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MARCOS VINICIUS SILVA DE ANDRADE

VIABILIDADE DE SEMENTES E PERFIS BIOQUÍMICO E METABOLÔMICO DE PLANTAS DE *STEVIA REBAUDIANA* (BERT.) BERTONI CULTIVADAS SOB DIFERENTES FOTOPERÍODOS

SALVADOR – BA
2022

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia do Instituto de Ciências da Saúde da Universidade Federal da Bahia (PPGBiotec/UFBA), como requisito parcial para obtenção do título de Doutor em Biotecnologia.

Orientador: Dr. Renato Delmondez de Castro

Coorientador: Dr. Paulo Roberto Ribeiro

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Dr. Valdir Gomes Neto
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Dedico a minha querida e amada mãe!
Por toda força, dedicação e apoio
Que representa.

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“Tudo o que um sonho precisa para ser realizado é alguém que acredite que ele possa ser realizado.”

Roberto Shinyashiki

RESUMO

A *Stevia rebaudiana* (Bert.) Bertoni tem se destacado nos últimos anos devido a características adoçantes proporcionadas pela presença de glicosídeos de esteviol (StGly – *Steviol Glycosides*), principalmente em suas folhas. Em razão do seu rico perfil nutricional e fitoquímico, a estévia também fornece efeitos benéficos contra uma infinidade de condições de saúde, sendo assim, um material de interesse no campo das plantas com propriedades medicinais. Entretanto, o cultivo em larga escala da estévia enfrenta alguns obstáculos, uma vez que esta espécie é altamente responsiva a estímulos ambientais, entre os quais o fotoperíodo, é um dos mais relevantes, afetando em suas características morfofisiológicas e bioquímicas. Portanto, torna-se de fundamental importância compreender os mecanismos subjacentes às respostas da estévia a diferentes condições ambientais. Neste sentido, o presente estudo teve como objetivo (*i*) caracterizar o perfil morfoanatômico da germinação de sementes e enzimático de plântulas, além de (*ii*) realizar uma caracterização bioquímica e metabolômica do desenvolvimento de plantas de *Stevia rebaudiana* (Bert.) Bertoni cultivadas sob diferentes fotoperíodos. No CAPÍTULO 1, reportamos inicialmente sobre a análise do perfil morfofisiológico da germinação dos diferentes aquênios comumente produzidos pelas plantas, onde definimos um padrão de viabilidade de sementes de estévia baseado no teste de tetrazólio. Posteriormente buscamos investigar o perfil das enzimas antioxidantes em plântulas submetidas a diferentes condições de fotoperíodo. Os resultados demonstraram que as enzimas antioxidantes podem ser uma ferramenta importante para a avaliação do estado fisiológico de plântulas/plantas de estévia sob as diferentes condições testadas, fornecendo informações muito úteis para o cultivo baseado em sementes. No CAPÍTULO 2 reportamos sobre o crescimento de plantas de estévia sob diferentes regimes de fotoperíodo, onde avaliamos o conteúdo de StGly e a composição química geral por Ressonância Magnética Nuclear, atividade antioxidante (SOD / DPPH) e atividade antimicrobiana de extratos fitoquímicos, bem como o teor de fenólicos totais.de extratos obtidos de amostras de folha e do caule. Percebemos que, os fotoperíodos com menos de 12h de luz induzem modo reprodutivo precoce indesejado reduzindo o conteúdo de StGlys em suas folhas. Em contrapartida, dias longos (modo vegetativo) aumentaram a produção de StGly e outros compostos bioativos produzidos pelas plantas. Assim, fornecendo informações valiosas potencialmente servirão para o

desenvolvimento de um protocolo de cultivo de estévia em larga escala. No CAPÍTULO 3, reportamos sobre o perfil fitoquímico com enfoque mais detalhado nos glicosídeos de esteviol (Esteviosídeo e Rebaudiosídeo A), em que promovemos a extração e quantificação destes metabólitos via RP–HPLC–UV (*Reversed-Phase High-Performance Liquid Chromatography*) para amostras de folha e caule de estévia. Como previsto, a folha sendo a matéria-prima de maior interesse na cultura da estévia, apresentou maiores teores dos StGly, sendo a síntese dos mesmos dependente das condições de luz testadas, onde plantas cultivadas sob 16/8h de luz apresentam um maior potencial para a síntese dos principais glicosídeos. Dentre tudo, vale destacar a presença de quantidades relevantes dos metabólitos de interesse também nas mostras de caule, podendo também apresentar propriedades potencialmente úteis para exploração futura.

Palavras-chave: Stevia, Luminosidade, Glicosídeos de Esteviol, Enzimas Antioxidantes, Compostos Fenólicos e Bioativos.

ABSTRACT

Stevia rebaudiana (Bert.) Bertoni has stood out in recent years due to the sweetening characteristics provided by the presence of steviol glycosides (StGly - Steviol Glycosides), mainly in its leaves. Due to its rich nutritional and phytochemical profile, stevia also provides beneficial effects against a multitude of health conditions, making it a biomaterial of interest in the field of plants with medicinal properties. However, the large-scale cultivation of stevia faces some obstacles, since this species is highly responsive to environmental stimuli, among which the photoperiod is one of the most relevant, affecting its morphophysiological and biochemical characteristics. Therefore, it is of fundamental importance to understand the mechanisms underlying stevia's responses to different environmental conditions. In this sense, the present thesis aimed to (*i*) characterize the morphoanatomical profile of seed germination and seedling enzymatic, in addition to (*ii*) perform a biochemical and metabolomic characterization of the development of *Stevia rebaudiana* (Bert.) Bertoni plants cultivated under different photoperiods. In CHAPTER 1, we report on the characterization of the morphophysiological profile of germination of the different achenes normally produced by the plants, where we could define a viability pattern for Stevia seeds based on tetrazolium test analysis. Further on, we report on the profile of antioxidant enzymes in seedlings submitted to different photoperiod conditions. The results showed that antioxidant enzymes can be an important tool for evaluating the physiological state of stevia seedlings/plants under the different conditions tested, providing very useful information for seed-based cultivation. In CHAPTER 2 we reported on the growth of stevia plants under different photoperiod regimes, where we evaluated the StGly content and the general chemical composition by Nuclear Magnetic Resonance, antioxidant activity (SOD / DPPH) and antimicrobial activity of phytochemical extracts, as well as the total phenolic content. of extracts obtained from leaf and stem samples. We noticed that photoperiods with less than 12h of light induce unwanted early reproductive mode by reducing the StGlys content in their leaves. In contrast, long days (vegetative mode) increased the production of StGly and other bioactive compounds produced by plants. Thus, providing valuable information will potentially serve for the development of a large-scale stevia cultivation protocol. In CHAPTER 3, we report on the phytochemical profile with a more detailed focus on steviol glycosides (Stevioside and Rebaudioside

A), in which we promote the extraction and quantification of these metabolites via RP–HPLC–UV (Reversed-Phase High-Performance Liquid Chromatographic) for stevia leaf and stem samples. As expected, the leaf being the raw material of greatest interest in the stevia culture, showed higher levels of StGly, and their synthesis depends on the light conditions tested, where plants grown under 16/8h of light have a greater potential for the synthesis of the main glycosides. Among all, it is worth mentioning the presence of relevant amounts of the metabolites of interest also in the stem samples, which may also present potentially useful properties for future exploration.

Keywords: Stevia, Luminosity, Steviol Glycosides, Antioxidant Enzymes, Phenolic and Bioactive Compounds.

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LISTA DE ABREVIATURAS E SÍGLAS

aa	Amino Acids
APX	Ascorbate Peroxidase
AsA	Ascorbate
ATCC	American Type Culture Collection
BA	N6-benzyladenine
BCAA	Branched-Chain Amino Acids
BOD	Biochemical Oxygen Demand
BSA	Bovine Serum Albumin
C	Celsius
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CAT	Catalase
CCT	Tropical Culture Collection
CL	Constant Light
cm	Centimeters
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO_2	Carbon Dioxide
CP	Component Principal
DAT	Days After Transfer
DHAR	Desidroascorbato-redutase
DMSO	Dimethylsulfoxide
DNP	Day-Neutral Plants
DPPH	2,2-difenil-1-picril-hidrazil
EDTA	Ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
EC	Enzyme Commission
FAO	United Nations Food and Agriculture Organization
FAPESB	Fundação de Amparo à Pesquisa do Estado da Bahia
FDA	Food and Drug Administration
FSANZ	Food Standards and the Food Standards of Australia and New Zealand
FW	Fresh Weight
g	Gram
GA	Gibberelli Acid
GA_3	Gibberelli Acid

GAE	Gallic Acid Equivalent
GPOD	Peroxidase Dependent Guaiacol
GPX	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione Reduced
GSSH	Glutathione Oxidized
h	Hours
HPLC	High-Performance Liquid Chromatography
HSD	Honestly Significant Difference
IAN	Instituto Agronómico Nacional
IC_{50}	Half Maximal Inhibitory Concentration
IPTA	Instituto Paraguayo de Tecnología Agraria
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	Kilogram
LED	Light Emitting Diode
LD	Long-days
LDP	Long-Day Plants
M	Methanol
M	Molar
m^3	Cubic Meter
MDHAR	Monodehydroascorbate Reductase
MIC	Minimal Inhibitory Concentration
mM	Milimolar
mm	Millimeter
Mg	Magnesium
mg	Milligram
µg	Microgram
mL	Milliliter
µL	Microliter
µmol	Micromolar
MS	Murashige and Skoog
mS	Micro-Siemens
n.a.	Not active
NADPH	Nicotinamide Adenine Dinucleotide Phosphate

NBT	Tetrazolium-Nitroblue Chloride
nm	Nanometer
NMR	Nuclear Magnetic Resonance
P A	For Analysis
pH	Hydrogen potential
PLS-DA	Partial Least Squares Discriminant Analysis
POD	Peroxidase
RAS	Regras para Análise de Sementes
RH	Relative Humidity
ROS	Reactive Oxygen Species
RPM	Rotações Por Minuto
SD	Short-days
SDP	Short-Day Plants
SG	Steviol Glycosides
SOD	Superoxide Dismutase
StGly	Steviol Glycosides
TCA	Tricarboxylic acid cycle
TDZ	Thidiazuron
TSC	Total Steviosides Content
UDP	Uridine 5'-diphospho-glucuronosyltransferase
UGT	Glucuronosyltransferase
UR	Relative humidity
USA	United States of America
US$	Dollars
VIP	Variable importance in the projection scores
v/v	Volume/Volume
w	Watts
WHO	World Health Organization

SUMÁRIO

REVISÃO DE BILIOGRAFIA

Alternativa de adoçantes naturais:
aprimorando o uso de *Stevia rebaudiana* como fonte de glicosídeos de esteviol.

Alternative for natural sweeteners: improving the use *Stevia rebaudiana* **as a source of steviol glycosides**

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Abstract

Stevia rebaudiana (Bert.) Bertoni is a shrub commonly called ‘stevia’ belonging to the Asteraceae family, which is cultivated in many regions of the world. Its main characteristic is the presence of metabolites with sweetening properties, i.e. the steviol glycosides (StGly), which are up to 300 times more sweetening capacity than sucrose. StGlys have made this species extremely relevant for the food and beverage industries, gaining greater industrial and scientific interest over the past 20 years. However, limited plant material production is not meeting the increased demand of the global market. In addition to its sweetening characteristics, stevia has a huge range of properties offering benefits against a multitude of health conditions. The main objective of the present review focused on the state of the art of the species, compiling studies from the characterization of seeds to their industrial and safety applications, addressing biotechnological strategies to understand, stimulate and improve the biosynthesis of stevia metabolites. In addition to providing useful information aiming for plant breeding and/or high-quality seeds**,** large-scale cultivation and leaf biomass production.

Keywords: Biotechnology; Diterpene glycoside; Germination; Large-scale cultivation.

1. Introduction

Stevia rebaudiana (Bert.) Bertoni has gained recognition in the industrial and scientific fields in recent years due to the presence of natural sweetening compounds in its leaves, i.e. steviol glycosides (StGly), which can be used as a healthier alternative to sucrose and artificial sweeteners in food and beverages (SAMUEL et al., 2018; TALEVI, 2016). Their purification, coupled with their commercialization as high purity sweeteners resulted in ca. US$ 492 million in 2018 with perspectives of ca. US$ 1.16 billion by 2026, thus making it nowadays a culture with great notoriety (GELSKI, 2020).

The market potential of stevia as a source of natural sweeteners has sparked the interest of producers and industry and led to an increasing amount of research in stevia since the 1970s when it began to be used as a sweetening agent in Japan for the first time (ABOU-ARAB; ABOU-ARAB; ABU-SALEM, 2010; CHATSUDTHIPONG; MUANPRASAT, 2009). It is currently a global crop grown commercially in countries in Asia, Africa, Europe, and Americas (CIRIMINNA et al., 2019; LEMUS-MONDACA et al., 2012; MADAN et al., 2010; PÓL; HOHNOVÁ; HYÖTYLÄINEN, 2007; SOEJARTO, 2002; YADAV et al., 2011).

The StGly compounds with sweetening properties are mainly present in stevia leaves and can be up to 300 times sweeter than sucrose and, therefore, much favorable for human consumption as they do not cause adverse health effects (KIM; CHOI; CHOI, 2002; MEGEJI et al., 2005; RAJASEKARAN et al., 2008; TALEVI, 2016; TANAKA, 1982). In addition to its sweetening features, there are reports demonstrating the medicinal use of stevia components in the treatment of diabetes, hypertension, myocardial and antimicrobial infections, tumors, and tooth caries. Such features make stevia an important plant in the current scenario in which people daily seek a healthier diet (GUPTA et al., 2013; JEPPESEN et al., 2002, 2003).

It is, therefore, of great importance the continuous evaluation of the properties presented by *Stevia rebaudiana*, as well as bring up issues of relevance for the development of proper stevia cultivation systems aimed at its large-scale production, e.g., high quality seeds and cultivation protocols under different biotic and abiotic stress aimed at the growing consumer demands and market production. In this context, the purpose of this review focused on the state of the art of the species, compiling studies from the characterization of seeds to their industrial and safety applications, addressing

biotechnological strategies to understand, stimulate and improve the biosynthesis of stevia metabolites. In addition to providing useful information aiming for plant breeding and/or high-quality seeds, large-scale cultivation and leaf biomass production.

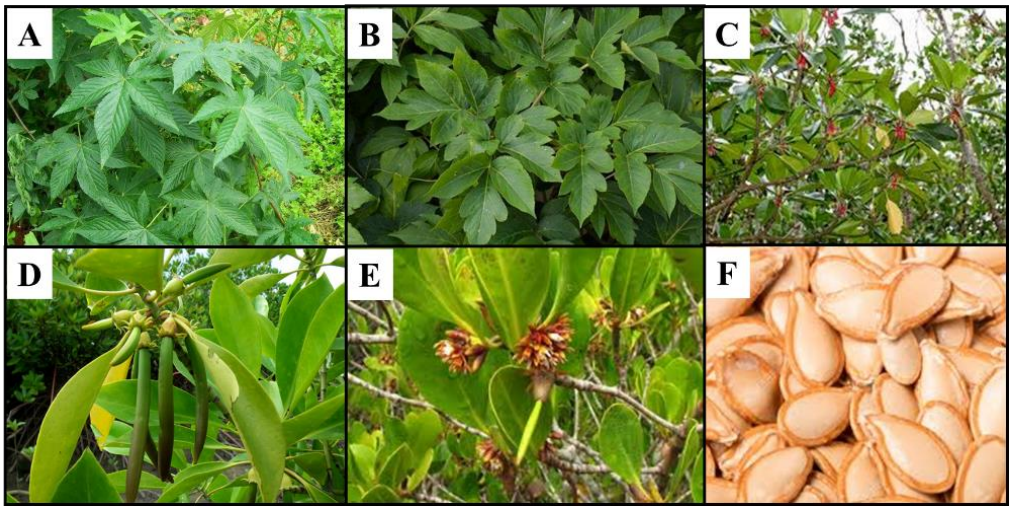
2. *Stevia rebaudiana* **(Bert.) Bertoni**

Stevia is a plant species that currently has a high commercial and nutritional value being endemic and native to the region of the Amambay Cordillera in Paraguay, with natural occurrence also along the border with Brazil and Argentina and composes one of the 950 genera of the Asteraceae (Compositae) family (MIZUTANI; TANAKA, 2002). The natural occurrence and distribution of the Stevia genus cover the tropical and subtropical regions of the Americas (LESTER, 1999; SOEJARTO, 2002; SOEJARTO et al., 1983). Evolutionarily, comparative genomic analyzes between Stevia and other plants of the Asteraceae family show that stevia and sunflower diverged approximately 29 million years ago (XU et al., 2021).

Due to its potential as a source of natural sweeteners administered by the components called steviol glycosides (StGly), stevia has aroused the interest of producers and industry in many countries since the 1970s. And so, lead to an increase in the investigation of its properties (ABOU-ARAB; ABOU-ARAB; ABU-SALEM, 2010; CHATSUDTHIPONG; MUANPRASAT, 2009). The species was initially domesticated in Japan (1960 - 1970s), but its commercial cultivation began in its endemic origin in Paraguay. It is currently a rapidly expanding global crop due to its important role in agriculture and bioindustry, therefore, with increasing worldwide bioeconomy relevance (CIRIMINNA et al., 2019; LEMUS-MONDACA et al., 2012; MADAN et al., 2010; PÓL; HOHNOVÁ; HYÖTYLÄINEN, 2007; SOEJARTO, 2002; YADAV et al., 2011). However, stevia has been so far cultivated under small-scale agricultural systems as it still presents inherent characteristics that are limiting to its large-scale cultivation and production (BRANDLE; STARRATT; GIJZEN, 1998; THIYAGARAJAN; VENKATACHALAM, 2012). Due to the presence of important secondary metabolites, stevia is considered an important medicinal and biologically valuable plant. The sweetening components present in *S. rebaudiana* are mainly present in its leaves, among which are stevioside, rebaudioside A, B, C, D, and E, and dulcoside A among others, which together can be approximately 300 times sweeter than sucrose and

suitable for human nutrition as they are natural and do not cause adverse effects in human health (CHOI et al., 2002; MEGEJI et al., 2005; RAJASEKARAN et al., 2008). Within the Stevia genus only *S. rebaudiana* and *S. phlebophylla* present the StGly (KINGHORN et al. 1984). The StGly has been identified in other species, such as in the leaves of *Rubus Suavissimus* S. Lee (Rosaceae) and *Angelica keiskei* (Miq.) Koidz. (Apiaceae), roots of *Bruguiera gymnorhiza* (Rhizophoraceae), *Bruguiera cylindrica* (Rhizophoraceae), *Ceriops decandra* (Rhizophoraceae) (E), and in the endosperm of seeds of *Cucurbita maxima* (Cucurbitaceae) (Figure 1), giving these species the same sweetening characteristic. However, only in *S. rebaudiana* such components are quantitatively and commercially viable to be isolated and used for human consumption (BLECHSCHMIDT et al., 1984; LIBIK-KONIECZNY et al., 2021; OHTANI et al., 1992; SUBRAHMANYAM et al., 1999; SULTANA, 2018).

Figure 1. Plants in which steviols has been identified. (A) *Rubus Suavissimus* S. Lee (Rosaceae); (B) *Angelica keiskei* (Miq.) Koidz. (Apiaceae); (C), roots of *Bruguiera gymnorhiza* (Rhizophoraceae); (D) *Bruguiera cylindrica* (Rhizophoraceae); (E) *Ceriops decandra* (Rhizophoraceae); and (F) endosperm of seeds of *Cucurbita maxima* (Cucurbitaceae).



In addition to its high sweetening potential, fresh stevia leaves have several properties with beneficial applications for the treatment of diabetes, hypertension, myocardial and antimicrobial infections, tooth decay, and tumors (CHAN et al., 1998; CHOI et al., 2002; JEPPESEN et al., 2002, 2003). Stevia plant extracts may have the presence of a wide range of metabolites, such as amino acids (alanine, aspartate and valine), organic acids (acetate, aconite, γ-aminobutyric acid, lactate, succinate and formate) phenolic compounds (gallate and kaempferol -3-Op-glucopyranosyl-7-Oa-ramnopyranoside) as

noted by Andrade et al. (2021) cultivating plants under different photoperiod conditions. In other studies, compounds such as austroinulin, β-carotene, dulcoside, nilacin, riboflavin, thiamine caffeic acid, chlorogenic acid, transferulic acid, and rutin have been identified (AHMAD et al., 2020; JAYARAMAN; MANOHARAN; ILLANCHEZIAN, 2008; LEMUS-MONDACA et al., 2016). Among the biocomposites found in the leaves, there is also the presence of assimilable carbohydrates, fibers, polypeptides; lipids, potassium; calcium, magnesium, phosphorus, chromium, cobalt, iron, manganese, selenium, silicon and zinc, traces of ascorbic acid, aluminum, beta-carotene C, tin, riboflavin, vitamin B1 and essential oils (CHOI et al., 2002; GUPTA et al., 2013; JEPPESEN et al., 2002, 2003).

