

Stijn CEUNEN & Jan M.C. GEUNS

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organised by EUSTAS 2015**

Stevia: Growth in Knowledge and Taste

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Stevia: Growth in Knowledge and Taste

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CHAPTER 1

Effects of Chemical Weed Management on the Quantitative and Qualitative Characteristics of *Stevia rebaudiana* Bertoni, under Mediterranean Conditions (Karditsa, Thessaly, Greece)

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ABSTRACT

After the use of steviol glycosides was approved by the European Union in December 2011, there has been a continuous interest in the cultivation of this plant in the Mediterranean region and especially in the mainland of Greece. One of the greatest problems in the cultivation of *Stevia rebaudiana* is weed management. In this experiment we focus on the effectiveness of certain active ingredients, from different herbicides, in *S. rebaudiana* cultivation.

Our preliminary results indicate the use of the active ingredient pendimethalyn as the most appropriate for the chemical weed management of *S. rebaudiana*. Positive results were also obtained from napronamide and dimethenamid. Our experiments provide the basis for further studies in the Southern Balkans, providing the necessary information for full-scale domestic *S. rebaudiana* cultivation, with significant economic, cultural, and social benefits for Greece.

CHAPTER 2

Floral phenology, insect pollinators and seed quality of 36 genotypes of *Stevia rebaudiana* Bert. cultivated in Italy

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ABSTRACT

The complex reproductive mechanisms and the poor seed germination represent serious obstacles towards large-scale cultivation of *Stevia rebaudiana* Bert. The main goal of this study was to evaluate differences in the reproductive development of 36 F1 open-pollinated genotypes during flowering formation, pollination and seed production in order to identify the best genotypes, environmental conditions and plant-pollinator interactions, for seed production and quality. Germination characteristics of seeds, obtained from different genotypes grown under glass without pollinators, were analysed. Reproductive phenology, from the beginning of flowering to seed maturity, was studied and the numbers of corymbs quantified for every genotype. Pollinators' diversity and abundance were quantified over the whole flowering period.

In Central Italy, the flowering stage spans from the beginning of September to the end of October, whereas seed formation and maturity lasts until the end of November. Flowering and seed formation overlap each other. The rainy conditions during flowering and seed formation hampered pollinators' activity

and seed filling. All F1 genotypes showed a large variability in terms of flowering time, length of flowering and number of corymbs per plant. All genotypes were visited by insects during anthesis. Main pollinators were Hymenoptera and Diptera. Honeybees and hoverflies represented the most abundant species, followed by sweat bees and blowflies. Seed germination rate and mean energy time differed among the genotypes. Further differences were found in the seed quality as consequence of pollinator abundance and spectrum. Overall, our findings suggest that the genotype choice, cross-pollination, and environmental conditions during the reproductive stage, together with support of pollinators are important parameters for achieving greater seed yield and quality in *S. rebaudiana*.

KEYWORDS

Floral phenology, Insect pollinators, Seed quality, *Stevia rebaudiana* genotypes

INTRODUCTION

Stevia rebaudiana is characterised by a complex reproductive mechanism, due to some peculiarities. It is a hermaphroditic species but also a highly cross-pollinated, photoperiod-sensitive crop that produces self-incompatible, tiny white florets borne in small corymbs of five florets small flowers (Miyagawa *et al.*, 1986; Macchia *et al.*, 2007; Abdullateef *et al.*, 2012; Yadav *et al.*, 2014). The fruit is an achene with a pappus, consisting of 13-15 appendices. The presence of a pappus and the extremely small size of the achenes (1000 seeds have a mass around 0.15-0.29 g; Carneiro, 2007) facilitate wind-borne dispersal. The biological cycle of *S. rebaudiana* is photoperiod-dependent, with latitude being one of the major factors influencing reproduction. Late flowering may adversely affect seed production when it occurs during a season that is unfavourable to pollination. In Italy, this species often gives scanty seed production, with poor germination rate and germination energy of seeds produced (Macchia *et al.*, 2007).

CHAPTER 3

Nitrogen Affects Growth, Steviol Glycosides, Leaf Gas Exchanges and Chlorophyll Fluorescence in *Stevia rebaudiana* Bert.

