sweetener compound concentration in mg/mL.

Rebaudioside A:

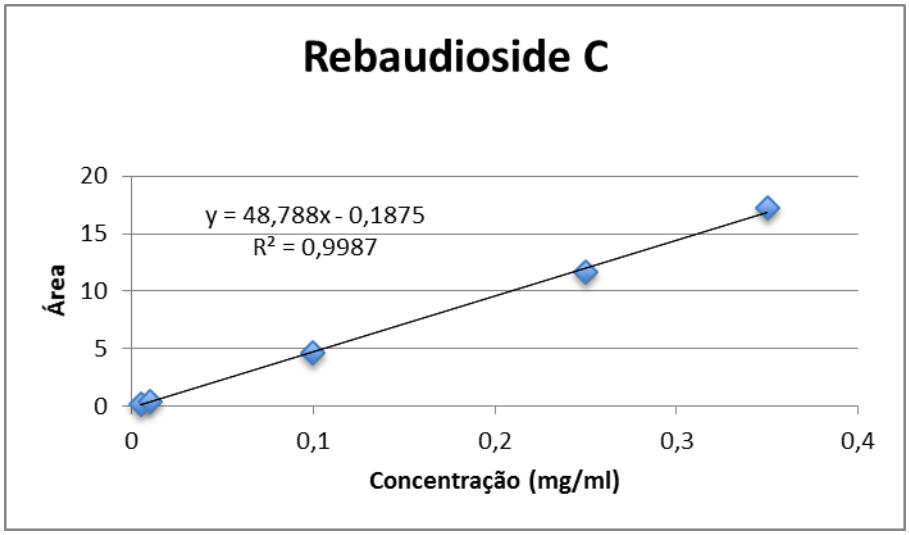


Calibration curve for determination of Rebaudioside A

Rebaudioside A:

Calibration curve function: y=45.46*x – 0.1387 (R^2=0.9914)

Rebaudioside C:

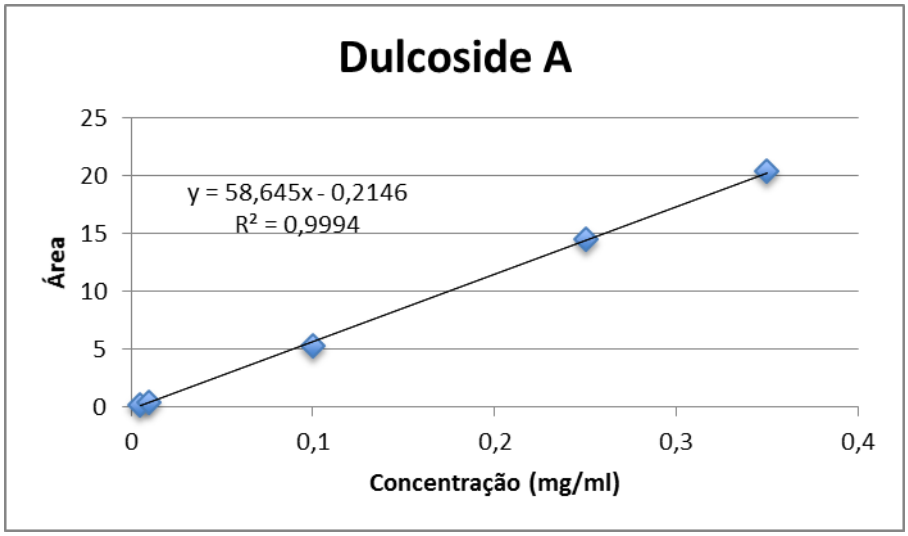


Calibration curve for determination of Rebaudioside C

Rebaudioside C:

Calibration curve function: y=48.788*x – 0.1875 (R^2=0.9987)

Dulcoside A:



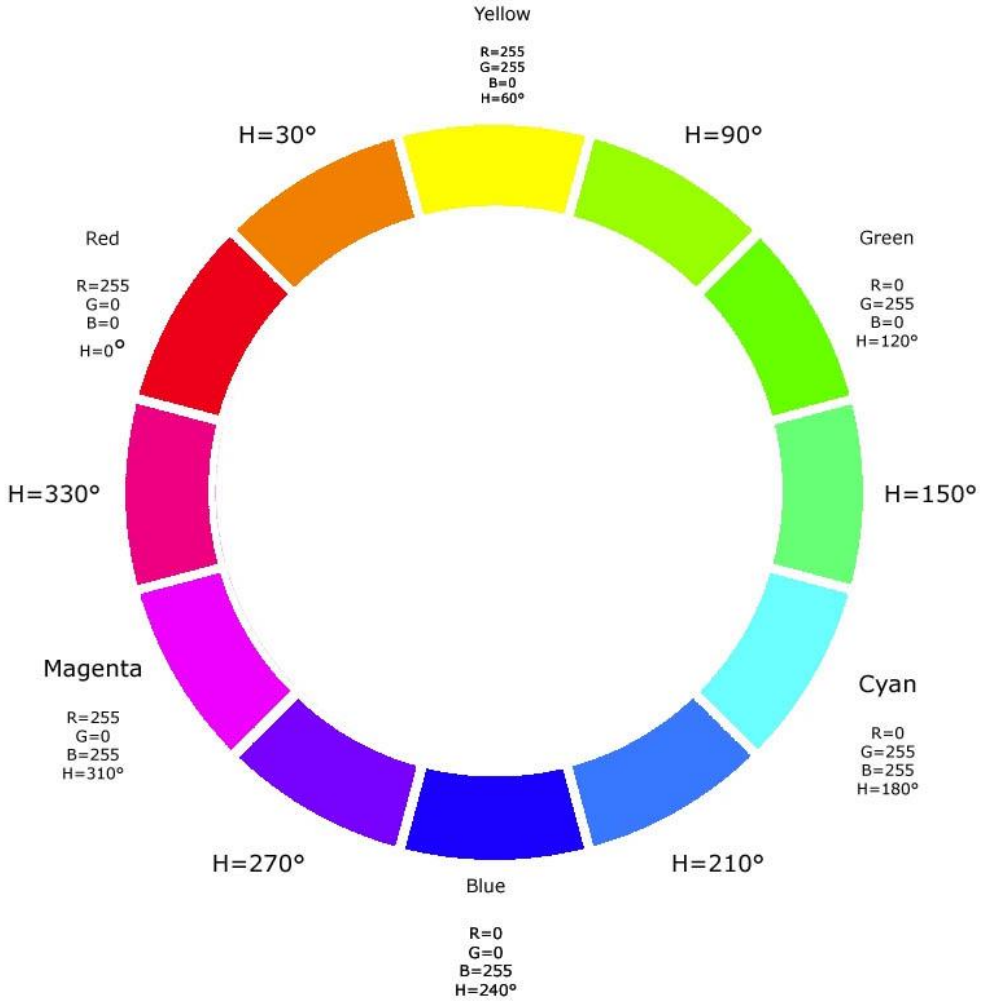
Calibration curve for determination of Dulcoside A

Dulcoside A:

Calibration curve function: y=58.645*x – 0.2146 (R^2=0.9994)

APPENDICE V: Colorimetric circle for Hue Angle

COLOR WHEEL



APPENDICE VI: Table of measured Drying parameters

	% RH - AMB	% RH - Chamber out	(ºC) T - AMB	(ºC) T1 - chamber in	(ºC) T2 - chamber out	(ºC) T - leaves	(m/s) Velocity air chamber out
9:30:00 AM	40.90	55.30	22.80	23.40	23.80	24.80	0.70
10:30:00 AM	35.00	35.60	24.00	23.90	24.00	24.80	0.70
11:50:00 AM	30.60	29.90	31.00	35.60	32.00	25.90	0.37
1:00:00 PM	35.40	36.20	32.00	37.70	41.10	29.50	0.89

2:20:00 PM	28.10	32.10	29.80	45.10	42.00	31.10	0.80
3:15:00 PM	30.20	29.50	27.00	42.50	39.90	31.10	0.23
4:40:00 PM	33.30	37.90	26.30	38.70	35.40	31.60	0.57
9:40:00 AM	50.00	53.0	24.00	27.00	29.40	23.30	0.70
10:05:00 AM	52.00	49.2	24.40	30.30	30.00	24.70	0.70
11:00:00 AM	55.70	58.6	23.40	26.50	26.80	25.10	0.65
11:20:00 AM	64.00	71.0	21.60	24.60	24.20	23.70	0.85
11:30:00 AM	65.40	64.3	23.60	25.00	25.40	23.20	1.05
12:10:00 PM	40.40	46.1	24.20	31.80	31.20	26.80	0.75
12:55:00 PM	53.20	53.6	25.40	30.70	29.40	26.90	0.35
2:00:00 PM	40.70	43.8	25.2	40.40	35.00	28.00	0.7
3:05:00 PM	44.60	34.6	26.20	52.00	39.30	34.60	0.75
3:35:00 PM	28.20	30.2	26.2	54.40	40.00	36.20	0.80
4:15:00 PM	31.60	48.4	26.2	54.90	41.40	36.20	0.76
4:40:00 PM	35.20	25.0	26	54.20	40.80	33.60	0.72
5:00:00 PM	36.50	24.4	25.8	51.50	40.2	33.00	0.68
10:00:00 AM	37.00	40.00	23.40	27.00	23.70	23.50	0.55
10:15:00 AM	37.00	46.20	23.60	34.50	30.60	23.70	0.60
10:30:00 AM	37.00	40.50	23.80	40.80	35.00	31.40	0.52
10:50:00 AM	35.00	33.50	24.40	48.00	38.20	34.60	0.55
11:40:00 AM	33.50	32.80	24.40	48.20	40.00	40.40	0.95
12:40:00 PM	42.70	29.10	24.60	47.50	40.70	40.70	0.90
1:45:00 PM	59.80	28.40	24.40	49.80	41.80	41.80	0.85
2:30:00 PM	55.70	25.30	25.40	51.40	42.80	42.40	0.60