Furthermore, to the leaves, studies with stem and root samples demonstrate the high medicinal potential of stevia for, phenolic, antioxidant and antimicrobial potential mebolites, besides the sweetening compounds (ANDRADE et al., 2021; BENDER; GRAZIANO; ZIMMERMANN, 2015; REIS et al., 2017). Given its characteristics, stevia has become a very important plant in the current scenario due to its overall medicinal properties in which consumers seek healthier foods daily.

3. Botanical Description

Phenotypically, *S. rebaudiana* is a perennial shrub with a woody and fragile stem, small elliptical leaves with a slightly granular pubescent surface, and the flowers are pentameric and small (Figure 2; Supplementary Figure 1) (GANTAIT; DAS; BANERJEE, 2018; SOEJARTO, 2002). It has slightly branched roots which are extensive and fibrous during the seedling stage, whereas in the adult plant predominates superficial branched roots without deepening (RAMESH; SINGH; MEGEJI, 2006). The plant can reach up to one-meter tall depending on the crop agronomic practices and the variety (RAKHRA et al., 2011; SHARMA et al., 2016; SIMLAT et al., 2016; SONI P K; G; SHARMA D K, 2015).

Figure 2. *Stevia rebaudiana* (Bert.) Bertoni plant.

It is observed great variability within the species making it difficult to characterize and determine the number of existing varieties. There is also a large variation in the number of chromosomes in the stevia genus, in which n = 11 (2n = 22) is preponderant, but also possible stevia strains with 2n = 33 and 2n = 44 which represent triploid and tetraploid cytotypes, respectively (FREDERICO et al., 1996; GALIANO, 1987; GALIANO; HUNZIKER, 1987; OLIVEIRA et al., 2004). In a study by Zhang et al. (2018), colchicine-induced polyploidy, originated tetraploid plants had the highest stomata and chlorophyll content, indicating that tetraploid stevia plants may have greater photosynthesis and transpiration capacity. In addition, tetraploidy in stevia plants provides more vigorous plants, with large and thick leaves, more robust stems, shorter internodes, and a greater density of glandular trichomes. Differences are observed in the levels of steviol, stevioside, and rebaudioside A glycosides, which were higher in tetraploid plants than in diploid controls (ZHANG et al., 2018).

Therefore, depending on the genotype, location, and cultivation conditions the varieties of stevia may differ in their vegetative characteristics such as in plant height/size, flowering period and consequently also in the content of phenolic compounds and the concentration of steviol glycosides in the leaves (OLIVEIRA et al., 2004; RAMESH;

SINGH; MEGEJI, 2006; SHARMA et al., 2016; SINGH et al., 2015; TATEO et al., 1998; WÖLWER-RIECK, 2012).

S. rebaudiana is more vigorous when grown as a crop than in its native habitat where latitude and longitude conditions are adequate providing values above at least 12 hours of daylight. However, due to its ability to adapt to various environmental conditions, it has now become successfully arable around the world (BRANDLE; STARRATT; GIJZEN, 1998; CIRIMINNA et al., 2019; GANTAIT; DAS; BANERJEE, 2018; KIM et al., 2011; LIBIK-KONIECZNY et al., 2018; RAMESH; SINGH; MEGEJI, 2006). Furthermore, *Stevia rebaudiana* is characterized by not having great needs for soil, moisture, and other characteristics. However, light is crucial, as its non-supply or partial shade results in slower growth, delayed flowering, lower leaf production, and consequently lower StGly content (RAMESH; SINGH; MEGEJI, 2006).

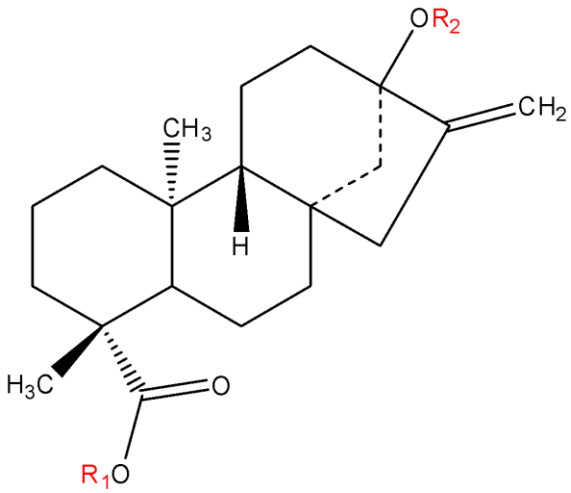
4. Steviol Glycosides (StGly)

4.1 Biosynthesis

Diterpenoids, a chemically heterogeneous group of compounds, secondary metabolites belonging to the class of terpenes constituted by 20 carbon atoms. Classified according to their skeletal core into linear, bicyclic, tricyclic, tetracyclic, pentacyclic, or macrocyclic (LUDWICZUK; SKALICKA-WOŹNIAK; GEORGIEV, 2017).

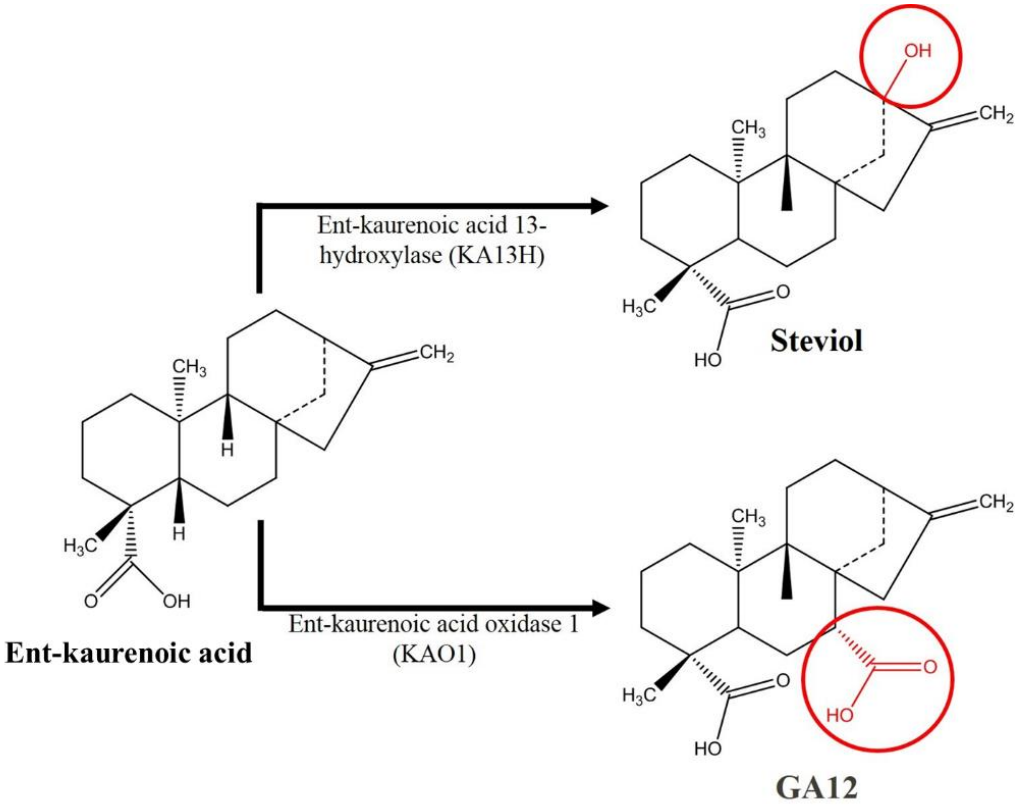
Steviol glycosides (StGly) are metabolites belonging to the class of tetracyclic diterpenoids, and their chemical structure is composed of an aglycone nucleus, steviol (ent-13-hydroxyur-16-en-19-oic acid) (Figure 3). The formation of the identified glycosides in stevia is established by glycosylation reactions starting with the production of steviol and ending with the formation of rebaudioside A (GERWIG et al., 2016; LIBIK-KONIECZNY et al., 2021). These compounds are found mainly in plants and may be converted by hydrolytic cleavage into sugar and non-sugar components. Its nomenclature is established according to the type of sugar they contain, such as glycosides (glucose), pentosides (pentoses), fructoses (fructose), among others (BERNAL et al., 2011; GERWIG et al., 2016; LIBIK-KONIECZNY et al., 2021).

Figure 3. Representation of the diterpene core skeleton of the steviol. R1 and R2 positions are substituted by glucose molecules (BRANDLE; TELMER, 2007).



Stevia evolved to synthesize StGly, it is believed that genetic modifications may have occurred to favor the biosynthesis of specialized metabolites, especially the biosynthesis of terpenoid structures, and for further oxidation and glycosylation of these compounds (LUCHO et al., 2021; BASHARAT et al., 2021; CEUNEN; GEUNS, 2013). These metabolites are mainly isolated from stevia leaves but can be found in other parts of the plant in smaller proportions, such as stems and roots. These compounds have the same skeleton as the diterpene nucleus and their biosynthesis shares the same pathway as gibberellic acid (GA) biosynthesis (Figure 4) (BRANDLE; TELMER, 2007; ESWARAN et al., 2012; HAJIHASHEMI; GEUNS, 2016, 2017; KUMAR et al., 2012; SINGH; RAO, 2005). Initially, StGly synthesis occurs in plastids, two isoprene units are produced from the 2-C-methyl-D-erythritol-4-phosphate (MEP), isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) derivative pathway of pyruvate and a glyceraldehyde-3-phosphate (BEHROOZI et al., 2017; BRANDLE; ROSA, 1992). Subsequently, IPP + DMAPP originates kaurenoic acid through the action of five enzymes, namely, geranylgeranyl diphosphate synthase (GGDPS), copalyl diphosphate synthase (CPPS), kaurene synthase (KS), kaurene oxidase (KO) and 13-kaurene hydroxylase (KO) (BRANDLE; TELMER, 2007; GERWIG et al., 2016; GULERIA; YADAV, 2013; KIM et al., 2019; YADAV et al., 2011).

Figure 4. Point of differentiation between steviol glycoside biosynthesis and gibberellin biosynthesis.



The ent-kaurenoic acid is the last common intermediate in the synthesis of steviol glycosides and gibberellic acid. In the endoplasmic reticulum, the derivation and differentiation between the two metabolites occur with the introduction of the hydroxyl group (-OH) provided by two proteins belonging to the cytochrome superfamily (CYP P450) in different positions. The KA13H located in the membrane of the endoplasmic reticulum introduces the -OH group at position 13C thus forming the steviol through the UDP-glycosyltransferases catalyzed glycosylations, giving rise to the different steviol glycosides. Meanwhile, the insertion of a -OH group at position 7C of ent-kaurenoic acid promoted by the enzyme KAO1 results in the synthesis of metabolite GA12 which acts as a precursor to all reported gibberellins (Figure 4) (RICHMAN et al., 2005, 1999; SINGH et al., 2017). This sharing is important as a way of regulating StGly biosynthesis. Studies carried out with the application of exogenous hormones demonstrated the role in controlling the biosynthesis of GA and consequently modulating the yield of StGlys. As it was possible to observe in studies carried out with the application of GA3, DAM, and NAA, demonstrating a regulation in the genes that

encode the transcription of enzymes in the pathway and in the total number of stevioside and rebaudioside A (YONEDA et al., 2018).

The formation of glycosides takes place in cellular cytosol, is established by glycosylation reactions at points of attachment, the R1 and R2 positions (Figure 3), from enzymatic reactions originate the different StGlys identified, starting with the production of steviol and ending with the formation of rebaudioside A,, considered to date the most important compound of commercial interest present in the biosynthetic pathway of steviol glycosides, since it has no residual bitterness (HANSEN et al., 2003; PRAKASH et al., 2015; PRAKASH; MARKOSYAN; BUNDERS, 2014). Each component of the StGly biosynthetic pathway is derived from carbohydrate residues, mainly glucose, but rhamnose compounds and xylose residues linked to the molecular structure of steviol are found. The final phase of StGly biosynthesis is their translocation and storage in the vacuole (GERWIG et al., 2016; LIBIK-KONIECZNY et al., 2021).

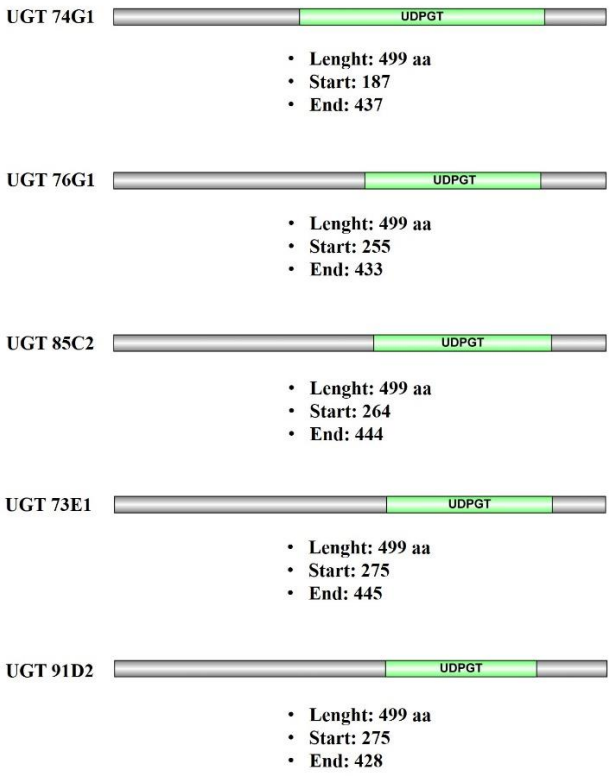
Enzymes of the UGT family are responsible for the formation and accumulation of StGlys, the UDP-glycosyltransferases. A divergent group of enzymes whose function is to transfer a sugar residue from an activated donor to an acceptor molecule. The UGT superfamily is conceptualized as a protein structure characterized by 44 amino acids responsible for joining the UDP fraction to the sugar donor. In general terms, UGTs participate in various functions, including human detoxification, homeostasis, and myelin leaf synthesis, while in plants they participate in growth, homeostasis by means of the balance between auxins and abscisic acid, detoxification, pigment stabilization, synthesis of glucosinolates, sweeteners, flavonoids, and reserves of phytoalexin. They also participate in fungi homeostasis, deactivation of pheromones and detoxification in insects, the regulation of antioxidant defense in nematodes, antibiotic resistance in bacteria (BOCK, 2016). In stevia, the sugar molecule acceptor is the steviol which is responsible for the acquisition of the sweet taste, besides greater stability, and solubility of the compounds (HANSEN et al., 2003; PRAKASH et al., 2015; PRAKASH; MARKOSYAN; BUNDERS, 2014).

Five UGTs are responsible for the glycosylation reactions that formed steviol glycosides. But currently, only four glycosyltransferases are confirmed to participate in the constitution of the metabolites present in stevia, that is, UGT 85C2, UGT 74G1,

UGT 76G1 and UGT 73E1. Although inconclusive, it is suggested that the fifth enzyme in the pathway is UGT 91D2, which may also be related to the conversion of steviolmonoside to steviolbioside (HANSEN et al., 2003; LUCHO et al., 2021; PRAKASH et al., 2015; PRAKASH; MARKOSYAN; BUNDERS, 2014; WANG et al., 2016).

The UGT described has similar domains and number of amino acids (Figure 5), UGT 85C2 is an enzyme responsible for the constitution of steviolmonoside. UGT 74G1 generates three other compounds present in the biosynthetic pathway of glycosides, rubusoside, stevioside, and rebaudioside A (Figure 6 and Supplementary figure 2).

Figure 5: Representation of known UGTs present in the steviol glycoside biosynthetic pathway. The domains were located through the Pfam platform (https://pfam.xfam.org/).

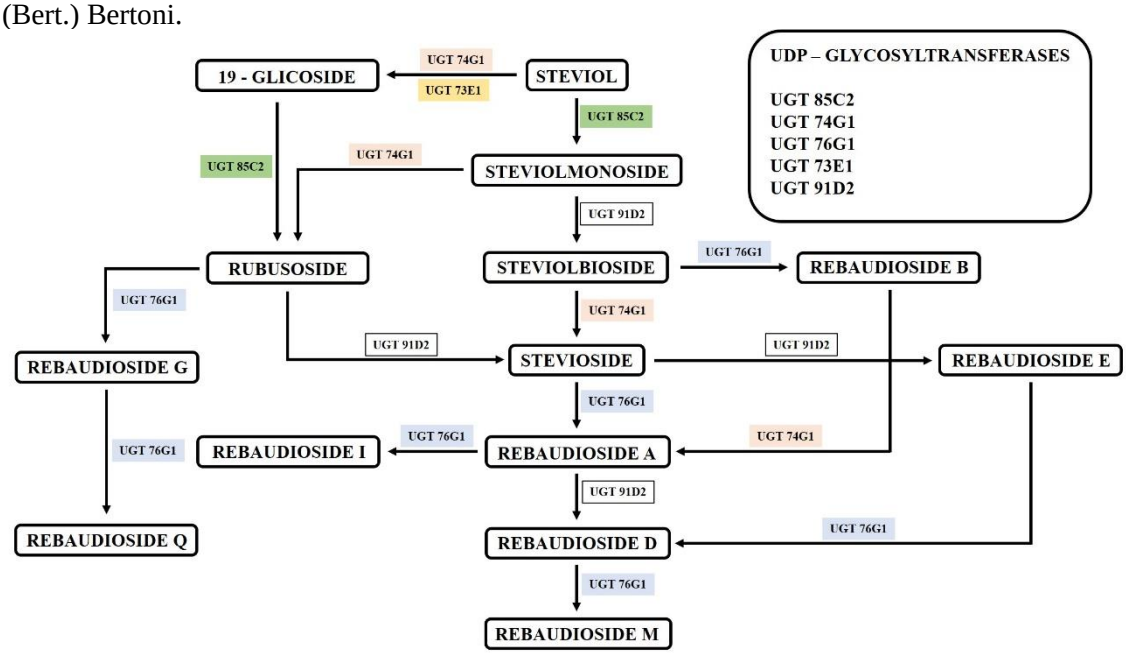


Finally, the enzyme UGT 76G1 is also responsible for the formation of rebaudioside A and three other compounds, i.e. rebaudiosides B and C, in addition to other rebaudiosides that were more recently discovered such as Rebaudioside I, D, and M (IBRAHIM et al., 2016; PERERA et al., 2017; PRAKASH et al., 2017; STARRATT et al., 2002; WEN et al., 2019), which have a sweetening intensity similar to rebaudioside

A, but with lower astringency (PRAKASH et al., 2015; PRAKASH; MARKOSYAN; BUNDERS, 2014).

More recently, the enzyme UGT 73E1 is responsible for catalyzing the formation of 19-O-β glucopyranosol steviol from steviol by the addition of a glucose molecule (LUCHO et al., 2021; LI et al., 2018). The UGT 91D2 enzyme plays an important role in the synthesis of stevioside, rebaudioside E and rebaudioside D (ZHANG et al., 2021).

Figure 6 – Biosynthetic pathway of the main steviol glycosides present in *Stevia rebaudiana* (Bert.) Bertoni.



4.2 Changes in the expression level of genes key to the synthesis of StGly and in their content

Photoperiod, temperature, and water stresses are among the several factors that can influence gene expression and the accumulation of StGly in stevia (TOTTÉ et al., 2003). Photoperiodic differentiation can influence the accumulation of these metabolites, and the accumulation of glycosides is greater in plants grown on long-days, compared to plants grown on short-days demonstrating a highly significant correlation between UGT 85C2 transcription and total steviol glycoside synthesis in younger leaves, suggesting that steviolmonoside formation is the crucial step in steviol glycoside formation (ANDRADE et al., 2021; MOHAMED et al., 2011). It is observed that high expression of genes encoding the enzymes KA13H, UGT 85C2, UGT 74G1, and UGT

76G1 have been reported during the vegetative phase with a high subsequent expression of relative GA genes (KAO, GA20O, and GA3O), followed by a reduction in steviol glycoside levels near the flowering period due to a change in the flow of ent-kaurenoic acid from steviol glycoside synthesis to GA synthesis (MOHAMED et al., 2011; SINGH et al., 2017). Moreover, it has been shown in plants grown at the same time of day that the synthesized steviol glycoside levels and transcription of the three genes responsible for glycoside formation are higher in the younger upper leaves than in the lower older leaves (MOHAMED et al., 2011). It is observed that high expression of genes encoding StGly biosynthetic pathway enzymes (KA13H, UGT 85C2, UGT 74G1, and UGT 76G1) were reported during the vegetative phase with a subsequent high expression of relative GA genes (KAO, GA20O, and GA3O), followed by a reduction in steviol glycoside levels near the flowering period due to a change in the synthesis flow of ent-kaurenoic acid that will give rise to StGly for the synthesis of GA (MOHAMED et al., 2011; SINGH et al., 2017). Furthermore, it has been shown in plants grown at the same time of day that the levels of synthesized StGly and the transcription of the three genes responsible for the formation of glycosides are higher in younger upper leaves than in lower older leaves (MOHAMED et al., 2011). It is suggested that there is a correlation between the greater accumulation of StGly in the upper leaves and the transcription of certain UGT genes depending on the photoperiod at which the plants are grown. Apparently, in upper leaves, the transition can be stressful for plants (MOHAMED et al., 2011), possibly resulting in greater production of more reactive oxygen species (ROS). The increase in StGly production may then be a reaction of the plants to increase their ability to eliminate ROS generated by photoperiodic variation.