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ABSTRACT

This study focused on the agronomic, physiological and biochemical response of *Stevia rebaudiana* Bertoni grown under different nitrogen (N) rates. A pot trial experiment in open air conditions was set up with the aim to define the effects of four different N rates on leaf gas exchanges, chlorophyll fluorescence, Rubisco activity, photosynthetic pigments and photosynthetic N use efficiency (PNUE) as well as on productive characteristics and steviol glycoside content. The results demonstrate that using an appropriate N rate it is possible to improve photosynthetic CO₂ assimilation, stomatal conductance, PNUE, Rubisco activity and PSII efficiency. In addition, it is possible to modulate steviol glycoside biosynthesis through N, with a significant increase in the rebaudioside A content and in the ratio between rebaudioside A and stevioside.

KEYWORDS

Chlorophyll fluorescence; gas exchange; nitrogen use efficiency; photosynthesis; steviol glycosides

CHAPTER 4

***Stevia rebaudiana* Bertoni Could be an Alternative Crop in the Mediterranean Region of Turkey**

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ABSTRACT

Stevia rebaudiana Bertoni is an herbaceous perennial plant belonging to the Asteraceae family, the leaves of which contain steviol glycosides, a family of zero calorie natural sweeteners that are 200-300 times sweeter than table sugar. This study was done in the experimental field of the Agriculture Faculty at Akdeniz University in Antalya, located in the Mediterranean Region of Turkey, between 2011-2013. This location is characterised by a Mediterranean climate with very warm, dry summers and mild, wet winters. Terra-rossa-type soil characteristics of the experimental field were clay loam, with large amounts of lime, little salt and very alkaline. The aim of the study was to investigate the possibility of *S. rebaudiana* production in the Mediterranean climate of Turkey. Initially, seed germination of cross-pollinated plants were examined under different light and temperature regimes. The greatest germination percentages were determined at 25 °C (71 %) and 20 °C (68 %) in darkness. In the field experiments, *S. rebaudiana* plants were planted at 65 cm row and 45 cm row intra-row spacing, respectively. Plants were irrigated by drip irrigation during the

CHAPTER 5

Agronomic and Phytochemical Evaluation of *Stevia rebaudiana* Genotypes

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ABSTRACT

In Switzerland, the agronomic potential and the phytochemical variability of 18 genotypes of the Paraguayan plant *Stevia rebaudiana* have been investigated to identify the best genotype for local cultivation. Yields, as in dry leaves, ranged from 10 to 170 g m⁻², with a percentage of leaves ranging from 53 to 74 %. HPLC analyses showed a notable variability in phytochemical composition, with stevioside content ranging from 0.3 to 7.9 % w/w and rebaudioside A from 0.3 to 6.5 % w/w. Cultivation of *S. rebaudiana* in Switzerland is feasible. With a density of 10 plants per m², the potential yields of dry matter are approximately 1-2 t ha⁻¹. The most productive genotypes (Pharmasaat, Hem Zaden, Stepa and Mediplant 3 and 11) will be submitted to the industry for organoleptic evaluation.

CHAPTER 6

A Danish Research Project for the Development of ‘Green Stevia’ – a Natural Sweetener for Organic Food Products

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ABSTRACT

The Department of Food Science at Aarhus University, Denmark, is collaborating with Organic Denmark, organic growers and several industrial partners in developing a Danish-grown product of *Stevia rebaudiana* Bertoni that can be used as a sweetener in organic food products in the EU. A first year screening trial of 12 different varieties and clones of *S. rebaudiana* revealed a satisfactory yield of biomass and steviol glycoside content when crops were transplanted early in the season, albeit with large differences between the genotypes. Preliminary analysis of steviol glycosides in dried leaves found about 14 % (w/w) and yields up to 520 kg ha⁻¹. In three varieties, the proportion of rebaudioside A varied between 23 and 56 % of the total steviol glycoside content during the first planting. These preliminary results of the cultivation of *S. rebaudiana* under Danish conditions seem promising.

CHAPTER 7

Agronomy of *Stevia rebaudiana* (Bertoni) in a Location of Southern Brazil

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ABSTRACT

The aim of this study was to evaluate the influence of climatic conditions (solar radiation, air temperature and degree-days) on *Stevia rebaudiana* and the best time for harvest in order to obtain greatest productivity in dry mass and sweetener concentration. The trial was carried out during the agricultural years of 2011/2012 and 2012/2013. One hundred twenty (120) plants were selected and divided in six groups of 20 plants each. Canopy height and diameter were measured weekly. Plants were harvested at the beginning of the flowering period. Dry leaf samples were used to determine steviol glycoside levels. The results show that solar radiation and degree-days influenced the growth of each cycle and the total yield of each group. The greatest dry mass yield was obtained between November 11, 2012 and January 17, 2013 (669 g m⁻²; harvested in January) and between December 3, 2012 and February 26, 2013 (599 g m⁻²; harvested in February). The cycle lengths for both were 77 and 85 days,

CHAPTER 8

Response of *Stevia rebaudiana* Bertoni to Polyethylene Glycol Treatment under *In Vitro* Culture Conditions

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ABSTRACT

This investigation was done with the aim of determining the effect of polyethylene glycol 6000 treatments (PEG6000; 0, 2, 4 and 6 % w/v) on *Stevia rebaudiana* Bertoni under *in vitro* conditions. Drought stress reduced the fresh and dry masses, water content, chlorophylls and carotenoids. Water soluble carbohydrates, reducing sugar and proline amounts showed a significant reduction at 6 % PEG. Electrolyte leakage, as well as malondialdehyde, α -tocopherol and glycine betaine contents increased in drought-stressed plants. Enzyme activities of 1-pyrroline-5-carboxylate synthetase and proline dehydrogenase as well as the protein content showed no significant changes under drought stress. SDS-PAGE analysis revealed that drought stress increased the amount of a 25 kD protein with a critical function in plant development under stress. PEG treatment significantly increased hydrogen peroxide, phenol content, total antioxidant capacity, catalase, ascorbate peroxidase, polyphenol oxidase and peroxidase activities, while it inversely decreased glutathione reductase activity. Superoxide dismutase activity was not affected by PEG treatment. PEG treatment increased the ascorbate pool, whereas the total glutathione level was

CHAPTER 9

Insights into the Bitter/Sweet Taste Profile of Steviol Glycosides by a Combination of Human Psychophysical and Cell-Based, Taste-Receptor, Studies

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Due to a high number of undesirable health effects such as obesity, dental caries, type-2 diabetes and cardiovascular diseases and its risk factors, which are associated with increasing sucrose consumption, high potency, low-calorie sweeteners, in particular, found widespread use in the USA food industry, as well as in the European Union. Since consumers are becoming more aware of what ingredients go into their foods, special emphasis was given by producers to find natural, non-nutritive sweet tasting compounds, the most prominent example being steviol glycosides, the sweet principle of *Stevia rebaudiana* Bertoni (Bertoni). Apart from the desirable sweetness, the taste profile of those diterpene *ent*-kaurene glycosides is characterised by an unpleasant, lingering bitter aftertaste which often causes consumer complaints. The development of

CHAPTER 10

Insight into Trade-Offs with the Sensus Sweetness Tool

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ABSTRACT

While the number of product launches with steviol glycosides is increasing, their after-taste still remains a key challenge for manufacturers and an obstacle for consumers. Oligofructose is a natural ingredient that is able to mask the after-taste of steviol glycosides. However, due to the differences in commercial steviol glycoside sources and differences in synergy at different additions, sugar replacement with steviol glycosides without compromising taste is not straightforward. Therefore, an insight into the trade-offs is important. In this article we present a visual modelling tool that enables manufacturers to optimise the taste with oligofructose when replacing sugar with steviol glycosides. The tool is based on sensory response surface models for relevant attributes in plain spoonable yoghurt. Much emphasis was given to the validation of the predictive models in order to present a reliable tool for a successful implementation of sugar-reduced products based on natural

CHAPTER 11

Enzyme Membrane Reactor-assisted Derhamnosylation of Steviol Glycosides

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ABSTRACT

Since even greatly purified steviol glycosides possess taste attributes such as liquorice flavour or bitterness, their acceptance by consumers is still limited. Therefore, new biotechnological conversions are being developed besides the selection and breeding of suitable *Stevia rebaudiana* plants. The most common bitter steviol glycosides detected in its leaves are rebaudioside C (or dulcoside B; 1-2 % w/w) and dulcoside A (0.2-1.5 % w/w) which comprise a terminal rhamnose in the sugar residue chain (Figure 1).