Stevia rebaudiana leaves accumulate a range of at least twelve more abundant glycosides (Table 2), among those mentioned for the whole plant, whereas traces of 40 other glycosides present were identified (Supplementary Table 1) (BASHARAT et al., 2021; LIBIK-KONIECZNY et al., 2021). Each of these metabolites has its own flavor profile and varies between 4 and 20% of the leaf dry weight depending on the genotype, growing conditions, cultivated variety, and leaf collection period. However, the most representative StGlys are stevioside (up to 13%), rebaudioside A (up to 4%), rebaudioside C (up to 2%) and dulcoside A (up to 0.7%) (GUPTA; SHARMA; SAXENA, 2016; MYINT et al., 2020; YADAV et al., 2013). It is advisable to collect

from the emission of the first flower buds, which can allow higher yields of the compounds of interest (BRANDLE; STARRATT; GIJZEN, 1998; BRANDLE; TELMER, 2007; METIVIER; VIANA, 1979; MOHAMED et al., 2011; PÓL et al., 2007; RICHMAN et al., 1999; SHOCK, 1982; TOTTÉ et al., 2003). In addition to variations in the level of total StGly, different genotypes, ontogeny, biotic and abiotic factors can influence the composition of these compounds. Some varieties of stevia plants with higher amounts of specific glycosides, with higher leaf biomass yields, have already been patented worldwide, in countries such as Korea, Japan, Indonesia, China, USA, Canada, Taiwan, Russia, and India (RAMESH; SINGH; MEGEJI, 2006; YADAV et al., 2011). With the development of these new varieties, a higher yield is sought, mainly of rebaudioside A, when compared to other steviosides, which is the most desirable, as it has a less bitter taste, so the focus of current research on this plant is to develop varieties with high content of rebaudioside A and lower content of stevioside (KHAN et al., 2021).

Table 1 - List of main steviol glycosides (StGly) extracted from leaves of *Stevia rebaudiana* (Bert.) Bertoni.

Diterpenic Glycosides	Sweetening power	Chemical formula	Molecular weight	Exact Mass	Adduct $[M+H]^+$
Steviol	-	$C_{20}H_{30}O_3$	318.45	318.219495	317.2122
Steviolmonoside	-	$C_{26}H_{40}O_8$	480.598	480.272320	-
Steviolbioside	100-125	$C_{32}H_{50}O_{13}$	642.33	642.325145	641.3179
Stevioside	250-300	$C_{38}H_{60}O_{18}$	804.87	804.377970	803.3716
Rebaudiosídeo A	250-450	$C_{44}H_{70}O_{23}$	967.01	966.430795	965.4242
Rebaudiosídeo B	300-350	$C38H_{60}O_{18}$	804.87	804.377970	803.3716
Rebaudioside C (Dulcoside B)	50-120	$C_{44}H_{70}O_{22}$	951.01	950.435880	949.4280
Rebaudioside D	250-450	$C_{50}H_{80}O_{28}$	1129.20	1128.483620	1127.4771
Rebaudioside E	150-300	$C_{44}H_{70}O_{23}$	967.01	966.430795	965.4241
Rebaudioside F	200	$C_{43}H_{68}O_{22}$	936.99	936.420230	935.4129
Rubusoside	114	$C_{32}H_{50}O_{13}$	642.73	642.325145	641.3179
Dulcoside A	50-120	$C_{38}H_{60}O_{17}$	788.17	788.383055	787.3758

4.3. Biological characteristics of StGly

Compared to sucrose, glycosides have a potential sweetener that may vary according to the pathway component evaluated, as shown in Table 2 (LEMUS-MONDACA et al.,

2012; MADAN et al., 2010b; PÓL et al., 2007; SOEJARTO et al., 1983; YADAV et al., 2011). Additionally, the glycosides may also provide protection against possible plant pathogens, attract pollinating insects and seed dispersers, and prepare plants for possible environmental variations (OROZCO; TATIS; LEGUÍZAMO, 2011). Several benefits are also associated with steviol glycosides and their human consumption since they are non-caloric compounds without contraindications, a characteristic that has attracted increasing attention from the market.

In addition to sweetness, StGly can present some important characteristics, such as having a remarkable thermal and pH stability in which aqueous stevioside extracts appears to be stable in pH ranging between 2 and 10 and temperature up to 80°C allowing its use in various food and drink formulations. In soy isolates, the emulsion prepared with all StGly exhibited stability in the soy isolate over 120 days, in addition to reaffirming the tolerance of the emulsion in relation to temperature, pH, and electrolyte (MOHAMED et al., 2011).

Studies show that rebaudioside A has greater stability when compared to stevioside. In formulations offering strong acidic conditions for more than 2 days at a temperature of 50° C caused the formation of steviol glycoside isomers (GERWIG et al., 2016). On the other hand, at high temperatures, beverages such as carbonated soft drinks stored for up to 72 hours at 80°C, can cause the degradation of StGly (KROYER, 2010). When assessed photostability at pH 2.5, both rebaudioside A and stevioside showed a low level of degradation when exposed to sunlight (CLOS; DUBOIS; PRAKASH, 2008).

In foods such as soy beverages, skim and fermented milk, and yogurt, no signs of decomposition of the different StGly were found, in addition to not altering the quality or normal shelf life of the food (JOOKEN et al., 2012). Numerous studies aim to investigate the use of StGly as an aggregator in the most diverse food products. In addition to sweetness, StGly have properties that can allow their use in various food and beverage formulations (ZHANG et al., 2021). The addition of StGlys to foods has been suggested to prevent obesity, not only by replacing sugar, but also by reducing appetite and therefore reducing food intake. StGlys can effectively prevent and treat type II diabetes. Such metabolites do not increase postprandial glucose levels, showing the ability to increase the activity of the TRPM5 taste transduction channel, in addition to increasing glucose-induced insulin secretion. Therefore, StGlys can be explored in the

pharmaceutical and nutritional areas as an antidiabetic functional food ingredient. Several experiments in cell models (human gastrointestinal cancer cells, breast cancer cell lines) have demonstrated the in vitro antitumor potential of StGlys (PHILIPPAERT et al., 2017). Beyond, the food association and StGly can offer a number of associated benefits such as antihyperglycemic, antihypertensive, anti-inflammatory, anti-tumor, anti-diarrheal, diuretic, and immunomodulatory effects (BENDER; GRAZIANO; ZIMMERMANN, 2015; BHASKER; MADHAV; CHINNAMMA, 2015; CHATSUDTHIPONG; MUANPRASAT, 2009; GUPTA et al., 2013; MADAN et al., 2010; PÓL; HOHNOVÁ; HYÖTYLÄINEN, 2007).

4.4. Steviol Glycoside Metabolism

Toxicological studies demonstrate that StGly is poorly absorbed by the body, which is partially responsible for the food safety of stevia consumption, as these metabolites pass completely intact through the upper gastrointestinal tract (GU et al., 2019). Studies by Gardana et al. (2003), demonstrate that steviol, like the final stevioside and rebaudioside A, remained unchanged during 72 hours of incubation with human microflora, indicating that bacterial enzymes are not able to cleave the steviol structure. The degradation of the main StGly, stevioside, and Rebaudioside A incubated with human intestinal microflora demonstrates that such compounds were completely degraded to steviol in about 10 and 24 hours respectively. It is suggested that stevioside is initially hydrolyzed to steviolbioside, then this intermediate is rapidly metabolized to steviol (GARDANA et., 2003).

Briefly, each metabolite is hydrolyzed by bacteria in the colon, the glycosidic bonds are cleaved. Steviol, the initial compound of the biosynthetic pathway, is initially metabolized in the liver to steviol glucuronide and excreted in the urine. Thus, there is no retention of any stevia-related by-product in the body. There are also no reports of associated mutagenic or carcinogenic effects, therefore, the product obtained from stevia can be used commercially as a natural and healthy sugar for foods and beverages. They are even used in the diet of male, female and pediatric people, where a similar metabolic structure and fate could be observed in different situations (PURKAYASTHA; KWOK, 2020). Therefore, the implementation of the stevia product becomes an alternative to the use of sucrose mainly for people with type II diabetes,

thus making it a possible candidate for conventional sugar replacement (PÓL et al., 2007; SHARMA et al., 2016; ZUBIATE, 2007).

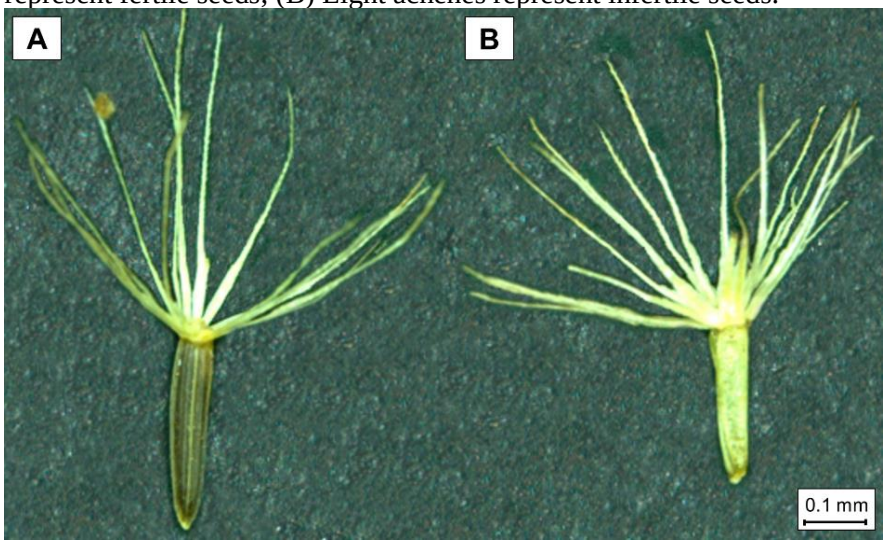
5. Morphophysiology of *Stevia rebaudiana* (Bert.) Bertoni

5.1. Germinative features

Although stevia is naturally propagated through seeds, this is not the widespread method for its large-scale commercial production due to its low germination performance (less than 50%) in addition to requiring an extended period of development of seedlings suitable for transplanting to the field (can last up to 60 days) (GOETTEMOELLER; CHING, 1999; LEMUS-MONDACA et al., 2012).

Each stevia seed is contained in an achene-type fruit, which represents the dispersal and propagative unit of stevia, and which has structures called arists (pappus) designed to promote wind dispersal. Stevia achenes have phenotypic color characteristics that can be used as a parameter for selection between dark-colored achenes containing "fertile" embryos/seeds, while the light/brown-colored achenes are generally "infertile" containing no embryos/seeds (Figure 9) (YADAV et al., 2011). However, plants can still produce unfertilized seeds through apomixis, which have the same characteristics as a viable seed (dark achene) but are not viable and therefore do not germinate (GOETTEMOELLER; CHING, 1999; MADAN et al., 2010).

Figure 7. Representation of the stevia achenes. (A) Dark achenes represent fertile seeds; (B) Light achenes represent infertile seeds.



Another relevant problem to produce stevia seeds is the self-incompatibility which seems to be also inherent to the species as an adaptation within the Asteraceae family, considered an important limitation for reproductive success (GANTAIT; DAS; BANERJEE, 2018; TULEJA et al., 2021). Studies show that this characteristic may be related to protandry, in which there is differentiated maturation between the male (androceus) and female (gynoecium) reproductive structures during the flowering of allogamous plants, which is a natural mechanism for reducing the rate of self-fertilization in favor of cross-pollination and fertilization. In addition, self-incompatibility in the production of stevia seeds may also be related to specific 'S' alleles and their correlations between different parental genotypes, as a phenomenon that does not allow the proper development of the pollen tube, which consequently prevents ovarian fertilization (CASTAÑEDA-SAUCEDO et al., 2020; GANTAIT; DAS; BANERJEE, 2018; TAMURA et al., 1984; YADAV et al., 2011). New research indicates that the problem with the propagation of *S. rebaudiana* via seeds may be caused by the inadequate level of gibberellins (GA), hormones commonly related as signaling factors for the transition of the vegetative and reproductive phases. Tuleja et al. (2021) verified the existence of a decrease in the concentration of GA during the flowering of stevia plants, suggesting that such an event may lead to the inability of pollen tube germination. In this sense, high concentrations of StGly, since there is sharing in the biosynthetic pathway at some stages with the formation of GA, maybe a reason for the malfunction of the stevia pollen grains. Thus, the production of high-quality seeds depends on further studies regarding the regulation of the life cycle and maturation of the species' reproductive organs (MADAN et al., 2010; MIDMORE; RANK, 2002; TULEJA et al., 2021).

The germination rate of stevia seeds can be influenced by several factors. The seeds germination itself is considered light-dependent or positive photoblastic. However, considerable germination rates can occur when subjected to germination in the dark (ABDULLATEEF; OSMAN, 2011; KUMAR, 2013). Some studies have shown that the use of different light wavelengths regulates not only germination but also plant growth and development in which stevia seed germination appears to be regulated by an association between light quality, morphological and biochemical parameters (ABDULLATEEF; OSMAN, 2011; KUMAR, 2013; KUMAR; SHARMA; PRASAD, 2013; SIMLAT et al., 2016). Blue LED light appears to induce a higher germination

rate and speed besides more vigorous seedlings with higher fresh weight, greater number of leaves, roots and stomatal frequency, pigment concentration and high activity of antioxidant enzymes (SIMLAT et al., 2016).

Stevia seed germination may also be influenced by the type of substrate used and the way the achenes are sown or deposited in it, i.e., when the achenes are not in total contact with the substrate there may be insufficient seed water absorption for the reactivation of cellular metabolism (CARNEIRO; GUEDES, 1992; KUMAR et al., 2013). The use of phytohormones such as gibberellin, thidiazuron, kinetin, and N6-benzyladenine may also play an important role in both germination and growth of stevia seedlings, given that gibberellin can improve seed germination, while kinetin provides seedling development by regulating the height of the above-ground parts, the number of leaves, and the length of the roots, along with the level of chlorophylls and antioxidant compounds (SIMLAT et al., 2019). Melatonin seems to act to favor a better germination performance as observed by Simlat et al. (2020), demonstrating the positive effect on stevia seed germination at different concentrations (5 and 20 μM) under normal and low conditions salinity. Thus, the use of phytohormones at appropriate levels can serve as an alternative for regulation of both stevia germination and plant development.

Thus, to obtain high quality seeds for large-scale cultivation, several suitable procedures must be considered by the producer, ranging from pollination, environmental conditions during seed formation and maturation, harvesting time and the characteristics of matrix plant cultivation and until the proper time for harvesting the seeds (CARNEIRO; MUNIZ; GUEDES, 1997; GOETTEMOELLER; CHING, 1999; LESTER, 1999; RAMESH; SINGH; MEGEJI, 2006; SHARMA et al., 2016; SHOCK, 1982).

5.2. Propagative features

Stevia is a plant which adapts easily to different environments, what provided the expansion of your cultivation. However, there are essential characteristics to be followed for its establishment, wet soils, with average organic matter content, reasonable permeability, and drainage, in a pH range of 5.5 to 7.5, are of paramount importance for obtaining vigorous plants (ABDULLATEEF; OSMAN, 2011; CORTÉS, 2012; PANDE; GUPTA, 2013). According to Cortés (2012), factors such as

temperature and light mainly influence both its development and the accumulation of components responsible for its sweetness.

Stevia biomass production (leaves) can be influenced by specific characteristics of perennial crops, such as crop establishment in the first year, plant age in the field and hibernation, for example. The age at which the stevia plants are harvested impacts biomass production, for example, it is possible to obtain a large increase in yield from the first to the fifth year of cultivation (LE BIHAN et al., 2021). In a study under mild climate conditions, the number of potential crops per year was tested, noting that a single crop at the end of the growing season leads to a higher yield than two or three crops in the same growing season (MORAES et al., 2013; SERFATY et al., 2013). Despite being a species that can be easily cultivated, stevia may respond negatively to improper cultivation methods such as exposure to salinity, drought, or flooding conditions affecting the ability of plants to absorb nutrients (ANDRADE et al., 2021; DEBNATH; ASHWATH; MIDMORE, 2019). In general, the literature shows that there is a limit to the growth and development of plants in stressed crops, e.g. plants treated with NaCl reduced (RAMEEH et al., 2017) or increased (GUPTA; SHARMA; SAXENA, 2016; PANDEY; CHIKARA, 2015) their StGly content depending on the molarity to which they were subjected, possibly affecting (LUCHO et al., 2019) or exhibiting a negative effect on growth parameters such as the number of roots. The effect of saline solutions on the development of the stevia outbreak and root may be due to the water deficit generated by NaCl, as well as its possible ion toxicity which causes imbalances in nutrient absorption and assimilation (FALLAH et al., 2017; GUPTA; SHARMA; SAXENA, 2016; PANDEY; CHIKARA, 2015). To obtain a higher yield in the stevia crop, hours of sunshine, humidity and temperature are very important to obtain an ideal stevia yield. The exogenous supply of foliar application (B, Se, and Fe) can favor plant height, number of branches, leaf production, for example. In addition, the plants show a certain adaptation to moderate stress levels of NaCl (30 mM) with the use of the aforementioned tools (AGHIGHI SHAHVERDI; OMIDI; TABATABAEI, 2018).

Currently, there is a constant search for alternatives that can favor crops with greater yield and yield and thus meet the growing market demand for stevia. Alternatives like the use of asexual reproduction, such as tissue culture, cuttings, etc., have been the most

used alternatives for stevia crops, even if such methods require a high financial investment and generate small-scale production (DEBNATH; ASHWATH; MIDMORE, 2019; KUMAR et al., 2013; SIVARAM; MUKUNDAN, 2003). In this perspective, large-scale stevia cultivation is dependent on stevia genotypes with superior capacity for high-quality seed production and/or the development of proper seed production systems aimed at high-quality seeds, both of which coupled with a proper ratio of rebaudioside-A/stevioside aimed at the elimination or lesser content of residual bitter flavor (SHARMA et al., 2016; YADAV et al., 2011).

5.2.1. Stem cuttings propagation

The production of stevia on a world scale is still limited due to the low germination percentage of its seeds. Thus, the priority propagation system used is the vegetative or clonal one, where mother plants are used to produce seedlings, producing more uniform and vigorous crops and in a short period of time, representing a potential alternative for the propagation of the species (LÓPEZ MEDINA; GIL RIVERO; LÓPEZ ZAVALETA, 2016). However, several factors affect the adventitious rooting of stem cuttings, such as the concentration of endogenous hormones, plant regulators, juvenility, seasonality, and environmental conditions during rooting are the most important (CASTAÑEDA-SAUCEDO et al., 2020; LÓPEZ MEDINA; GIL RIVERO; LÓPEZ ZAVALETA, 2016).

An alternative to establishing stakes is the use of hormones. Studies show that the use of IBA can promote better rooting in stevia cuttings (KASSAHUN; MEKONNEN, 2012). In addition, characteristics such as plant height, number of leaves, leaf dry weight, leaf area, total biomass, leaf area ratio and specific leaf area were statistically higher than the control. On the other hand, the treatment with 6.4 mM NAA + 0.3 mM IBA presented better results for the variable parameters root length, root dry weight and biomass partitioning of root (CASTAÑEDA-SAUCEDO et al., 2020).

For the establishment of stevia cuttings, it is also necessary a comprehensive study regarding the month or season of propagation of the species, a characteristic that varies depending on the genotype and climatic conditions (CASTAÑEDA-SAUCEDO et al., 2020; KASSAHUN; MEKONNEN, 2012; KHALIL; ZAMIR; AHMAD, 2014). Thus, it

is important to study the effect of hormones on rooting seedlings and the best month for stevia propagation. The most suitable establishment months for stevia propagation are February to July, except for June. Cuttings can be established in November, but the percentage of seedlings rooted in our study was 93.17%. Establishing the stevia spread in June or August is not recommended (CASTAÑEDA-SAUCEDO et al., 2020).

Studies carried out by Galo (2019) showed significant results regarding the multiplication of stevia via cuttings, where the highest percentage of seedling survival was obtained in cuttings originating from the tips of the stems (93.92%), which differed statistically from the intermediate cuttings (91%) and basal stem cutting (85.51%). This high percentage is directly associated with the region of acquisition of these cuttings, highly meristematic tissues, associated with the use of the IBA hormone (5.5 g/L).

It is expected that the production of stevia can supply the world demand for the sweeteners present in its leaves. Therefore, it is important to find alternatives for large-scale stevia production to obtain leaves, where vegetative propagation can be an important tool, however further studies are needed for its use.

5.2.2. Micropropagation

The application of in vitro cultivation techniques is a powerful vegetative proliferation tool for many plant species. It is considered a faster and more efficient method of obtaining a large number of plants from an industry point of view. When it comes to stevia, it is possible to obtain plants that are homogeneous in composition and StGlys content, for example (SIVARAM; MUKUNDAN, 2003). Multiple studies prove that tissue culture techniques are effective methods for propagating stevia, mainly because they use different parts of the same plant, such as leaves, shoot primordia, auxiliary shoots and multimodal explants (YADAV et al., 2011). Therefore, the best option for plant multiplication and healthy biomass production.