Many processes for the transglycosylation (sucrose as donor of saccharides) and deglycosylation (production of saccharides) of steviol glycosides are known so far (Kochikyan *et al.*, 2006; Uchiyama *et al.*, 2010; Wan *et al.*, 2012). In our laboratory we developed a new strategy for the specific trimming of rhamnose residues from steviol glycosides. This enzymatic treatment decreases bitterness and enhances the sweetness of steviol glycoside mixtures. A fungal α -L-rhamnosidase was applied to derhamnosylate steviol glycosides.

CHAPTER 12

Evaluation of *Stevia rebaudiana* Bertoni Antioxidant Properties at Cellular Level, Cytotoxicity and Proliferative Effects

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ABSTRACT

The aim of this study was to investigate the antioxidative effect of *Stevia rebaudiana* Bertoni in a cellular model from human origin. In addition, the modulation of catalase, cytotoxicity as well as the proliferative properties of *S. rebaudiana* water extracts have been determined. The antioxidant properties of *S. rebaudiana* leaf extracts as well as purified steviol glycosides were determined *in vitro*, through the oxygen radical absorbance capacity (ORAC) and the cellular antioxidant activity (CAA) assays. The activities of endogenous catalase, lactate dehydrogenase as well as the cellular proliferation were assessed spectrophotometrically in treated cells. The results demonstrate that *S. rebaudiana* leaf extracts can increase the cellular activity and simultaneously decrease the intracellular oxidation compared to control cells. ORAC values were positively correlated with CAA values. Furthermore, we observed that purified steviol glycosides (stevioside and rebaudioside A) exhibited a low ORAC value in comparison with plant extracts, and could not elicit any intracellular antioxidant activity. The extracts proved non-toxic for the treated

CHAPTER 13

Effects of *Stevia rebaudiana* Extracts on Oral Biofilms

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ABSTRACT

Biofilm control is fundamental to oral health. Fermentable carbohydrates are implicated in the etiology of biofilm-associated diseases such as gingival inflammation and dental decay. Therefore, sugar substitutes, such as sole sweeteners or mixtures with carbohydrates, were introduced into preventive dental care to replace sucrose. The cariostatic potential of sweeteners is well accepted in dentistry. Steviol glycosides as natural sweeteners are said to have non-cariogenic effects and a potential to affect structure, growth and pH-decline of complex oral biofilm communities.

To evaluate the preventive potential of steviol glycosides, especially in the presence of carbohydrate sources, the growth, fermentation of nutrients and biofilm formation was examined in mono- and mixed cultures of oral Streptococci. All bacteria were cultured under optimised growth conditions in brain-heart-infusion with or without the addition of sweeteners from *Stevia rebaudiana*. Bacterial growth was determined by measuring the optical density of the liquid cultures and in addition, pH values were recorded. To calculate glucose and sucrose consumption under the influence of *S. rebaudiana* extracts,

CHAPTER 14

ROS - Scavenging Capabilities of Steviol Glycosides and Derivatives Thereof

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ABSTRACT

Steviol glycosides (SVGlys) are well-known as natural, non-calorific sweeteners. Their use as a sugar substitute is not only interesting, but also positive pharmacological effects to the human health were observed after intake of larger doses, particularly stevioside (Stev). These effects are possibly related to the scavenging activity of SVGlys on reactive oxygen radicals.

Two aspects were examined with regard to the scavenging capabilities of SVGlys. The first relates to the fact that SVGlys are composed of sugars, while the other aspect is related to the steviol (SV) scaffold containing a double bond. In this project, scavenging activities of rebaudioside A (RebA), Stev and a leaf extract were compared to those of glucose (Glc) and sucrose (Suc). Experiments were also done to investigate the correlation between scavenging activity and sugars or the number of OH-groups. Experiments with the hydrogenated compound dihydrostevioside (DH-Stev) were done to see whether the double bond of the SV scaffold is responsible for the scavenging activity. The latter was

CHAPTER 15

Effects of Rebaudioside A on Adipocyte Function

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Malfunction of adipocytes is a major health risk leading to the metabolic syndrome which causes diabetes and cardiovascular diseases. Adipose tissue has an endocrine function and releases specific hormones, called adipokines, which affect energy metabolism, immune response, and insulin sensitivity. Most adipokines are pro-inflammatory, whereas a small number of anti-inflammatory adipokines, including adiponectin, exert beneficial actions on metabolic complications. Thus, release of adiponectin from fat tissue is reduced in conditions of diabetes or obesity. Rebaudioside A, a steviol glycoside occurring in leaf extracts of *Stevia rebaudiana*, possibly has positive health benefits, such as anti-inflammatory and anti-microbial effects, although more research is required to confirm these effects.