In a study by Rokosa and Kulpa (2020) with nodal fragments, it was possible to observe that the use of cultures enriched with cytokinins (KIN, BAP and 2iP) and auxins (IAA, IBA and NAA) had a very significant impact on the growth and development of stevia in in vitro cultures. vitro. On the other hand, obtaining stevia plants using RITA® temporary immersion systems result in a large number of *Stevia rebaudiana* plants with

high genetic fidelity. Therefore, it is also an effective procedure for the commercial micropropagation of S. rebaudiana, as well as for the rapid multiplication of new stevia varieties or elite genotypes (RAMÍREZ-MOSQUEDA et al., 2016).

A peculiar point in the micropropagation of stevia in vitro is the physiological state of micropropagated plants during acclimatization to ex vitro conditions, especially regarding the availability of light and its intrinsic relationship with the species. Acosta-Motos et al. (2019) noted that there is a huge contribution of antioxidant mechanisms and photosynthesis in the acclimatization of stevia plants, providing very useful information to monitor plant stress during the acclimatization process (ACOSTA-MOTOS et al., 2019).

The micropropagation and acclimatization of stevia plants can be an excellent tool to guarantee the production of clonal plant material, uniform and faithful to the type. This can be used as a diversion to the problems associated with the low germination rate and the wide variability in the steviol glycoside profile in S. rebaudiana plants. As consequence, the production of uniform plants of *S. rebaudiana* with the same steviol glycoside profile is of important commercial interest. This is particularly interesting for rebaudioside A, which provides superior flavor to food products compared to other steviosides. For these reasons, tissue culture techniques are widely used to produce maximum mass from a single plant in a short time and also provide opportunities for the conservation of important plant germplasm (ANBAZHAGAN et al., 2010; HASSANEN; KHALIL, 2013; JAGATHEESWARI; RANGANATHAN P., 2012; SIVARAM; MUKUNDAN, 2003; TAWARE et al., 2010).

5.3. Photoperiod and the relationship with stevia

Stevia is a plant highly responsive to the length of day/night, depending on the purpose of its cultivation. The critical photoperiod for stevia is defined between 12/13 hours of light, below which the plant enters a reproductive or flowering mode (seed production), whereas above which the plant favors its vegetative mode (leaf and steviol glycoside production) (Figure 8) (DEBNATH; ASHWATH; MIDMORE, 2019; METIVIER; VIANA, 1979; VALIO; ROCHA, 1977).

Characterized as a strictly short-day plant, *S. rebaudiana* blooms from January to March in the southern hemisphere and from September to December in the northern hemisphere. Plants subjected to long-day photoperiods generally present leaves with larger leaf area and dry weight resulting in higher rates of photosynthetic activity and leaf biomass (YONEDA et al., 2017b). On the other hand, plants submitted to short-day photoperiods tend to accumulate higher nutrient contents in the reproductive organs, resulting in a decrease in vegetative growth.

Figure 8. Influence of photoperiod on the morphophysiological characteristics of *Stevia rebaudiana* (Bert.) Bertoni plants; (A) Plants grown under short-day, and (B) long-days.

Therefore, plants grown in long-days photoperiods have higher steviol glycoside contents in the leaves compared to plants in shorter light periods. Thus, the longest photoperiod is the most indicated when the objective of the harvest is the of leaf matter as the source of steviol glycosides for economic gain (BRANDLE; ROSA, 1992; CEUNEN; GEUNS, 2013; CEUNEN; WERBROUCK; GEUNS, 2012; METIVIER; VIANA, 1979). Similar results could be observed by Andrade et al. (2021), where the submission of stevia plants under artificial conditions of long photoperiod days (15/9h and 16/8h, light/dark) demonstrated the most promising antimicrobial activity, in addition to as the most promising conditions are maximizing the content of compounds

with phenolic and antioxidant properties, in addition to the content of rebaudioside A and total StGly.

Currently, for use in the sweetener market, production should be aimed at the production of foliar bioassay. In this sense, the use of alternatives that can favor the vegetative growth of the species have been sought. The use of artificial light has been a chosen option, either increasing the duration of the day light or applying light at night in favor greatest vegetative development and largest accumulation of the sweetening compounds. This was demonstrated by Ceunen et al. (2012) when applying red LED light at night as alternative to prolong vegetative growth causing stimulation and accumulation of steviosides during short-day periods. Obtaining a 55% increase in StGly content compared to control plants. The use of LED has also been shown to regulate enzymes of the steviosides biosynthetic pathways, e.g. upregulation of UGT85C2 (YONEDA et al., 2017a).

In this context, proper light and/or light duration can be used as a tool to regulate the physiological and biochemical characteristics in stevia due to its high sensitivity to this abiotic factor, especially regarding the production of glycosides of commercial interest that plants may have. On the other hand, a shorter light duration is required for flowering and seed production. Overall, it must be assumed that specific stevia cropping systems, such as the use of artificial light sources, especially in regions where latitude conditions do not provide a longer duration of days, are required to produce leaves as the source of stevioside sweeteners, or to produce high-quality seeds aimed at large-scale production.

5.4. Studies with different varieties of S. rebaudiana

Currently, several varieties of *S. rebaudiana* are cultivated worldwide, thus highlighting the ease of adaptation to cultivation in different environments which the species presents. The genetic improvement for the species is mainly focused on the production of the aforementioned steviol glycosides, present mainly in its leaves, in order to supply the current market demand for natural sweeteners (ANGELINI et al., 2016).

The Criolla and Morita II varieties are the most popular, the former having Paraguay as its center of origin, while the Morita II genotype has high levels of rebaudioside A. In

this universe, some varieties were also selected because they have a low need for water for cultivation and are suitable for cultivation in arid soils, Morita III and Katupyry respectively (ANGELINI et al., 2016). Bogado-Villalba et al. (2020) used ten varieties of *S. rebaudiana* in their studies: Eirete, Katupyry, VC-IAN-259, T60, VC-IAN-228, 25A, F1-Eirete, IKPS-49, 8A and IKPS-67. The Eirete variety had the highest content of stevioside and rebaudioside A, followed by the Katupyry variety. However, a promising strain was identified, VC-IAN-259 for the production of the two main StGlys of market interest (BOGADO-VILLALBA et al., 2020).

Regarding cultivation and associating it with the photoperiod in which the species presents high sensitivity, Hernández et al. (2022) and Andrade et al. (2021) studied light intensity on the production of StGlys in leaves of different varieties of *S. rebaudiana*, Morita II/Criolla and Eirete II respectively. In both studies it was possible to observe that the intensity of light applied in the cultivation of the species can drastically influence the production of StGlys, being important an in-depth study on the characteristics of the varieties to be used (ANDRADE et al., 2021; HERNÁNDEZ et al., 2022).

Despite its observable ease of cultivation, studies on the adaptation of different *S. rebaudiana* varieties to different environmental conditions and how such conditions influence plant characteristics are necessary (ANGELINI et al., 2016). Khiraoui et al. (2021) studied four varieties of *S. rebaudiana* ('Pop INRA', 'Sug-High 3' from Canada, 'Gawi' from Germany and 'Candy' from Israel) were cultivated under conditions in Morocco, with the aim of developing the local agriculture. In that study, it was possible to observe that the geological and edaphoclimatic factors of the region altered the quantity and quality of stevia leaves, and consequently the production of StGlys. However, it is notable that stevia can be grown in different soil types and nutritional conditions (KHIRAOUI et al., 2021). As can be observed for the Sugar High A3 variety, being salt tolerant and obtaining higher rates of callus survival and regeneration when compared to the Spanti variety (AZZAM et al., 2021).

S. rebaudiana proves to be a crop with great potential, but research is needed to develop specific systems for its cultivation, whether for leaf/biomass production and consequent production and extraction of StGlys, or with the objective of producing seeds. on a large scale and thus reduce costs for the implementation of the culture, since worldwide a

large part of stevia production is carried out by small producers (ANGELINI et al., 2016).

6. Use of Sweeteners - Stevia in the Sweetener Scene

There is growing interest in the food industry to replace sucrose in food, beverages and other inputs due to its concern for public health. However, it is a difficult task to reduce sucrose in foods as it has a unique sweetness profile that is difficult to replace and is crucial to the texture, structure, flavor, and preservation of food products. Currently, there is a tendency to replace sucrose in food products with non-nutritive sweeteners, mainly from natural sources such as stevia (LUO et al., 2019). Stevia has made a significant contribution to the beverage industry, specifically in sports, functional and soft drinks. Diterpene glycosides isolated mainly from leaves are currently used in the food industry as sugar substitutes and sweeteners, because of their unique organoleptic properties, namely their sweetness and the virtual absence of bitterness and adventitious flavors, as well as their low calorific value (LEMUS-MONDACA et al., 2012).

Sweeteners from stevia, StGly, are currently part of the composition of a range of food products. However, a series of studies were needed to regulate the use of such metabolites, demonstrating that there are no contraindications attributed to their use. Hundreds of studies have been carried out to analyze the action of the high purity extract from stevia against conditions such as diabetes, allergies, and cancer (RUIZ-RUIZ; MOGUEL-ORDOÑEZ; SEGURA-CAMPOS, 2017). Organizations responsible for regulating the use of purified stevia products include various bodies such as the Joint Committee on Food Additives (JECFA) of the Food and Agriculture Organization of the United Nations (FAO), the European Food Safety Authority (EFSA), the Food and Drug Administration (FDA), Food Standards, and the Australian and New Zealand Food Standards (FSANZ) have determined that consumption of high purity stevia leaf extract is safe for children, adults and special populations (EFSA, 2011; FSANZ, 2008; JECFA, 2016).

The crude extract (dry leaf), although marketed, is not approved for consumption in food and beverages. Thus, only the high purity extract (≥ 95% steviol glycosides)

contains safety parameters that allow its consumption (PERRIER; MIHALOV; CARLSON, 2018).

Since its regulation, the consumption and trade of stevia has been growing around the world, where several world-renowned companies have invested in the production and improvement of the stevia crop. Among them are PureCircle, Coca-Cola, Stevia Corp, PepsiCo, Nestle, Tate & Lyle, GLG Life Tech Corp and Evolva Holding S.A. (HARTMAN, 2017).

There are several sweeteners on the market, among which they can be classified in different ways. The current market has a wide variety of sweetening compounds (Table 2). These compounds can be classified in several ways, among which are artificial sweeteners; modified sugars; natural caloric sweeteners; no-calorie natural sweeteners; sugars; and sugar alcohols. They can also be categorized according to their properties, nutritional value, sweetening power, or origin (Table 2) (CAROCHO; MORALES; FERREIRA, 2017).

Table 2. List of sweeteners currently on the market.

Nutritive Sweeteners	Non-nutritive Sweeteners		
Glucose Isoglucose Isomaltulosis Trehalose Erythritol Isomaltitol Lactitol Maltitol Xylitol Fructooligosaccharides Sorbitol Mannitol	Intensive Sweeteners		Bulk Sweeteners
	Natural	Synthetic	Carbohydrates
	Steviol glycosides Neohesperidin dihydrochalcone Tagatosis Glycyrrhizinic acid	Acesulfame X Aspartame Sodium cyclamate Saccharin Sucralose Neotame	Fructose Sucrose Hydrogenated Fructose Corn Dextrose Syrup

Artificial sweeteners are non-nutritive sweeteners, but on the other hand they have a very strong sweet taste. They are compounds that are not responsible for the appearance of tooth decay, without having any effect on blood glucose or calories. Modified sugars are cariogenic products and have a high glycemic index, commonly used in confectionery and processed foods, for example. Natural caloric sweeteners are mainly

derived from beetroot, sugarcane, maple, etc., and some nutrients are also added to their formulation. These compounds make up most sweeteners because their caloric level is reduced. These sweeteners can be purchased from the most diverse raw materials, thaumatin, glycyrrhizic acid, brazein, rebaudioside A, stevioside are some examples. Sugars are conventional carbohydrates (monosaccharides, disaccharides) that occur naturally in fruits, vegetables, milk, and cereals and have a high glycemic index. Polyols, polyalcohols, or sugar alcohol are naturally available in vegetables, plants, and cereals. They are safe to consume and used in the composition of sweets, cookies, and sugar-free chewing gum (NG et al., 2019). Non-nutritive artificial sweeteners are generally considered to be sugar substitutes for human consumption, covering several applications (PRAVEENA; CHEEMA; GUO, 2019). Currently, the main artificial sweeteners are commercially produced by chemical methods (AHMAD et al., 2020; DUBOIS; PRAKASH, 2012).

There are currently a range of artificial sweeteners on the market as listed in Table 3 (CHAKRABORTY; DAS, 2019). Even presenting a sweetening power, such substances can cause a series of harms such as carcinogenic potential, mutagenic damage, disturbances in the fecal metabolic profile, and causing serious side effects on the intestinal flora can be mentioned.

Table 3. Variety of artificial sweeteners on the market and sweetening potential compared to sucrose.

Artificial Sweetness	Sweetness Intensity Relative to Sucrose
Acesulfame K	200 x
Advantame	7000 – 47000 x
Alitame	2000 x
Aspartame	160 – 220 x
Cyclamate	30 x
Isomaltitol	45 - 60%
Lactitol	30 - 40%
Maltitol	80 - 90%
Neotame	7000 – 13000 x
Saccharin	300 x
Sucralose	500 x

On the other hand, StGly, in addition to being calorie-free, extractable sweeteners have several benefits, such as acting in the regulation of serum levels of glucose and insulin, which accounts for their use in several countries around the world for the natural control of diabetes. It also stands out because, after ingestion, its metabolites are completely eliminated in the urine, not being stored. In addition to the aforementioned characteristics, StGly can have a high sweetening power, characterizing it as a high potency sweetener and an alternative to the use in replacement of sucrose and the use of artificial sweeteners (CHÉRON; MARCHAL; FIORUCCI, 2019; MOORADIAN; SMITH; TOKUDA, 2017; ROSA RIBEIRO; F. FREDIANI PIROLLA; M. NASCIMENTO-JÚNIOR, 2020).

Food products include sweeteners in their formulation with the aim reducing the number of calories, in addition to diseases that can be caused by high consumption sucrose, such as obesity, which is an important risk factor for many other diseases, and also cardiovascular disease, diabetes, musculoskeletal disorders and some types of cancer (TAHERGORABI et al., 2016). Therefore, many studies have been reported to improve the biosynthesis of these compounds in order to meet the market demands, such as the use of biotechnological techniques and the use of low-cost substrates, such as molasses, crude glycerol or hemicellulosic and cellulosic hydrolysates, fermentation in conjunction with metabolic engineering, which leads to low production costs and, consequently, low market costs (RZECHONEK et al., 2018). In recent years, the demand for sweeteners has increased mainly in the food and pharmaceutical sectors. The global market for low-calorie sweeteners is booming and reaching an annual growth rate of approximately 5% (SYLVETSKY; ROTHER, 2016), among natural sweeteners, xylitol is also of plant origin and the most common and demanded, however, there has been an increasing demand in recent years for stevia sweeteners, both obtained from lignocellulosic biomass (HERNÁNDEZ-PÉREZ et al., 2020).

Although there is a wide variety of sweeteners available on the market, research is constantly looking for new ingredients that can satisfactorily replace sweeteners artificial and sucrose (KIM et al., 2015; MOORADIAN; SMITH; TOKUDA, 2017). Production from biotechnological tools can be a viable and sustainable way to meet the growing market demand. Both the natural extraction of plants, the use of lignocellulosic biomass and microbial production are pillars on which sweeteners can be produced.

However, there are great technologies to expand the production of sweeteners through biotechnological routes (CHAKRABORTY; DAS, 2019; HERNÁNDEZ-PÉREZ et al., 2020; PARK et al., 2016). The recovery of hemicellulose sugars from biomass for the commercial production of xylitol, the development of microbial strains for the commercial production of sweeteners, and the use of new methods such as CRISPR/Cas9 are future projections for this market (HERNÁNDEZ-PÉREZ et al., 2020; SCHALLMEY et al., 2014; SHALEM; SANJANA; ZHANG, 2015).

Even with all the notoriety obtained in recent years, products based on steviol glycosides still face great resistance from consumers, as they may have characteristic bitterness, which is a limiting factor for their trade. It appears that the sweetness of the various StGly increases with the number of β-glucosyl residues attached and the perception of bitterness also correlates with the total number of bonds. On the other hand, recent studies have shown that StGly specifically activates the bitter taste receptors hT2R4 and hT2R14, triggering this sensation in the mouth. In this sense, studies have been carried out in order to eliminate this characteristic , either by genetic improvement techniques, with the overexpression of enzymes from the biosynthetic pathway of steviosides, in order to eliminate intermediates of the biosynthetic pathway with the occurrence of a level of glycosylation reactions, as in the study carried out by Abdelsalam et al. (2019), evaluating different varieties of stevia, where they observed that the gene expression of the enzyme UDP-glycosyltransferase UGT76G1, responsible for the final product of the biosynthetic pathway, rebaudioside A, is greater for the variety Chinese 1 compared to Egy1 and Sponti, suggesting it as a promising variety. Considering that the accumulation of rebaudioside A is one of the most important characteristics that contribute to the economic value addition of stevia crops. The modification of the structures of the steviosides themselves, as performed by Bhardwaj et al. (2019), performing a molecular docking of ligands prepared with sweet and bitter taste receptors through 3D modeling, it was possible to create sweet and bitter receptors for steviosides, producing similar structures that are not represented by residual bitterness, potentially substitutive to StGly.

Another commonly used strategy to improve the flavor of stevioside or rebaudioside A is through glycosylation reactions. By enzymatic transglycosylation of stevioside using α-amylase from *Aspergillus oryzae*, stevioside was transformed into a product with

better sweetness characteristics and a notable decrease in bitterness (YE et al., 2014). Ye et al. (2013) performed the transglucosylation of stevioside using a-amylase from *Bacillus amyloliquefaciens* in starch solution, thus producing transglucosylated steviosides with reduced bitter aftertaste. Muñoz-Labrador et al. (2020), used three cyclodextrin glucosyltransferases (CGTases) from *Paenibacillus macerans*, *Geobacillus* sp. and *Thermoanaerobacter* sp. with CGTase transglucosylation they obtained the addition of up to 11 glucose units to the steviol aglycone, which meant obtaining profiles with a greater sweet taste and a decrease in bitterness.

Another recent method to mask the bitter taste and improve the sweetness potency of stevia is the biotransformation of stevioside to Rebaudioside A. In this method, a new enzymatic transglycosylation of stevioside by pretreatment of stevia leaves with cellulose and using soluble starch as a glucosyl donor increased rebaudioside A content from 4 to 66% (ADARI et al., 2016).

Identifying alternative sources, the replacement of sucrose and artificial sweeteners in food has been something very important for the current search for a healthy diet. In this scenario, stevia has stood out and been the subject of numerous studies which aim to enhance its use in this growing market.

7. Final Considerations

The safety of its consumption makes stevia an ideal raw material against artificial sweeteners, making it a more suitable substitute for sucrose as an ingredient in the food industry, for example. In addition to glycosides, the plant has the presence of several phytochemical constituents, which makes it suitable for the extraction of several by-products that can favor or be indicated in the pursuit of healthy eating. This compilation provides valuable information on the various facets that involve stevia, but it becomes necessary to think of cultivation strategies that seek exclusively two aspects, the mass production of leaves for the sweetener industry, and an exclusive system to produce high-quality seeds considering the high production demand through large scale plantations, as occurs with numerous cultivated species. To produce leaves and consequently a greater production of StGly, several techniques can be used, however, conventional methods still do not meet the continuously increasing demand for steviol

glycosides due to the lack of high-quality planting material. Plants with desirable characteristics are propagated by stem cuttings and tissue culture practices, strategies that limit large-scale production of planting material.

The search for solutions to problems related to the production of seeds of the species is something crucial to meet this growing demand for the StGly market. Poor germination is an already observed problem that limits the cultivation and marketing of many members of the Asteraceae family (BUNKER, 1994). For stevia, this problem is linked to self-incompatibility issues related to the species itself, since for a higher yield of viable seeds with a self-germinating index, cross-pollination is crucial. In addition, certain parameters such as achene deforestation and ideal temperature contribute to the high germination performance of the species, as observed by Takahashi et al. (1996), where deforestation and temperature maintenance promoted up to 99% of germinated seeds.

A system to produce plants aimed at seed production requires some prerogatives such as conditions of short days for flowering (12 to 13 hours), temperature around 25º C, considered ideal for cultivation, in addition to a healthy hive, it is necessary near the area for seed production for the occurrence of cross-pollination between different plants. More generally, this study provides information on possible cropping system strategies for developing stevia production. Future research should emphasize the development of new seed varieties with high adaptability to different climatic conditions, viable seed production, better germination, better leaf:stem ratio for successful harvests, and higher production of sweetening compounds.