To evaluate the effect of rebaudioside A on adipocyte function, the expression and secretion of adipokines as well as inflammatory cytokines and regulatory metabolic mediators were analysed in the established 3T3-L1 adipogenic cell line. Differentiated adipocytes were challenged with various concentrations of rebaudioside A in cell culture medium. Cell viability of 3T3-L1 cells was measured by a XTT-Assay. In addition, mRNA expression of adipokines and

CHAPTER 16

Evaluation of *Stevia rebaudiana* Genotypes at a Location in the Alentejo Region in Portugal

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ABSTRACT

Stevia rebaudiana genotypes previously evaluated at Bonn under European temperate zone climate conditions (Lankes and Zabala, 2011) were cultivated at a location in the Alentejo Central Subregion in Portugal under a hot-summer Mediterranean climate in 2012-2013. The experiment was done in a randomised block design with 270 plants per genotype with a planting density of 9 plants per m². The planting was realised in three rows on a raised bed covered with black plastic mulch foil. Irrigation was provided by drip hoses under the plastic mulch. The soil was loamy sand. No fertilisers or pesticides were applied. Genotypes were evaluated for vegetative performance and steviol glycoside levels.

The ranking of the genotypes at the location in the Alentejo corresponded well to the ranking at Bonn. Steviol glycoside levels were in accordance with the best results obtained in Bonn, though their greatest yields that were observed in the Alentejo in July 2013 were still smaller than the greatest amounts measured in Bonn in October 2009 for most of the genotypes under study. Only in genotype F

CHAPTER 17

***In vitro* Culture of *Stevia rebaudiana*: Principles and Possibilities**

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ABSTRACT

Tissue culture methods offer the possibility of establishing healthy plants as well as mass propagation and maintenance of a nuclear stock for selected genotypes. A general survey of the consecutive stages of *in vitro* propagation, i.e. establishment, propagation, rooting and acclimatisation of plants is given. For each stage, the importance for plant health, quality and stability of genetic traits will be stressed.

For the purpose of propagating young plants of *Stevia rebaudiana* for field tests on different locations, several selected genotypes were transferred to a commercial tissue culture laboratory in September 2011. Apical shoots from pot plants of different *S. rebaudiana* accessions provided by EUSTAS were used for establishing *in vitro* culture. Management and control of endophytic bacteria was necessary. Shoot quality and elongation during propagation was best on a simple MS medium without plant growth regulators. Grown shoots were cut into micro-cuttings and then subcultured on fresh media every two weeks. Propagation rates depended on the genotype. On average, about 2 to 2.5 times of the initial numbers could be achieved during a four-weeks subculturing. Rooting of shoots

CHAPTER 18

Comparison of Conventional and Biotechnological Approaches for *Stevia rebaudiana* Production with Elevated Rebaudioside A Content

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ABSTRACT

Stevia rebaudiana is an important medicinal plant used in the production of low-calorie sweeteners. Plant tissue culture techniques are useful for the propagation of elite plants. Due to the economical and medicinal importance of *S. rebaudiana*, establishment of an efficient *in vitro* technique might be applicable for commercial interests. In this study, propagation of *S. rebaudiana* via conventional and tissue culture techniques was compared. The seeds of two *S. rebaudiana* genotypes (GF and CB) were germinated with a success of 50 %

CHAPTER 19

Organ-specific Transcriptome Analysis In Leaves and Roots of *Stevia rebaudiana* Bertoni and Associations with the Steviol Glycosides Biosynthetic Pathway

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ABSTRACT

Stevia rebaudiana Bertoni is an important perennial herb which produces large amounts of natural, non-calorific sweetening compounds, known as steviol glycosides. Information about the transcriptome of *S. rebaudiana* is limited with the availability of few hundreds of expressed sequence tags in the public databases. Next-generation sequencing technology provides an opportunity to expand the identification of genes through in-depth transcript profiling. The present work describes the high-throughput *de novo* transcriptome sequencing and analyses of leaf and root tissues using paired-end (PE) Illumina sequencing

CHAPTER 20

Judicial Questions and Answers about *Stevia* in Europe

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ABSTRACT

Steviol glycosides have been commercialised legally as a food additive in the EU since December 2, 2011. There is still confusion about e.g., declaration, commercialisation of *Stevia* leaves, or organic *Stevia*, although several trials clarified these legal questions. Additionally, innovations like enzymatically modified steviol glycosides, rebaudioside M and biotechnologically produced steviol glycosides are commented on from a judicial point of view.

KEYWORDS

Commission Regulation, novel food, *Stevia* leaves, organic, natural, enzymatically modified, rebaudioside M, TTIP

December 2, 2011 is commonly recognised to be the legal breakthrough for *Stevia* in Europe: on this date, regulation 1131/2011 was enacted, allowing steviol glycosides as a food additive. In fact, *Stevia* leaves were already legally commercialised in Europe many years before, but this fact has been recognised only recently. In the following paper, we try to elucidate some of the legal confusion surrounding *Stevia* in Europe. Interestingly, there were several trials in

CHAPTER P1

The Effect of *Stevia rebaudiana* Extracts on Oral Biofilm Formation: Development of a Protocol to Quantify Biofilm Growth

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ABSTRACT

Modification or suppression of plaque growth by sweeteners, namely extracts of *Stevia rebaudiana*, have a huge impact on preventive dental care. To investigate the dynamics of biofilm formation, protocols to quantify the growth parameters are needed. The effect of carbohydrate sources and a *S. rebaudiana* extract on oral bacterial growth and biofilm formation was investigated by adding a *S. rebaudiana* sweetener to a BHI medium, which was used to grow the test species *S. mutans*, *S. salivarius*, *S. sanguinis* and *S. sobrinus*. Additionally, a mixed culture of the four test bacterial strains was used to simulate more closely an *in vivo* situation. To quantify biofilm formation, a crystal violet staining protocol was used. All cultures were grown in 96-well microplates. First, planktonic growth of the liquid cultures was recorded by measuring the optical density at 600 nm. After removing the media from the wells and excess washing, the formed biofilm at the wall of the microplate wells was stained with crystal violet. To quantify biofilm formation, the incorporated dye was eluted by ethanol and the optical density of the eluate was measured at 630 nm. The ratio between

CHAPTER P2

Towards Breeding of new *Stevia rebaudiana* Cultivars adapted to Organic Production in the South West of France: First Results on Genetic and Phenotypic Evaluation

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ABSTRACT

Studying phenotypic and genotypic diversity is a key step to develop a plant breeding programme. The genotypic and phenotypic variability of several genotypes of *Stevia rebaudiana* were studied with the aim of enhancing our knowledge about *S. rebaudiana* variability and to develop a breeding strategy. Phenotypic variability was measured through experiments done in partially controlled conditions. Plant development was evaluated at different phenologic stages through foliar biomass, leaf area, number of leaves, and plant height.

CHAPTER P3

Applications of Membrane-Based Technologies for the Extraction and Purification of Steviol Glycosides

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ABSTRACT

There are numerous patents and research articles published in the literature, dealing with the refinement of steviol glycosides from dried leaves using different technologies. Due to their complexity, these processes can be expensive and time-consuming. In addition, the use of organic solvents has many disadvantages, including environmental and toxicological risks. Due to their operational advantages, membrane-based technologies, such as micro- and ultrafiltration, can be a feasible alternative in extraction or purification processes.

KEYWORDS

Microfiltration, ultrafiltration, steviol glycosides, extraction

INTRODUCTION

Stevia rebaudiana Bertoni, belonging to the Asteraceae (Compositae) family, is a herbaceous perennial plant, native to the north of South America (Paraguay, Brazil). The sweet diterpene glycosides present in its leaves include stevioside, rebaudiosides A, B, D and E, and dulcoside A and B. Both powdered leaves and