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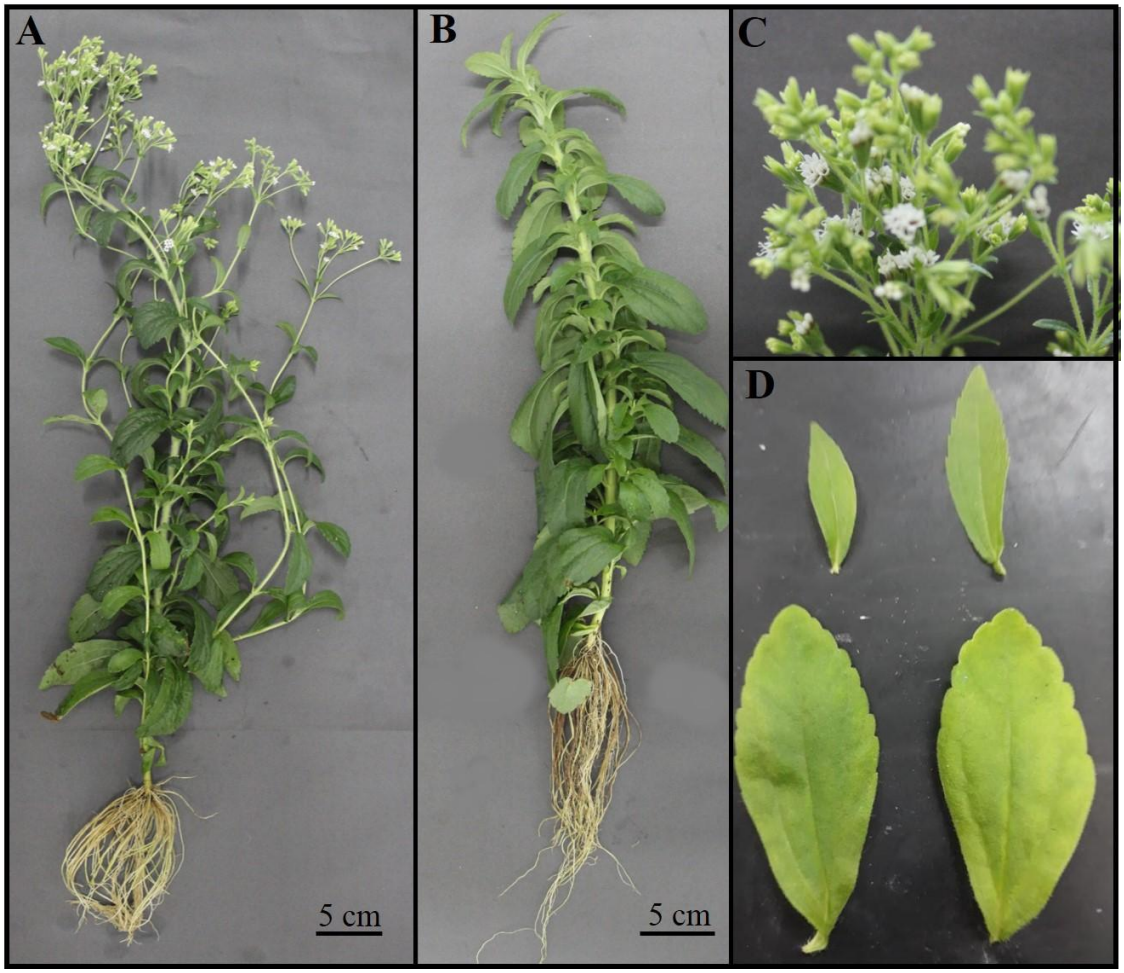
Supplementary Material

Supplementary Figure 1. Plant of *Stevia rebaudiana* (Bert.) Bertoni. (A) Reproductive stage; (B) Vegetative stage; (C) Small pentameric flowers; and (D) Elliptical leaves with a slightly granular pubescent surface.

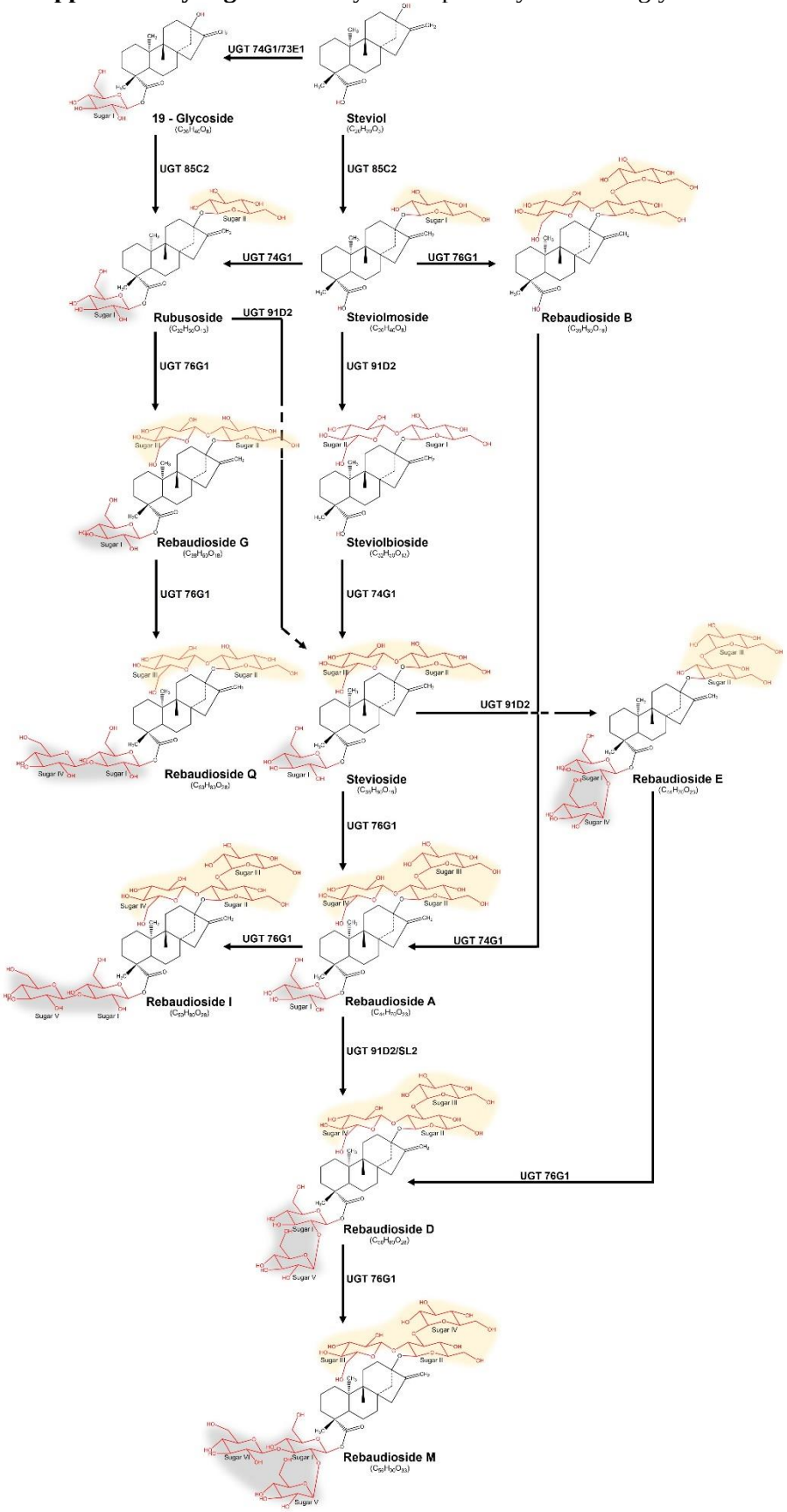


Supplementary Table 1. Steviol glycosides identified in *Stevia rebaudiana* (Bert.) Bertoni.

Diterpenic Glycosídes	Relative sweetening power	Formula	Molecular Weight	Exact Mass	Adduct $[M+H]^+$	Reference
Steviol	-	$C_{20}H_{30}O_3$	318.45	318.219495	317.2122	Minne et al (2004); Gardana et al (2018)
Steviolmonoside	-	$C_{26}H_{40}O_8$	480.598	480.272320	-	Ohta et al (2010)
Steviolbioside	100 – 125	$C_{32}H_{50}O_{13}$	642.33	642.325145	641.3179	Kohda et al (1976); Gardana et al (2018)
Stevioside	250 – 300	$C_{38}H_{60}O_{18}$	804.87	804.377970	803.3716	Kohda et al (1976)
Rebaudioside A	250 – 450	$C_{44}H_{70}O_{23}$	967.01	966.430795	965.4242	Kohda et al (1976)
Rebaudioside B	300 – 350	$C_{38}H_{60}O_{18}$	804.38	804.377970	803.3716	Kohda et al (1976)
Rebaudioside C/Dulcoside B	50 – 120	$C_{44}H_{70}O_{22}$	951.01	950.435880	949.4280	Sakamoto and Yamasaki (1977)b
Rebaudioside C2	-	$C_{44}H_{70}O_{22}$	951	950.435880		Purkayastha et al (2019)
Rebaudioside D	250 – 450	$C_{50}H_{80}O_{28}$	804.87	1128.483620	1127.4771	Sakamoto and Yamasaki (1977)a
Rebaudioside E	150 – 300	$C_{44}H_{70}O_{23}$	967.01	966.430795	965.4241	Sakamoto, Yamasaki (1977)a; Gardana et al (2018)
Rebaudioside F	200	$C_{43}H_{68}O_{22}$	936.99	936.420230	935.4129	Starratt et al (2002)
Rubusoside	114	$C_{32}H_{50}O_{13}$	642.73	642.325145	641.3179	Prakash et al (2012)
Dulcoside A	50 – 120	$C_{38}H_{60}O_{17}$	788.17	788.383055	787.3758	Englert et al (2016)
Rebaudioside M	200 - 350	$C_{56}H_{90}O_{33}$	1307.30	1290.536445	1289.5274	Prakash, Markosyan, Bunders (2014)
Rebaudioside S	-	$C_{44}H_{70}O_{22}$	950	950.435880	-	Wen et al (2020)
Rebaudioside U	-	$C_{50}H_{82}O_{26}$	1098	1098.509440	-	Perera et al (2017)a
Rebaudioside T	-	$C_{50}H_{82}O_{26}$	1099	1098.509440	-	Perera et al (2017)a
Rebaudioside G	-	$C_{38}H_{60}O_{18}$	805	804.377970	803.3716	Ohta et al (2010); Gardana et al (2018)
Rebaudioside A2	-	$C_{44}H_{70}O_{23}$	967	966.430795	967.4411	Chaturvedula et al (2011)
Rebaudioside D	-	$C_{50}H_{80}O_{28}$	1129	1128.483620	1127.4763	Gardana et al (2018)
Rebaudioside L	-	$C_{50}H_{80}O_{28}$	1129	1128.483620		Morita et al (2014)
Rebaudioside I	-	$C_{50}H_{80}O_{28}$	1129	1128.483620	1127.4770	Gardana et al (2018)
Rebaudioside I2	-	$C_{50}H_{80}O_{28}$	1129	1128.483620	1129	Chaturvedula, Upreti, Prakash (2011)
Rebaudioside I3	-	$C_{50}H_{80}O_{28}$	1129	1128.483620	-	Purkayastha et al (2016)
Rebaudioside Q	-	$C_{50}H_{80}O_{28}$	1129	1128.483620	-	Olsson et al (2016)
Rebaudioside Q2	-	$C_{50}H_{80}O_{28}$	1129	1128.483620	-	Chaturvedula, Upreti, Prakash (2011)

Rebaudioside Q3	-	$C_{50}H_{80}O_{28}$	1129	1128.483620	1129	Chaturvedula, Upreti, Prakash (2011)
Rebaudioside N	-	$C_{56}H_{90}O_{30}$	1274	1242.551700	1273.5345	Purkayastha et al (2019); Gardana et al (2018)
Rebaudioside O	-	$C_{62}H_{100}O_{37}$	1436	1436.594355	1435.0000	Ohta et al (2010)
Rebaudioside O2	-	$C_{62}H_{100}O_{37}$	1436	1436.594355	-	(FAO 2017)
Rebaudioside K	-	$C_{50}H_{80}O_{27}$	1112	1112.488705	-	Purkayastha et al (2019)
Rebaudioside K2	-	$C_{50}H_{80}O_{27}$	1112	1112.488705	-	Purkayastha et al (2019)
Rebaudioside H	-	$C_{50}H_{80}O_{27}$	1112	1112.488705	1111.4814	Purkayastha et al (2019); Gardana et al (2018)
Rebaudioside J	-	$C_{50}H_{80}O_{27}$	1113.2	1112.488705	-	Purkayastha et al (2019)
Stevioside F	-	$C_{37}H_{59}O_{17}$	775	775.375230	-	Prakash and Chaturvedula (2013)
Rebaudioside F	-	$C_{43}H_{68}O_{22}$	937	936.420230	935.4129	Gardana et al (2018)
Rebaudioside F2	-	$C_{43}H_{68}O_{22}$	937	936.420230	-	Prakash and Chaturvedula (2013)
Rebaudioside F3	-	$C_{43}H_{68}O_{22}$	937	936.420230	937.4316	Chaturvedula et al (2011)
Rebaudioside R	-	$C_{43}H_{68}O_{22}$	937	936.420230	-	Wen et al (2020)
Rebaudioside U2	-	$C_{50}H_{82}O_{26}$	1099	1098.509440	-	(EFSA 2020)
Rebaudioside V2	-	$C_{56}H_{92}O_{31}$	1261	1260.562265	-	(EFSA 2020)
Rebaudioside V	-	$C_{56}H_{92}O_{31}$	1261	1260.562265	-	Perera et al (2017)b
Rebaudioside W	-	$C_{50}H_{82}O_{26}$	1098	1098.509440	-	Perera et al (2017)b
Rebaudioside W2	-	$C_{50}H_{82}O_{26}$	1098	1098.509440	-	(EFSA 2020)
Rebaudioside W3	-	$C_{50}H_{82}O_{26}$	1098	1098.509440	-	Wang et al (2019)
Rebaudioside Y	-	$C_{56}H_{92}O_{31}$	1260	1260.562265	-	(FAO 2017)
Rebaudioside T1	-	$C_{50}H_{80}O_{28}$	1128	1128.483620	-	(FAO 2017)
Rebaudioside A3	-	$C_{44}H_{70}O_{22}$	951	950.435880	805.3871	Chaturvedula et al (2011)
Stevioside D	-	$C_{38}H_{60}O_{17}$	789	788.383055	789.3917	Chaturvedula and Prakash (2011)
Stevioside E	-	$C_{44}H_{70}O_{22}$	951	950.435880	951.4460	Chaturvedula and Prakash (2011)
Stevioside E2	-	$C_{44}H_{70}O_{22}$	951	950.435880	-	Purkayastha et al (2016)
Stevioside A						Purkayastha et al (2019)
Stevioside B						Chaturvedula and Zamora (2014)

Supplementary Figure 2. Biosynthetic pathway of steviol glycosides.



CAPÍTULO 1

Viabilidade de sementes e comportamento fotoperiódico de mudas em *Stevia rebaudiana* **(Bert.) Bertoni**

Seed viability and seedling photoperiodic behavior in *Stevia rebaudiana* **(Bert.) Bertoni**

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Abstract

Stevia rebaudiana is an industrial crop that produces high amounts of natural sweeteners (steviol glycosides - StGly) in its leaves. However, its seeds (achenes) are known to have poor germinability limiting its large-scale cultivation. On the other hand, it is a short-day species with 12–13h critical photoperiod, below which induces precocious flowering. Therefore, we have conducted a morphophysiological investigation of viability in stevia seeds followed by the analysis of antioxidant enzymatic activity in seedlings under different photoperiods (12/12h, 15/9h, 16/8h light/dark). The morphophysiological analysis confirmed the poor quality of the stevia seed batch analyzed and that only dark seeds may be viable. The analysis of antioxidant enzymes indicated that seedlings at vegetative 16/8h photoperiod appeared to be under regular homeostatic development. The 15/9h condition seemed to represent a transition threshold in which catalase and peroxidase were induced. While the overall analysis of antioxidant enzymes showed a drastically altered profile in seedlings under the flowering 12/12h photoperiod, apparently due to exposure of reactive oxygen species (ROS)-inductive conditions probably resulting from precocious flowering. We may conclude that seed quality evaluation provided relevant information concerning peculiarities in using seeds for stevia cropping. While antioxidant enzymatic profile provided information in which seedlings grown in light conditions below the critical photoperiod appear to face drastic stress, possibly because of the induction of ROS in a stage in which the plants (seedlings) are still underdeveloped and immature for proper flowering. Overall, our results provide useful information apparently related to circadian clock alterations depending on life cycle stage and photoperiod. Therefore, determining the need for a proper cultivation system exclusively for high-quality seed production aiming at large-scale stevia crops for StGly production. While future molecular studies shall provide insights into the photoperiodic regulation of the circadian clock, flowering, and redox homeostasis.

Keywords: Luminosity, Antioxidant enzymes, Tetrazolium test, Viability, Natural sweeteners.

1. Introduction

Photoperiod is an extremely important environmental factor for plant growth, being responsible for the control of several processes, such as development, including photomorphogenesis, growth, flowering, stress tolerance, and circadian rhythms (JACKSON, 2009). *Stevia rebaudiana* (Bert.) Bertoni is known to be sensitive to different photoperiod regimes, being considered a strictly short-day plant, with a critical photoperiod defined between 12-13h of light (CEUNEN; GEUNS, 2013; SIMLAT et al., 2016). Thus, changing the light exposure to which the species is subjected can change its biochemical, metabolomic, and molecular profile, becoming an important tool for its commercial production, as observed by (YONEDA et al., 2017), where the use of experimental light treatments extending the photoperiod to which the plants were exposed resulted in an increase in all morphological characteristics analyzed, except for the total number of leaves.

S. rebaudiana is a native plant from Paraguay, inserted among the 154 members of the Stevia genus. It became known worldwide for its ability to produce steviol glycosides (StGly), mainly stevioside and rebaudioside A, compounds with high sweetening power that can represent between 4 and 20% of the weight of the leaf and are about 300 times sweeter than sucrose. In addition, StGly have low caloric content and resistance to high temperatures, characteristics that have contributed to a growing interest in stevia over the years and its use as a sweetener or flavor aggregator in food products, mainly used as sweeteners in soft drinks, sauce of soy, yogurt and other foods in Japan, Korea and Brazil (AGHIGHI SHAHVERDI; OMIDI; TABATABAEI, 2018; LEMUS-MONDACA et al., 2012; LESTER, 1999; MYINT et al., 2020; RAJASEKARAN et al., 2008; SIMLAT et al., 2016b; TADA et al., 2013; YADAV et al., 2011).

Belonging to the Asteraceae (Compositae) family, stevia embryos are characterized by being contained within structures called achenes (Cypsela), usually dry fruits of carpel origin (YADAV et al., 2011). Stevia, in the same plant, produces two types of achenes, dark and light/brown (GOETTEMOELLER; CHING, 1999; KUMAR, 2013; RAINA; BHANDARI, 2013; YADAV et al., 2011). The development of these achenes is still not well understood, but it seems to be controlled by a compatibility factor present in the species. Other studies attribute this characteristic to a decrease in the concentration of

GAs during plant flowering, thus causing the pollen tube's inability to grow. In this sense, high concentrations of StGly, since there is sharing in the biosynthetic pathway between metabolites, may be a reason for the malfunction of stevia pollen grains. Thus, to produce high-quality seeds, further studies are needed regarding the regulation of the life cycle and maturation of the species' reproductive organs (TULEJA et al., 2021). That way, dark achene, considered viable, is produced by cross-pollination, while achene with light/brown color originates from self-pollination and is not viable. This feature limits the spread of large-scale culture via seeds (ABDULLATEEF; OSMAN, 2011; SIMLAT et al., 2016a). In addition, the species is characterized by having a low germination percentage (less than 50%) and requires a long period to establish seedlings suitable for planting (up to 60 days) (ABDULLATEEF; OSMAN, 2011; CARNEIRO; MUNIZ; GUEDES, 1997; KUMAR, 2013; MADAN et al., 2010; MEGEJI et al., 2005; SIMLAT et al., 2016a). Alternatively, the production of seedlings by cuttings and micropropagation by tissue culture have emerged as techniques to solve such difficulties, and these tools are important for farmers, however, they can be very costly (GOETTEMOELLER; CHING, 1999; YADAV et al., 2011).

Adverse conditions can alter homeostasis and, consequently, the metabolism of reactive oxygen species (ROS). ROS are products of aerobic cellular metabolism, and their threshold levels are maintained through homeostasis between ROS generation and elimination systems (GANGULI; MUKHERJEE; SONAWANE, 2019). ROS in high concentrations exceeds the capacity of antioxidant defense enzymes, which can damage structures such as membrane lipids, proteins, and nucleic acids and, ultimately, can cause cell death (TRIPATHY; OELMÜLLER, 2012). The superoxide ion (O^{2-}), the hydroxyl radical (OH^{-}), the perhydroxyl radical (HO^{2-}), and the hydrogen peroxide (H_2O_2) are common examples of ROS presented in plants (KAUR et al., 2016). These forms have the ability to react with different biological targets, interrupting their function (Sharma et al., 2012; Huang et al., 2019) (MITTLER, 2017). Plants have an efficient antioxidant system to deal with oxidative stress induced by ROS, which involves enzymatic defense processes that include the enzymes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) and non-enzymatic such as ascorbate (AsA), glutathione

(GSH), carotenoids, tocopherols and phenolics (ANJUM et al., 2017; ASHRAF et al., 2015; HERNÁNDEZ et al., 2001). Several studies explore the enhanced activity of these antioxidant enzymes, which lead to ROS detoxification and reduced oxidative damage in many plants. The antioxidant potential is directly proportional to the detoxification of free radicals, providing protection against oxidative damage. The alternative way to combat ROS is the development of transgenic crops that can be tolerant to stress (FINKEL; HOLBROOK, 2000; KAPOOR et al., 2019; SINGH et al., 2019)

Due to its economic importance and its commercial characteristics, knowing how *S. rebaudiana* develops, from germination to seedling formation, is extremely important for the potential of cultivation, especially with the use of seeds. Several techniques can be used to study the germinative profile of plant species, biochemical and molecular techniques can be important tools in this context. Therefore, the present study carried out the morphophysiological investigation of viability in stevia seeds followed by the analysis of the antioxidant enzymatic activity in seedlings under different photoperiods.

2. Material and Methods

2.1. Sampling

The cultivar IAN/VC-142/Eirete (Stevia Eirete 2) used in this study was developed by the Instituto Paraguayo de Tecnología Agraria - IPTA.

2.2. Germination Test

Seed germination tests were carried out under laboratory conditions. The norms recommended by the Rules for Seed Analysis (RAS), published by the Ministry of Agriculture, Livestock, and Supply were followed with some adaptations (BRASIL, 2009). The germination test was conducted in a completely randomized design with four replications of 50 seeds. A first test with seeds without selection of achenes by characteristic color (mixture of dark and light/brown achenes) and a second test composed of seeds with dark achene and seeds with light/brown achene separately.

Initially, the seeds (achenes) were superficially disinfected in a solution with 0.5% active chlorine for five minutes under constant agitation and then subjected to four times washing with distilled water to remove all hypochlorite residue and placed on paper to dry. Subsequently, the achenes were deposited under the substrate, "germitest" paper (PROLAB) previously moistened with distilled water in gerbox-type plastic boxes (11 x 11 x 3.5 cm) followed by incubation for germination in a chamber (Panasonic / Sanyo, MLR-351H) with constant light (15 white fluorescent lamps, Mitsubishi / Osram, FL40SS-W/37) and temperature adjusted to 25 °C. For 7 days, successive counts of the number of germinated seeds were performed.

2.3. Stevia seeds viability

To assess the viability of different stevia achenes, the tetrazolium test was performed (FRANÇA-NETO; KRZYZANOWSKI, 2019; FRANÇA-NETO; KRZYZANOWSKI; COSTA, 1998) and embryo growth was performed in Murashige and Skoog (MS) culture medium (3 $mg.mL^{-1}$ - pH 5.8). Stevia achenes (dark and light/brown achenes) were previously soaked for 12h in a petri dish and “germitest” paper (PROLAB). Subsequently, the embryos were removed from the achenes, tegmen, and soaked in a 0.05% tetrazolium solution (w/v), being stored in a BOD oven at 40 °C (Panasonic / Sanyo, MLR-351H) for 3h. Concomitant to tetrazolium, embryo germination in culture medium was monitored to confirm the viability of selected embryos. Embryos were placed in a petri dish (25 mL) with MS culture medium and stored in BOD (Panasonic / Sanyo, MLR-351H) equipped with 15 white fluorescent lamps (Mitsubishi / Osram, FL40SS-W/37) at 25° C under constant light. Thus, the germination of the collected material was observed.

2.4. Behavior of stevia seedlings under different photoperiod conditions

Stevia seedlings were subjected to different photoperiod conditions. Initially, the dark achenes were previously germinated on “germitest” paper (Prolab) moistened with 8 mL of distilled water in gerbox-type plastic boxes (11 x 11 x 3.5 cm), being kept under constant light in a germination chamber (Panasonic / Sanyo, MLR-351H). After 7 days

(beginning of germination) the most vigorous stevia seedlings were collected and transferred to MS culture medium in Petri dishes (60 x 15 mm) where they were subjected to different photoperiod regimes (12/12h, 15/9h and 16/8h light/dark) and under constant light (Control), stored in BOD (Panasonic / Sanyo, MLR-351H) equipped with 15 white fluorescent lamps (Mitsubishi / Osram, FL40SS-W/37) at 25 °C and 70% RH.

The entire experiment was carried out in three repetitions per analyzed photoperiod, using 15 seedlings per repetition. All samples were lyophilized, ground, and kept frozen (-80 ºC) until further use for preparation of extracts and analysis. The evaluation of the activity of antioxidant enzymes was determined spectrophotometrically (Varian - Cary Microplate Reader 50 MPR) in a 96-well microplate (ANDRADE et al., 2021; BEAUCHAMP; FRIDOVICH, 1971; DROTAR; PHELPS; FALL, 1985; GARCÍA-LIMONES et al., 2002; GIANNOPOLITIS; RIES, 1977; GOMES NETO et al., 2018; SCHAEDLE; BASSHAM, 1977).

2.4.1. Protein extraction and quantification

To extract the total proteins from the stevia seedling samples, 5 mg of the samples ground in liquid nitrogen were weighed into individual 1.5 mL conical-bottomed microtubes, then the samples were homogenized in 125 µL of potassium phosphate buffer 0.1 M (pH 7.8) (SAMBROOK; FRITSCH; MANIATIS, 1989). The samples were kept in containers with ice on a shaker table (Labnet International, Inc. ORBITTM 300) for 1h, under constant agitation. Subsequently, the homogenate was centrifuged at 14.000 rpm for 10 minutes at 4 °C. The supernatant, hereinafter called crude extract (protein extract) was collected and stored at -20 °C for further analysis.

The quantification of total proteins was performed according to the method proposed by (BRADFORD, 1976), using a standard curve of bovine serum albumin - BSA (Molecular Probes) for the construction of the analytical curve. The bradford reagent (Sigma Aldrich – B 6916) was used as a color reagent. To determine the concentration of total proteins, the BSA stock solution (1440 $\mu g.mL^{-1}$) was used. An analytical curve was constructed, in which the BSA concentration range ranged from 0.0 to 1.4 $mg.mL^{-1}$. For the quantification of total proteins in the samples, an aliquot of 5 µL of the protein

extract mixed with 250 μL of Bradford's reagent (Sigma Aldrich – B6916) was used. The reading of the absorbance of the samples was performed in a spectrophotometer (Varian Cary 50 Microplate Reader), at a wavelength of 595 nm. The experiment was carried out using three biological repetitions and three technical repetitions. The concentration of total proteins in the samples was calculated from the equation obtained in the linear regression calculation of the analytical curve with standard BSA, through the equation generated in the calibration ($R^2 = 0.99$).

2.4.2. Superoxide dismutase (SOD, EC 1.15.1.1)

The evaluation of the detection of the antioxidant enzyme SOD was based on the identification of the tetrazolium-nitroblue chloride (NBT) reduction product, formazan, at 560 nm (ANDRADE et al., 2021; BEAUCHAMP; FRIDOVICH, 1971; GIANNOPOLITIS; RIES, 1977; GOMES NETO et al., 2018). Initially, 175 μL of the reaction mixture [50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA] was mixed with 15 μL of diluted enzyme extract 30 times with 50 mM potassium phosphate buffer (pH 7.8). The reaction was started by placing the samples under a 27 W fluorescent lamp at 25 °C for 12 minutes. Enzyme activity was expressed in units (U) SOD.mg^{-1} of protein. As a unit of SOD activity, it is expressed as the amount of enzyme needed to cause 50% inhibition of NBT photoreduction under experimental conditions.

2.4.3. Catalase (CAT, EC 1.11.1.6)

CAT activity was determined using the reaction medium used consists of 232.5 μL of the reaction mixture containing 100 mM potassium phosphate buffer (pH 7.8), 0.2 mM EDTA, H_2O, 3% H_2O_2, and 17.5 μL of the extract is grown enzyme diluted 20-fold with 100 mM potassium phosphate buffer (pH 7.8). The reaction was started with the addition of H_2O_2, and the enzymatic decomposition of H_2O_2 was observed by measuring the decrease in absorbance at 240 nm (ε=36 $mM^{-1}cm^{-1}$) for 4 minutes. A unit of CAT activity is expressed as the amount of enzyme needed to break down one μmol.min^{-1} of H_2O_2 under experimental conditions (GARCÍA-LIMONES et al., 2002).

2.4.4. Ascorbate Peroxidase (APX, EC 1.11.1.11)

Total APX activity was measured from a reaction medium consisted of 125 µL of 100 mM potassium phosphate buffer (pH 7.0), 25 µL of 0.1 mM EDTA, 12.5 µL of 0.5 mM ascorbate, 12.5 µL of 20 diluted sample times with 100 mM potassium phosphate buffer (pH 7.0) and 25 µL of 1 mM H_2O_2 to start the reaction. APX activity was expressed in mM ascorbate (AsA) $min^{-1}.mg^{-1}$ of proteins, monitoring the ascorbate oxidation which occurs with the decline in absorbance at 290 nm (ε=2.8 $mM^{-1}cm^{-1}$) as the ascorbate was oxidized for 7 minutes (NAKANO; ASADA, 1981). One unit of APX activity is expressed as the amount of enzyme needed to oxidize one µmol.min^{-1} of AsA under experimental conditions.

2.4.5. Peroxidase Dependent Guaiacol (GPOD, EC 1.11.1.7)

The GPOD activity was measured according to the method proposed by (GARCÍA-LIMONES et al., 2002) with modifications. We used a reaction medium containing 100 mM potassium phosphate buffer (pH 6.5), 15 mM Guaiacol, 0.05% (v/v) H_2O_2 and enzyme extract diluted 80 times with 100 mM potassium phosphate buffer (pH 6.5). Enzyme kinetics assessed by the increase in absorbance at 470 nm (ε=26.6 $mM^{-1}cm^{-1}$) was measured for 10 minutes.

One unit of peroxidase was defined as the amount of enzyme that caused the formation of 1 mM tetraguaiacol per minute under experimental conditions.

2.4.6. Glutathione Reductase (GR, EC 1.6.4.2)

GR activity was measured according to (SCHAEDLE; BASSHAM, 1977) and (GARCÍA-LIMONES et al., 2002) with modifications. We used a reaction medium containing 50 mM potassium phosphate buffer (pH 7.8), 0.5 mM oxidized glutathione, 3 mM $MgCl_2$, 0.15 mM NADPH and 25 µL of protein extract diluted 10 times with potassium phosphate buffer. 50 mM potassium (pH 7.8). Oxidation was measured by decreasing the absorbance at 340 nm (ε=6.2 $mM^{-1}cm^{-1}$). A unit of glutathione reductase

is defined as the amount of enzyme that oxidizes 1 μmol.min^{-1} of NADPH under experimental conditions.

2.4.7. Glutathione Peroxidase (GPX, EC 1.11.1.9)

GPX activity was measured from the reaction mixture (250 μL) contained 100 mM potassium phosphate buffer, pH 7.0, 2 mM glutathione, 1 mM NADPH, 1 mM EDTA, 0.5 U glutathione reductase, 2 mM H_2O_2 and 15 μL extract protein diluted 10-fold with 100 mM potassium phosphate buffer, pH 7.0. The oxidation rate of NADPH was measured at 340 nm (GARCÍA-LIMONES et al., 2002).

2.5. Data processing and statistical analysis

Statistical analysis was performed using the SISVAR 5.6 software. The dataset was subjected to a normality test, the Shapiro-Wilk test, to verify whether the probability distribution associated with a dataset can be approximated by the normal distribution (Supplementary table 1). Analysis of variance was used to identify statistically significant differences between samples ($p < 0.05$) followed by Tukey's multiple comparison tests. Results are presented as the mean of the replicates ± standard deviations. Multivariate analysis by unsupervised and supervised techniques was applied to descriptive and predictive models (https://www.metaboanalyst.ca) (CHONG; WISHART; XIA, 2019).

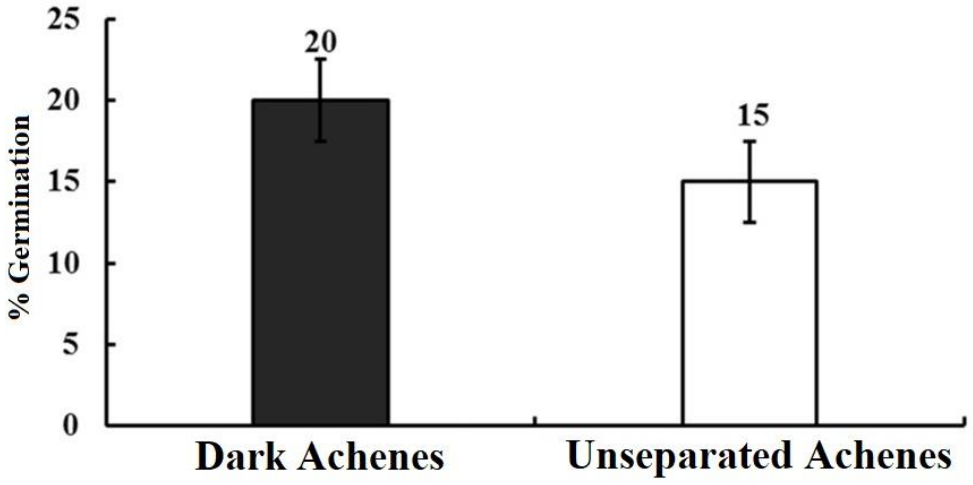
3. Results and discussion

3.1. Germination profile and seedling establishment in stevia

Stevia germination is a factor that is currently considered a limiting factor for its large-scale cultivation (RAINA; BHANDARI, 2013; SIMLAT et al., 2019; YADAV et al., 2011). In the present study, the germination of stevia achenes was potentially low regardless of the type of selection used. Germination with only dark achenes reached about 20%, while for achenes without color selection (dark and light/brown color) germination reached 15%, which indicates the poor quality of the seed lot (Figure 1).

The period for establishing a seedling was around the sixth and seventh day, when the greenish cotyledons, tissues responsible for photosynthesis, are visible.

Figure 1. Percentage of germination of *Stevia rebaudiana* (Bert.) Bertoni achenes (seeds).



The low seed germinability is currently a restrictive factor for stevia crop implantation and expansion, and consequently for supplying the growing market. Therefore, the research and/or breeding focused on the reproductive system and/or seed production is seen as of paramount importance and thus facilitate the expansion and large-scale production aimed at the growing stevia market (MARTINI et al., 2017; RAINA; BHANDARI, 2013; SIMLAT et al., 2019; YADAV et al., 2011).

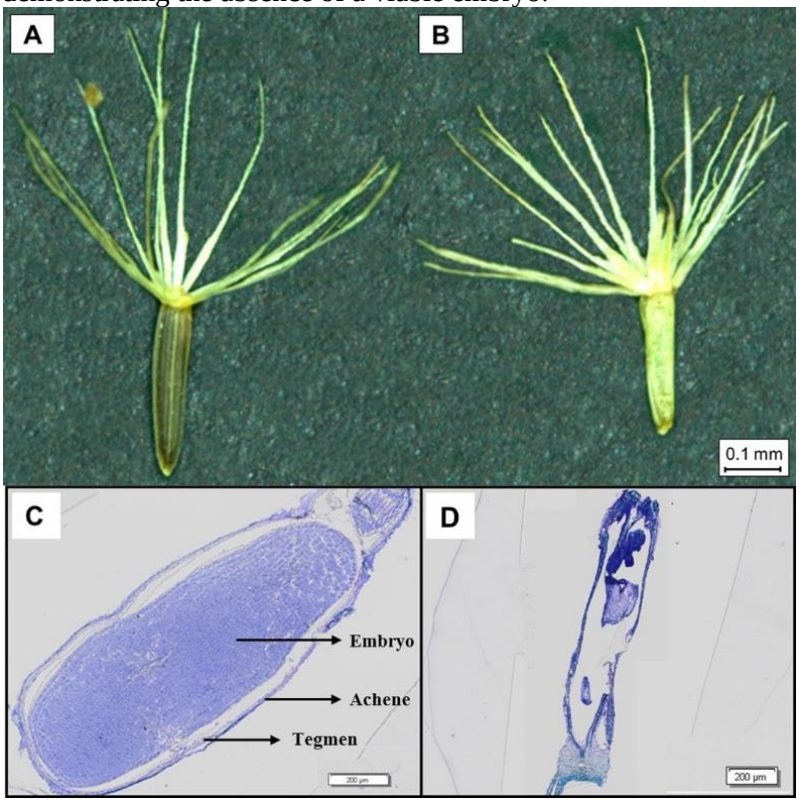
In addition to the factors intrinsic to the culture, other factors can act on stevia seeds and consequently on their germination. Temperature, luminosity, salinity, "*nutri-priming*", storage and harvesting can be mentioned. (SIMLAT et al., 2019) indicated that hormones such as GA3, TDZ, KN, and BA influence both germination and development of stevia seedlings. While (SHAHVERDI; OMIDI; TABATABAEI, 2017) evaluated the effect of "*nutri-priming*" and salinity on stevia germination, where they observed that the nutritional supplementation offered by "*nutri-priming*" can favor the germination of the species and, concomitantly with this, allow the establishment of the same against high saline conditions.

Another factor observed is the existence of a physiological dormancy mechanism intrinsic to the culture itself, as mentioned by (MAGANGANA; MAKUNGA, 2016). In this sense, a series of alternatives that can enhance germination and consequent implementation of stevia plants. Several studies have aimed to develop alternatives to the use of seeds, currently the main stevia propagation technique is via stem cuttings. However, it is a technique that has limitations due to the number of individuals that can be obtained from a single plant (CASTAÑEDA-SAUCEDO et al., 2020; GANTAIT; DAS; BANERJEE, 2018). In vitro regeneration studies performed on stevia which focus mainly on its organogenesis have also been developed. In vitro culture technology has become an important method which allows the production of plants on a large scale within a short period of time. In a study by (YÜCESAN et al., 2016), it was possible to observe through in vitro multiplication, the occurrence of direct regeneration of multiple nodal stevia explants lasting about 65 days. Thus, in vitro propagation can be commercially viable for stevia, using simple and low-cost methods that can provide the production of uniform plants in a relatively short period and with a high rate of multiplication.

3.2. Viability of stevia seeds

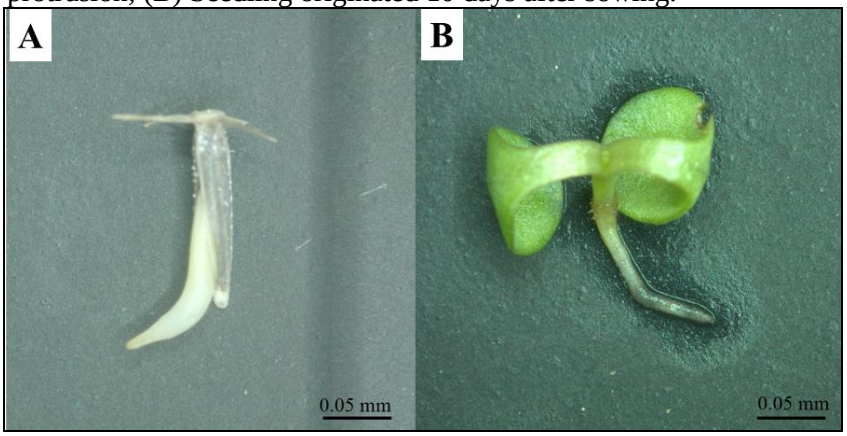
Stevia is a plant that needs cross-pollination to produce fertile seeds. Even if it presents the two sexual organs in the same flower, being a hermaphrodite, this need is reflected in the formation of its seeds, causing the same plant to originate seeds (achenes) with different morphophysiological aspects that will indicate their viability, the dark achenes and light/brown (Figure 2) (YADAV et al., 2011, 2013).

Figure 2. Morphoanatomy of *Stevia rebaudiana* (Bert.) Bertoni achenes (seeds). (A) Dark achene, viable embryo; (B) light/brown achene, nonviable embryo; (C) longitudinal anatomical section of a dark achene demonstrating the embryo and tegmen; (D) longitudinal anatomical section of a light/brown achene, demonstrating the absence of a viable embryo.



In our study we observed that the dark achene has a fertilized embryo inside which will give rise to a new seedling/plant, an event that occurs around the 7th day of the germination test performed, whereas the greening of the cotyledons and tissues with photosynthetic properties around the 10th day (Figure 3 A and B). However, even being considered fertile, dark achene may contain an embryo that does not have germination capacity, indicating some objection that may be linked to its formation, whether fertilization or development, resulting in a problem that is directly linked to the difficulty for propagation via seeds, i.e its low viability. The light/brown achenes that do not have the embryo inside, on the other hand, originate from self-pollination (GOETTEMOELLER; CHING, 1999; KUMAR, 2013; MARTINI et al., 2017; RAINA; BHANDARI, 2013).

Figure 3. Representation of *Stevia rebaudiana* (Bert.) Bertoni germination and seedling formation. (A) Germination, i.e. radicle protrusion; (B) Seedling originated 10 days after sowing.



Several factors can act in decreasing the viability of stevia seeds, malformation, predation, presence of damaged tissues, mechanical damage or even the conditions in which the mother plant was cultivated. According to (OLIVEIRA et al., 2004), the low germination level of stevia may be linked to the physiological phenomenon of hormonal imbalance that leads to the disposal of malformed eggs by plants, where hormonal precursors, such as steviol itself, a prominent sweetening compound in the crop, can cause such abnormality. In this sense, there is a discontinuity in the growth of the pollen tube or a degeneration of the endosperm and the consequent absence of embryos. On the other hand, (MARZINEK; OLIVEIRA, 2010) correlates this phenomenon with the scarcity of reserves, resulting in the abortion of eggs and cypsela. Thus, crop reproduction via seeds, low viability, and germination percentage become an obstacle to the expansion of stevia culture (CARNEIRO; MUNIZ; GUEDES, 1997; MARTINI et al., 2017; MEGEJI et al., 2005).

3.2.1. Tetrazolium test

In studies to estimate seed viability, the tetrazolium test is routinely used. Its employability ranges from the species in which the seeds may have dormancy or slow germination and to species of commercial importance (LAMARCA; BARBEDO, 2014).

The test is based on the activity of dehydrogenase enzymes that catalyze respiratory reactions in mitochondria during glycolysis, citric acid cycle, or Krebs cycle. In glycolysis, the activity of the enzyme glyceraldehyde-3-phosphate dehydrogenase occurs, and in the citric acid cycle, five other enzymes are activated: pyruvate dehydrogenase; isocitrate dehydrogenase; α-ketoglutarate dehydrogenase; succinate dehydrogenase; and malate dehydrogenase. Among such enzymes, malate dehydrogenase, performs the reduction of the tetrazolium salt (2,3,5-triphenyl tetrazolium chloride) in living tissues. When a seed is immersed in the colorless solution, the tetrazolium salt penetrates the seed tissues, where it interferes with the reduction processes of living cells by accepting a hydrogen ion (FRANÇA-NETO; KRZYZANOWSKI, 2019).

When the tetrazolium salt is reduced to form triphenylformazan in tissue, this indicates that respiratory activity is taking place in the mitochondria of the seed tissue cells, which are considered to be alive. Therefore, the red color resulting from the reaction in seed tissue is a positive indicator of viability, indirectly detecting respiratory activity at the cellular level. While non-viable seed tissues do not react with the tetrazolium salt and consequently do not stain (DE BARROS FRANÇA-NETO; KRZYZANOWSKI, 2019).

Differences in red color or lack of color allow a distinction between damaged and dead tissues and the classification of seeds as viable or non-viable (BRASIL, 2009). The main advantages of this test are the speed and effectiveness of providing the results. In addition, in some cases, the test allows the diagnosis of the causes of reduced physiological potential of seeds (DE BARROS FRANÇA-NETO; KRZYZANOWSKI, 2019; MERCADO; CALEÑO; ROZO, 2020; SANTOS DAS VIRGENS; ALMEIDA CONCEIÇÃO; MARANI BARBOSA, 2019).

In our work we can describe the profile of the tetrazolium test for stevia. As it is a species that has a series of factors which limit its cultivation via seeds, the application of this test becomes necessary due to its speed and ease in obtaining results. The classification was based on the intensity of red coloration presented by the embryos, as well as on the location and extent of the decayed areas, represented by certain color changes. Thus, three viability classes were successfully identified in stevia embryos.

The first (I), with embryos considered viable, is represented by a total coloration of the embryonic tissue, indicating the existence of intense metabolic activity in the entire tissue; a second aspect (II) is represented by a partial coloration of the tissues of the embryos, which can be indicative of lesions or dead tissues, which can affect the formation and development of the plant; the third aspect (III) identified are the embryos without color, indicating the infeasibility of the evaluated tissue, dead tissue (Figure 4).

Figure 4. Tetrazolium test pattern for *Stevia rebaudiana* (Bert.) Bertoni embryos. (I) Viable embryos; (II) Partial staining, indicative of lesions or dead tissue; (III) colorless embryos, dead tissue.

However, to observe these results, it is necessary to remove the membrane that covers the embryo, the tegmen (Figure 2C), which facilitates the contact of the tetrazolium solution with the embryonic tissue. In addition to viability, the tetrazolium test provides valuable information about vigor, which enables the diagnosis of the main problems that can affect seed quality, such as mechanical damage, field deterioration, storage, and damage caused by insects. In addition to being associated with the emergence of field

quality seedlings (MILOŠEVIĆ; VUJAKOVIĆ; KARAGIĆ, 2010) and obtains answers in a shorter time than traditional tests (MARIN et al., 2017).

Embryos classified as viable based on the tetrazolium test were germinated in culture medium (MS) and subjected to different photoperiods where the pattern of antioxidant enzymes for the different treatments was evaluated.

3.3. Exposure to different photoperiod conditions induces stress in stevia seedlings

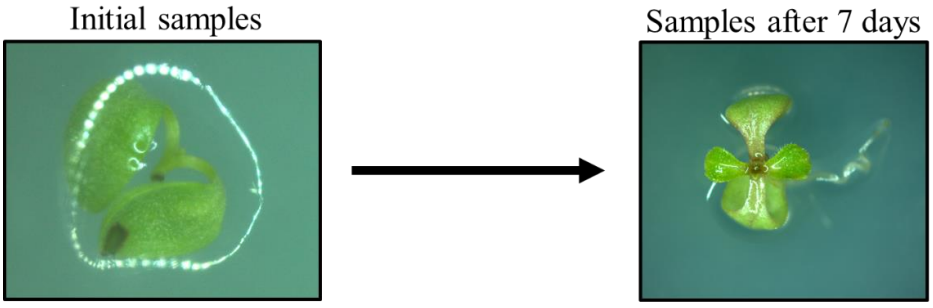
Reactive oxygen species (ROS) are naturally produced during photosynthesis and respiration through electron transport chains. Furthermore, abiotic, and biotic stresses can dramatically increase ROS production and accumulation. In addition to their roles in oxidative stress, these molecules act in signaling or alter the cellular oxidative environment to influence downstream gene expression and regulate the organism's development (MUNNÉ-BOSCH; QUEVAL; FOYER, 2013). In plant cells, the lifetime of ROS is mainly determined by catalases and enzymes of the glutathione-ascorbate cycle (GUETA-DAHAN et al., 1997).

Plants are exposed to a daily light-dark cycle, daily light duration, the photoperiod, has a strong impact on many processes during the plant's life, including abiotic and biotic stress responses (GREENHAM; MCCLUNG, 2015; SHIM; IMAIZUMI, 2015). For example, short photoperiods cause greater resistance to freeze than long photoperiods (LEE; THOMASHOW, 2012), whereas long photoperiods cause a stronger pathogenic defense than short photoperiods (ABUELSOUD; CORTLEVEN; SCHMÜLLING, 2020; GRIEBEL; ZEIER, 2008). Several metabolites are crucial for the role they play during plant growth and development, whether in integrating stress-indicating signals, controlling downstream stress responses, modulating gene expression machinery, and regulating various transporters or pumps and biochemical reactions (TUTEJA; SOPORY, 2008).

On the other hand, ROS are continuously produced as by-products of metabolism, some are highly toxic and rapidly detoxified by various enzymatic and non-enzymatic cellular mechanisms. Plants have a series of mechanisms acting to combat the increase in ROS levels during conditions of abiotic stress and consequently the maintenance of the

organism's homeostasis (ABUELSOUD; CORTLEVEN; SCHMÜLLING, 2020). On the other hand, based on the results found for the analysis of tetrazolium viability, facilitating the identification of embryos that gave rise to viable seedlings, we are demonstrating how certain enzymes, superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), guaiacol peroxidase (GPOD, EC 1.11.1.7), glutathione reductase (GR, EC 1.6.4.2) and glutathione peroxidase (GPX, EC 1.11.1.9), are highly involved in the ROS detoxification process in ROS plantlets stevia submitted to different photoperiod conditions (Figures 5 and 6).

Figure 5. Sample of *Stevia rebaudiana* (Bert.) Bertoni seedlings subjected to different photoperiod conditions.



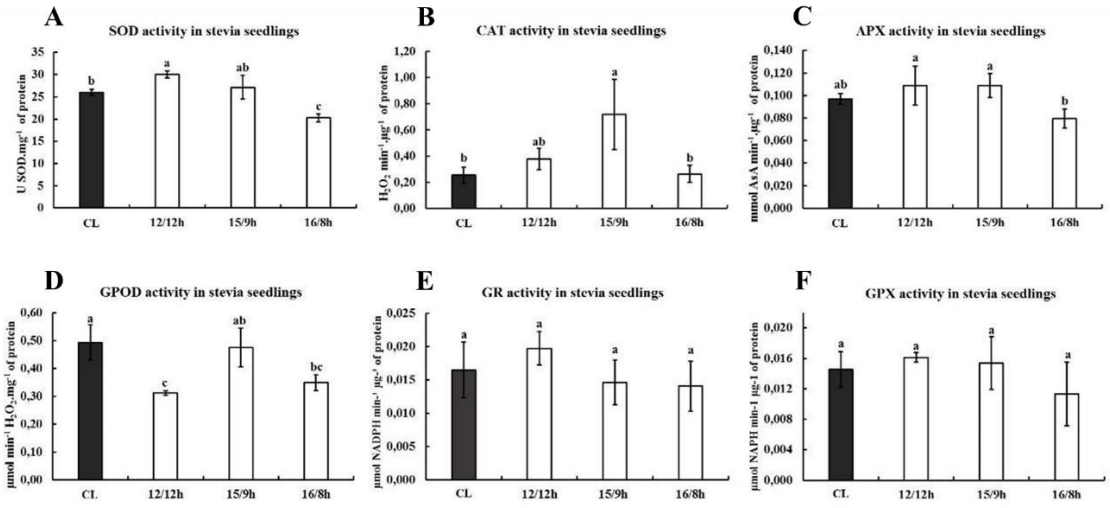
- Superoxide dismutase (SOD, EC 1.15.1.1)
- Catalase (CAT, EC 1.11.1.6)
- Ascorbate Peroxidase (APX, EC 1.11.1.11)
- Guaiacol Peroxidase (GPOD, EC 1.11.1.7)
- Glutathione Reductase (GR, EC 1.6.4.2)
- Glutathione Peroxidase (GPX, EC 1.11.1.9)

SOD is considered the main intracellular antioxidant defense against free radicals. This enzyme plays an important role in the defense against oxygen radical-mediated toxicity in aerobic organisms. In plants, environmental adversities such as drought, high or low temperature, flooding, presence of heavy metals, and macronutrient deficits often lead to increased generation of reduced oxygen species and, consequently, it is suggested that SOD plays an important role in tolerance to plant stress (STEPHENIE et al., 2020).

In this study, the total SOD activity of the stevia seedling extracts ranged from 20.29 ± 0.86 to 30.01 ± 0.78 U SOD.mg^{-1} of protein (Figure 6 A). The total SOD activity of different stevia seedling extracts showed a very peculiar variation in response to increased exposure time to different photoperiod conditions. It is noticed that the seedlings exposed to 12/12h photoperiod condition showed superior activity to the other

treatments. It appears that seedlings growing in the 12/12h photoperiod condition perceive this as a stress condition, even when compared to the constant light (CL) condition. While the seedlings submitted to the photoperiod of 15/9h is a transitional condition, in which there is a decrease in the total activity of SOD. While the seedling samples, when submitted to the 16/8h photoperiod, do not seem to consider this a stress condition, as can be seen in the SOD results. It seems, however, that seedlings tend to respond more quickly to sudden changes in photoperiod, because even with only seven days of stress submission, there was an increase in total SOD activity for the 12/12h photoperiod condition when compared to the others. treatments. Stevia has a close relationship with exposure to different photoperiod conditions, with a sensitivity spectrum that can range from 8 h to 14 h (VALIO; ROCHA, 1977; ZAIDAN; DIETRICH; FELIPPE, 1980). In our study, we considered the 12h photoperiod as a short-day photoperiod, while the 15-16h conditions as a long-day photoperiod. Thus, we can observe that 12/12h photoperiod conditions can induce early flowering of stevia seedlings, without adequate physiological development and, thus, trigger the generation of ROS, as can be seen in our results with stevia seedlings, where this induction of early flowering may have triggered the generation of excess ROS.

Figure 6. Antioxidant enzyme activity for *Stevia rebaudiana* (Bert.) Bertoni seedling extracts submitted to different luminosity stresses. The different letters indicate significant differences between the samples taken by Tukey's HSD ($P < 0.05$); Supplementary Table 2.



With increased stress, the formation of ROS is intensified, and the expression of superoxide dismutase (SOD) is altered, the first enzyme to act in the antioxidant system,

performing the dismutation of the superoxide radical ($O_2^{-\bullet}$) to hydrogen peroxide (H_2O_2). SODs are able to detect subtle differences in metabolism both in the cell cytoplasm and in the mitochondrial matrix, so the formation of free radicals activates superoxide dismutase as a protective and superoxide scavenger mechanism (CORTE et al., 2010; STEPHENIE et al., 2020).

Catalase is an excellent enzyme that makes up the antioxidant system, which promotes the degradation of H_2O_2 into $2H_2O + O_2$ at a rapid rate and without consuming cellular energy. Playing a central role in maintaining the balance of cellular hydrogen peroxide in plants, catalase was the first documented antioxidant enzyme and appears in all prokaryotes and eukaryotes (SHARMA; AHMAD, 2014).

The importance of catalases in the antioxidant defense system of plants has been proven by several studies and their role against the most diverse oxidative stresses (BOČOVÁ et al., 2012; SHARMA; AHMAD, 2014; SHARMA; TRAVLOS, 2012; YOUSSEF; AZOOZ, 2013).

For the present study, the total CAT activity of the seedling extracts ranged from 0.25 ± 0.06 to 0.72 ± 0.27 H_2O_2 $min^{-1}.\mu g^{-1}$ of protein (Figure 6 B). The total CAT activity of the different stevia seedling extracts showed a significant variation in response to exposure to different photoperiod conditions. It is noted, in a peculiar way, that samples of seedlings that were exposed to the 15/9h photoperiod condition showed superior activity to the other treatments. Our results suggest that the seedlings that grow in the 12/12h and 15/9h photoperiod conditions perceive such conditions as stress, even when compared to the control condition, of constant light (CL). Seedlings submitted to photoperiod conditions of 16/8h showed a strong reduction in the total CAT activity as seen for the SOD activity. The results demonstrate the modulation of such enzymes by light and highlighting those long photoperiods can provide better conditions for the establishment of seedlings for the species, as can be seen for the variations in CAT activity for control samples (CL) and samples under 16/8h photoperiod.

Ascorbate peroxidase (APX) belongs to class I heme-peroxidases and is found in higher plants, chlorophytes (CAVERZAN et al., 2012; TAKEDA et al., 2000), red algae (SANO et al., 2001) and members of the kingdom Protista (WILKINSON et al., 2002).

The expression of genes that code for APX can be modulated by a series of environmental stimuli, such as drought and salt stress, light, temperature, pathogen attacks, H_2O_2 and abscisic acid. Furthermore, the transcriptional expression of APX genes is influenced by the tissue and stage of development of the organism (BONIFACIO et al., 2011; CAVERZAN et al., 2012; ROSA et al., 2010).

In the present work, the activity of the APX enzyme ranged from 0.079 ± 0.01 to 0.11 ± 0.02 $mmolAsA.min^{-1}.\mu g^{-1}$ of protein (Figure 6 C). Note that the activity of the enzyme mentioned was influenced by the different photoperiods to which the stevia seedling samples were submitted. Furthermore, the results showed a trend as observed for the SOD activity. The seedling samples exposed to 12/12h and 15/9h photoperiod conditions showed higher activity than the control samples, under constant light. It seems that the seedlings that remained in these photoperiod conditions perceive this as a stress condition, even when compared to the constant light (CL) condition, as observed for the SOD activity, considering that the H_2O_2 generating from the superoxide radical dismutation is one of the substrate sources for the activity of this enzyme in the antioxidant chain. Seedlings submitted to photoperiod conditions of 16/8h showed a decrease in total APX activity, which apparently can be a less aggressive condition when compared to other conditions, taking into account the sensitivity of stevia and the modulation of APX activity to light, even in young seedlings. In spinach studies, APX isoenzyme responses to photooxidative stress were observed. It was seen that cAPX activity and transcripts increased during light stress. While the activities of the chlAPX isoforms showed a gradual decrease, while the other isoenzymes showed no significant variation in the levels of transcription and protein, as well as in activities (YOSHIMURA et al., 2000). *Arabidopsis thaliana* mutants without the isoenzyme tAPX or sAPX showed higher levels of H_2O_2 and higher levels of oxidized proteins than wild plants when exposed to high light and methyl viologen stresses. The strongest effect of photooxidative stress was observed in plants without tAPX, which showed increased accumulation of H_2O_2 and oxidized proteins (MARUTA et al., 2010).

Currently, APX is seen as enzymes of great importance not only as classic antioxidant enzymes that prevent oxidative damage in plant cells but also as modulators of an H_2O_2 signal to adjust abiotic and biotic stress responses (MARUTA et al., 2016).

Class III peroxidases (POD – EC 1.11.1.7) are involved in a series of functions, which include dealing with physical stress, pathogens or auxin, and lignin metabolism (MARJAMAA; KUKKOLA; FAGERSTEDT, 2009). In our study, we focused on investigating the group of guaiacol peroxidase (GPOD – EC 1.11.1.7) which has high activity using guaiacol as a substrate. To estimate its activity, it is possible to monitor the formation of guaiacol into tetraguaiacol (VAN DOORN; KETSA, 2014). Guaiacol peroxidases are heme-containing proteins that preferentially oxidize aromatic electron donors such as guaiacol and pyrogallol at the expense of H_2O_2. They are active in important biosynthetic processes and defense against abiotic and biotic stresses or referred to as a stress "enzyme" (SHARMA et al., 2012).

GPOD activity ranged from 0.31 ± 0.01 to 0.49 ± 0.06 μmol.min^{-1}H_2O_2.mg^{-1} of protein (Figure 6 D). The samples of stevia seedlings kept under constant light (CL) apparently showed a higher level of stress regarding the GPOD enzyme when compared to the other studied photoperiods. This highlights the different effects that light such as abiotic stress can have. However, for the different photoperiods analyzed, the samples submitted to the 12/12h treatment provided a lower stress condition for the samples.

Due to the fact that GPOD plays a fundamental role in the response to environmental stress and can be easily determined by the colorimetric method, it can be a good parameter in assessing the plant's response to environmental stress (GALLIE, 2013).

Glutathione is a strong antioxidant and has a vital role in antioxidant defense, being important in the role of plant tolerance to different abiotic stresses, such as salinity, drought, high and low temperature, and toxic metal stress (HASANUZZAMAN et al., 2012, 2017, 2020; HOSSAIN et al., 2017). For the protective activity of glutathione expressed by the reduction of oxidizing species, the activity of the enzymes glutathione reductase (GR) and glutathione peroxidase (GPX) must be considered, these enzymes that make up the glutathione cycle (HASANUZZAMAN et al., 2017; QIN et al., 2018). Glutathione reductase (GR) is an essential enzyme that recycles oxidized glutathione (GSSG) back to the reduced form (GSH) (PASSAIA; MARGIS-PINHEIRO, 2015; QIN et al., 2018). In the present study, it was observed that the activity ranged from 0.014 ± 0.001 to 0.0183 ± 0.001 μmol NADPH.min^{-1}.μg^{-1} of protein (Figure 6 E). Apparently, the results followed the same trend observed for the SOD evaluation, however we did

not obtain a significant variation between treatments for different photoperiods and treatment under constant light. Even so, for samples submitted to a photoperiod of 16/8h, it is possible to notice a lower activity, which may reflect a lower level of photooxidative stress for them.

Plants have developed very efficient antioxidant defense systems and GSH is one of the main molecules that form an integral part of the AsA-GSH cycle that eliminates H_2O_2. The various biochemical properties of GSH give it the potential to be involved in plant growth and development, both under normal growing conditions and under different stress conditions (PASSAIA; MARGIS-PINHEIRO, 2015; QIN et al., 2018). The increase in GR activity, as observed for the samples under 12/12h photoperiod, can provide tolerance to the stress to which the seedlings were submitted and have the ability to change the redox state of the important elements of the electron transport chain. Considering GR, it promotes the recycling of GSH in the plant cell via maintenance of the GSH/GSSH ratio (HAMEED et al., 2014; HASANUZZAMAN et al., 2017). GPX are enzymes that have the ability to remove hydrogen peroxide indirectly, using it as a substrate for the oxidation of reduced glutathione (GSH) (HAMEED et al., 2014; HASANUZZAMAN et al., 2017). Even without obtaining a significant difference in the treatments (Figure 6 F), we can observe that the results were similar to those found for GR because the enzymes share the same cycle.

Enzyme activity data were correlated using the Pearson correlation coefficient. High correlations were found between GPX - SOD ($r = 0.87648$, $p = 0.0002$) and GPX - GR ($r = 0.85987$, $p = 0.0003$) followed by an again high correlation between SOD - GR ($r = 0\ .95507$, $p = 1.3364e\text{-}06$). Negative correlations were mostly presented for GPOD and the other analyzed enzymes (Table 1).

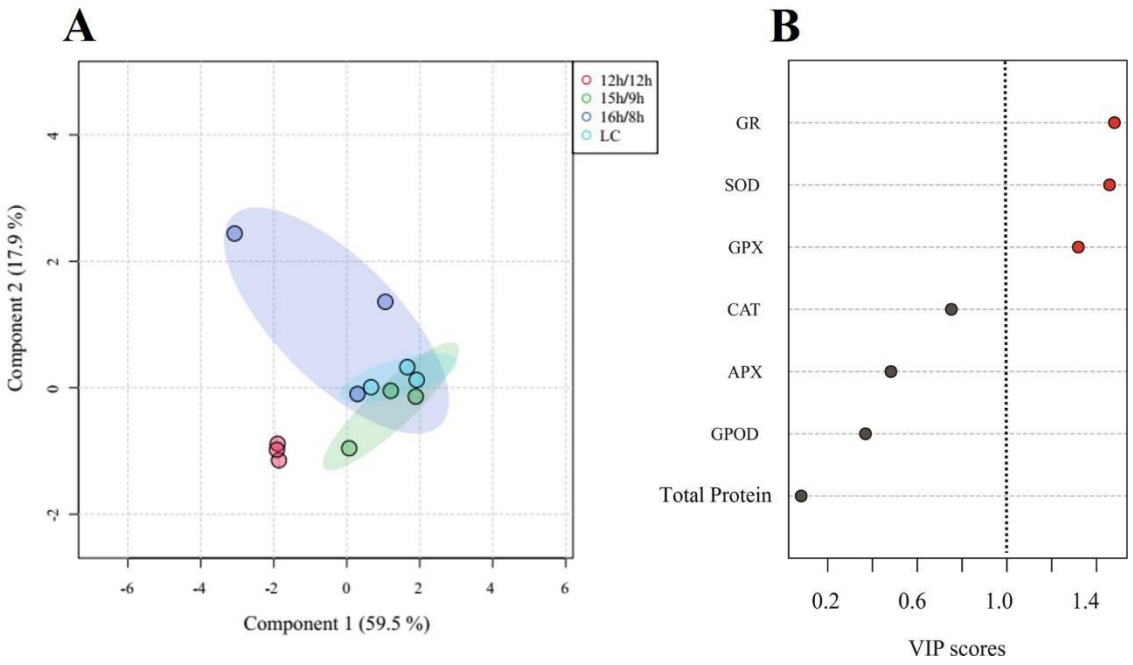
Table 1. Correlations and p-value of antioxidant enzymes analyzed for stevia seedling samples submitted to different photoperiod conditions.

	GPOD	CAT	GPX	SOD	GR	Total protein	APX
GPOD	1	-0.19669	-0.5727	-0.60634	-0.65371	-0.72331	-0.48494
CAT	0.54008	1	0.61734	0.61675	0.48939	0.39135	-0.030485
GPX	0.051628	0.032459	1	0.87648	0.85987	0.38274	0.40914
SOD	0.036606	0.032674	0.00018338	1	0.95507	0.62648	0.59249
GR	0.02113	0.10635	0.00033474	1.3364e-06	1	0.67885	0.6771
Total Protein	0.0078471	0.20839	0.21946	0.029277	0.015207	1	0.61801
APX	0.11005	0.92507	0.18661	0.042353	0.015575	0.032219	1

The observed correlations emphasize that a group of enzymes are acting more strongly in order to mitigate the ROS produced by exposure to different photoperiods, enzymes that we can highlight in this context are SOD, GR and GPX, enzymes with an indicative correlation coefficient that are positively related. The SOD, GPX and CAT are antioxidant enzymes that not only play a fundamental role, but also an indispensable role in the antioxidant protection capacity of biological systems against the attack of free radicals, being called first-line antioxidant defense (IGHODARO; AKINLOYE, 2018). Because they have a greater distribution between the SOD and GPX cellular compartments, they justify their greater correlation for the present work. Studies also demonstrate that, in plants under abiotic stress, the defense mechanism can be based on the enzymes of the AsA-GSH cycle, which in the present study shows a high correlation, represented by GR and APX.

The enzyme activity data in this study were submitted to supervised and unsupervised methods to better understand the data distribution and classify the samples according to their biochemical behavior (Figure 7). In addition, we use PLS-DA as the best-unsupervised method. The total variance explained by the PLS-DA was 77.40%, with 59.50% and 17.90% for PC1 and PC2, respectively. As a result, a similar profile was detected in the samples submitted to treatments with the LC photoperiod, 15/9h, and 16/8h, with a distinct group formed by the samples at 12/12h photoperiod exposure, thus demonstrating the sensitivity of stevia to exposure to various photoperiods (Figure 7A).

Figure 7. Enzyme profile of *Stevia rebaudiana* (Bert.) Bertoni seedling samples grown under different photoperiods. (A) Partial least squares discriminant analysis (PLS-DA); (B) Variable importance in the projection scores (VIP).



Variable importance in projection scores (VIP) was used to assess the contribution of each enzyme in separating groups in the PLS-DA analysis. For that, we set the VIP threshold to one. The projection scores indicate that the enzymes GR (MIC1 1.4803; CP2 1.0338), SOD (CP1 1.4585; CP2 1.0189) and GPX (CP1 1.3191; CP 2 0.92817) were mainly responsible for the variation in the data observed in the PLS-DA chart (Figure 7 B). From these results, we can assume that, in addition to SOD, characterized as the main enzyme of the antioxidant apparatus, GR and GPX are the main players in the redox cycle in stevia seedlings, where reactions are regulated by glutathione synthesis dependent on the concentration of GSH and GSH/GSSG ratio, mainly in stevia samples submitted to long-day photoperiods (15h and 16h of light).

The most varied factors can cause the alteration of homeostasis in stevia seedlings, such as photoperiod variations which were conducted in the present study. Studies with stevia also portray that melatonin applied in the germination phase altered the activities of CAT, POD and SOD in seedlings. CAT activity was higher in seedlings obtained from seeds germinated in 500 mM melatonin. As also observed for the increase in POD activity. Among the various concentrations of melatonin tested, in that study, the

activity of the enzyme superoxide dismutase significantly decreased according to an increase in the concentration of melatonin, which can be seen as a sign that ROS are being generated by other pathways in cells (SIMLAT et al., 2018).

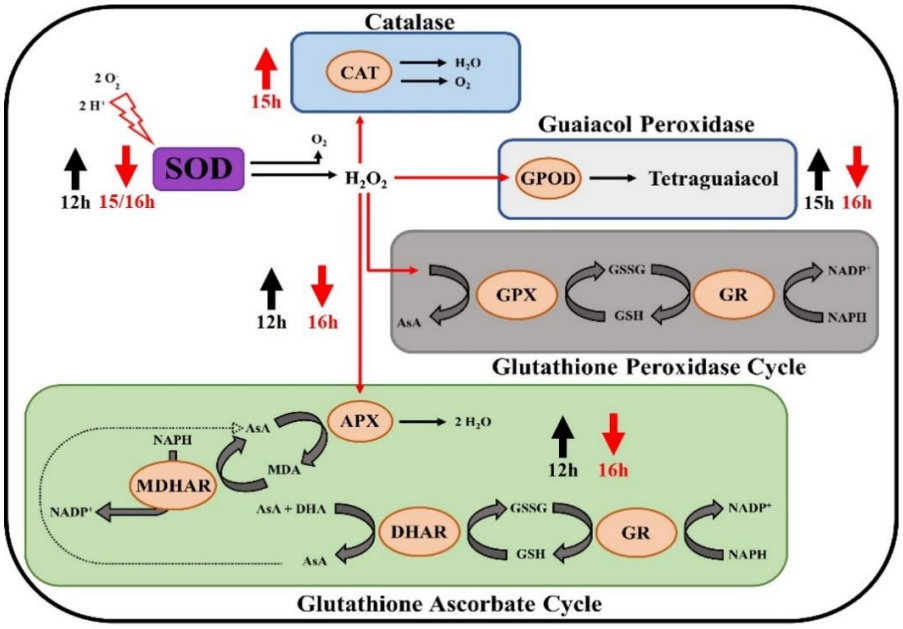
Acosta-Motos et al. (2019) in a study with acclimatization of stevia seedlings realized how this parameter can influence enzyme activity, also considering the sensitivity of stevia to different photoperiod conditions and light intensity. In that work, using different light intensities, during the acclimatization of stevia seedlings, it was noted the occurrence of oxidative stress, indicated by the levels of lipid peroxidation, this variable is a marker of oxidative stress. However, due to its sensitivity to different photoperiodic conditions, once this change in growing conditions occurred, the plants progressively adapted to the new condition, which is reflected in the decrease in lipid peroxidation (ACOSTA-MOTOS et al., 2019).

Antioxidant enzymes may have their activity altered in response to a wide range of oxidative stress. Environmental stresses lead to the abnormal generation of ROS in plants due to disruption of cellular homeostasis. ROS are extremely harmful to organisms in high concentrations and when the level generated exceeds the defense mechanisms, the cell is said to be in a state of “oxidative stress”. This exaggerated production of ROS poses a threat to cells, causing lipid peroxidation, protein oxidation, nucleic acid damage, enzyme inhibition, and activation of the programmed cell death pathway, thus leading to cell death. However, ROS may have a signaling function for a variety of cellular processes, including tolerance to environmental stresses (KAPOOR et al., 2019; STEPHENIE et al., 2020). The enzymatic and non-enzymatic antioxidant systems are responsible for the elimination or detoxification of excess ROS. Enzymatic antioxidants include SOD, CAT, APX, POD, GR, and GPX, etc. Several studies have reported increased activities of many enzymes of the antioxidant defense system in plants to combat oxidative stress induced by various environmental stresses. Considering developing plants, and establishing all their defense apparatus, the simultaneous expression of multiple antioxidant enzymes has been shown to be more effective (KAPOOR et al., 2019; STEPHENIE et al., 2020).

Apparently, the path of elimination of ROS by plants depends, at times, on the joint action of some enzymes that make up this enzymatic antioxidant defense apparatus. For

plants, the joint action of this large complex can be seen in Figure 8, where the action of APX is dependent on the ascorbate supplied by DHAR and consequently generates MDHAR. While GPX indirectly removes hydrogen peroxide, using it as a substrate for the oxidation of GSH, forming GSSG that will act as a substrate for GR, configuring the Glutathione Peroxidase cycle (KAPOOR et al., 2019; MITTLER, 2002). Regarding this joint action of antioxidant enzymes, from the results obtained we can infer that the subcellular location of each enzyme reflects how they act. Predominantly, CAT seems to act independently, being mostly peroxisomal, while the other enzymes SOD, APX, GPX, GR have a greater distribution in cell compartments, for example, in mitochondria, chloroplasts, and in the cytoplasmic region. Thus, it can be observed that the elimination of ROS from, mainly, this set of enzymes from the antioxidant enzyme complex.

Figure 8. Elimination of reactive oxygen species (ROS) in *Stevia rebaudiana* (Bert.) Bertoni seedlings.



Adapted from (KAPOOR et al., 2019; MITTLER, 2002).

4. Conclusions

We can conclude that seed viability/quality assessment provided relevant information about the peculiarities of using seeds for stevia cultivation. While the antioxidant enzyme profile provided information that seedlings grown in light conditions below the critical photoperiod appear to face drastic stress, possibly because of ROS induction at a stage where the plants (seedlings) are still underdeveloped and immature for proper

flowering, driving the seedlings there is a photo-oxidative stress. Overall, our results provide useful information apparently related to circadian clock changes depending on lifecycle stage and photoperiod. In this sense, our study indicates that long photoperiod conditions (16/8h) can lead to the establishment of healthier plants. Therefore, when it comes to stevia, it is necessary to determine a suitable cultivation system exclusively to produce high-quality seeds or aiming at large-scale stevia cultures to produce StGly.

5. Acknowledgement

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6. Conflict of interest

The authors declare no conflict of interest.

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Supplementary Material

Supplementary Table 1. Normality test for the results of the analysis of antioxidant enzymes from different extracts of *Stevia rebaudiana* (Bert.) Bertoni seedlings cultivated under different photoperiods.

Variable	n	W	Pr<W
SOD	12	0.9167785651754	0.2603435
CAT	12	0.7764069110403	0.0051147
APX	12	0.9859673634640	0.9976446
GPOD	12	0.8880805321542	0.1113156
GPX	12	0.9000283502546	0.1587531
GR	12	0.9747038346762	0.9533287

n – number of samples; W - value resulting from the applied test that statistically measures whether a random sample comes from a normal distribution or not. When W is closer to 1, it indicates that the sample is more likely to come from a normal distribution.

Supplementary Table 2. Antioxidant enzyme activity for *Stevia rebaudiana* (Bert.) Bertoni seedling extracts submitted to different luminosity stresses. The different letters indicate significant differences between the samples taken by Tukey's HSD ($P < 0.05$).

	SOD (U SOD.mg^{-1} protein)	CAT (U CAT.µg^{-1} protein)	APX (µmol AsA min^{-1}.µg^{-1} protein)	GPox (µmol min^{-1} H_2O_2.mg^{-1})	GR (µmol min^{-1}µg^{-1} protein)	GPx (µmol min^{-1} µg^{-1} protein)
CL	25,98±0,68^{b}	0,03±0,01^{b}	0,08±0,00^{ab}	0,49±0,06^{a}	0,02±0,00^{a}	0,01±0,00^{a}
12h	32,68±0,41^{a}	0,04±0,01^{ab}	0,11±0,01^{a}	0,31±0,01^{ab}	0,02±0,00^{a}	0,02±0,00^{a}
15h	27,41±0,53^{ab}	0,08±0,03^{a}	0,10±0,01^{a}	0,47±0,07^{bc}	0,01±0,00^{a}	0,02±0,00^{a}
16h	19,66±0,78^{c}	0,03±0,01^{b}	0,06±0,00^{b}	0,35±0,03^{c}	0,01±0,00^{a}	0,01±0,00^{a}

CAPÍTULO 2

Stevia rebaudiana (Bert.) Bertoni cultivada em diferentes condições de fotoperíodo: melhorando as características fisiológicas e bioquímicas para aplicações industriais

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Stevia rebaudiana **(Bert.) Bertoni cultivated under different photoperiod conditions: improving physiological and biochemical traits for industrial applications**

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Abstract

Stevia rebaudiana is an important industrial crop due to the accumulation of high amounts of steviol glycosides (SG - natural sweeteners) in its leaves. *S. rebaudiana* cultivation has faced some pushbacks since this species is highly responsive to environmental factors, such as light availability. Sixty days after sowing, plants were transferred to different photoperiod conditions (12/12h, 15/9h, and 16/8h of light/dark). Leaf extracts of plants growing at the 16/8h photoperiod showed greater accumulation of antioxidant-like metabolites as compared to the other two photoperiods, which might be explained by the total phenolic content of the extracts. Additionally, plants growing at the 16/8h photoperiod showed increased SOD activity as compared to plants growing at the 15/9h photoperiod, which in turn showed higher SOD activity than plants growing at the 12/12h photoperiod. It seems that SOD isoforms act synergically with phenolic compounds to prevent possible damages caused by reactive oxygen species that are produced in plants growing at long-day photoperiods. Sixteen metabolites were identified by Nuclear Magnetic Resonance in the leaf and stem extracts. Alanine, formate, choline, kaempferol-3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside, and gallic acid seems to contribute to maintain stevia homeostasis under unfavorable conditions. Furthermore, the accumulation of SGs and other bioactive compounds in *S. rebaudiana* in response to different photoperiods provides important leads for the improvement of its large-scale cultivation, as well as for the extraction and purification of phytochemicals with industrial interest.

Keywords: Luminosity, Metabolomics, Sweetening compounds, Steviol glycosides

1. Introduction

Stevia rebaudiana (Bert.) Bertoni belongs to the Asteraceae family and it is originally found at the border between Brazil and Paraguay (Mizutani and Tanaka, 2002; Wölwer-Rieck, 2012). This species has been recognized as an important industrial crop due to the natural occurrence of high amounts of compounds with sweetening properties in its leaves. The so-called steviol glycosides (SG) are up to 300 times sweeter than sucrose, conferring high economic potential to the species (Lemus-Mondaca et al., 2012; Samuel et al., 2018). Additionally, stevia has important therapeutical and pharmacological applications as their extracts are metabolically stable and non-toxic to the human body, exhibiting several biological activities (Ruiz-Ruiz et al., 2017). Thus, stevia has become a viable alternative to sucrose and artificial sweeteners. Stevia leaves contain a myriad of more than 30 different SGs, which may account for up to 13% of the dry weight of the leaves (Brandle et al., 1998a; Myint et al., 2020). Nevertheless, the most representative SGs are stevioside (up to 13%), rebaudioside A (up to 4%), rebaudioside C (up to 2%), and dulcoside A (up to 0.7%) (Gupta et al., 2016; Yadav et al., 2013). Since SGs are not metabolized by the human body, they have been widely used by patients with type II diabetes, hypertension, myocardial and antimicrobial infections, dental caries and tumors (Gupta et al., 2013; Ruiz-Ruiz et al., 2017; Samuel et al., 2018; Lemus-Mondaca et al., 2012; 2018; Zhao et al., 2019). Currently, China is the main producer of stevia accounting for up to 80% of the total worldwide production, whereas Japan and Korea represent the largest stevia consumer market worldwide (Myint et al., 2020). Nevertheless, stevia cultivation has quickly spread over several countries due to its plasticity to different climates and feasible crop management requirements (Lemus-Mondaca et al., 2012). Besides their use as sweeteners, SGs have prominent physicochemical properties for several industrial branches, such as acid resistance and thermostability. SGs can act neutralizing free radicals and, therefore, they can be used as food and beverages additives, increasing their shelf-life. Several studies have suggested that stevia leaves are a multifunctional source of natural bioactive compounds (Ruiz-Ruiz et al., 2017; Shivanna et al., 2013), which include vitamins, phytosterols, triterpenes, and polyphenols. These compounds are believed to be responsible for the wide range of pharmacological activities of the extracts (Gaweł-Bęben et al., 2015; Molina-Calle et al., 2017).

Environmental factors such as high and low temperatures, water and light availability affect the biosynthesis and accumulation of SGs during stevia growth and development. Light availability (photoperiod) is one of the most relevant environmental factors affecting stevia growth and cultivation (Debnath et al., 2019; Lemus-Mondaca et al., 2012; Metivier and Viana, 1979; Valio and Rocha, 1977). Additionally, the period of the year in which harvest is performed, along with the pre- and post-harvest techniques that are used during its cultivation may affect SGs content in stevia leaves (Cantabella et al., 2017; Goyal et al., 2010; Megeji et al., 2005; Tavarini et al., 2016; Zeng et al., 2013).

Photoperiod has become a reliable indicator of the seasons throughout the year, which allow the plants to adapt their physiological and developmental events within particular environmental conditions. The response of plants to different photoperiods is a complex trait that encompasses the initial detection of the light by the leaves, followed by the involvement of the circadian clock, culminating with a signal response transmitted throughout the plant. Light availability affects vegetative growth, flowering, leaf area, dry matter, total carbohydrate and protein levels (Brandle and Rosa, 1992; Ceunen and Geuns, 2013a). Although photoperiod is a crucial environmental cue for stevia large-scale production, very few studies have investigated the influence of light availability on the accumulation of SGs (Yadav et al., 2013). *S. rebaudiana* grown under field conditions showed considerable differences in terms of growth and steviol glycoside content in response to environmental factors. Briefly, plants were grown at two different years (2014 and 2015), in which plants that were grown in 2015 experienced lower precipitation, higher maximum temperature, and higher solar radiation than plants grown in 2014. As result, an accumulation of rebaudioside A on the leaves of plants grown in 2015 was observed as compared to those plants grown in 2014 (Munz et al., 2018). In a more recent study, night-interruption treatments (8h light, 6h dark, 4h light interruption, 6h dark) led to higher SGs content than that observed under 12h photoperiod (Yoneda et al., 2017b). Nonetheless, the optimal photoperiod regime required to enhance total SGs production in stevia leaves is still controversial. Herein, we have conducted the in-depth characterization of *S. rebaudiana* leaf and stem physiological and biochemical responses to different photoperiods.

2. Material and Methods

2.1. Plant growth and sampling

In this study, we used the cultivar IAN/VC-142/Eirete (Stevia Eirete 2) that was developed by the Instituto Paraguayo de Tecnología Agraria - IPTA. Plants were grown in a plant growth chamber (Panasonic/Sanyo, MLR-351H) equipped with 15 white fluorescent lamps (Mitsubishi/Osram, FL40SS-W/37) and adjusted to constant 25°C and 70% RH. Selected fertile seeds (darkish achene fruits) (Gantait et al., 2018) were previously germinated over germination paper moistened with 8 mL distilled water on plastic germination boxes (10 x 10 cm). These plastic germination boxes were incubated in the growth chamber for 10 days, after which the most vigorous seedlings were transplanted to plastic pots (700 mL volume, 13.7 cm high x 10 cm diameter) filled with commercial growth substrate (Carolina Soil, pH 5.5, electrical conductivity 0.7 $mS.cm^{-2}$, maximum humidity 60% $m.m^{-1}$, dry density 130 $kg.m^{3}$, water holding capacity 350% $m.m^{-1}$). These seedlings were allowed to grow for 60 days at constant to favor vegetative growth, after which plants were transferred to different photoperiods (12/12h, 15/9h, and 16/8h of light/dark). Leaves and stem of 5 individual plants were sampled in triplicate at 4, 8, 12, and 16 days after transfer (DAT) to the different photoperiods (12/12h, 15/9h, and 16/8h). Plants were phenotyped at each sampling point by measuring plant height (distance in centimeters from the stem bottom to its apex), stem diameter, number of leaf pairs per plant, and dry matter. For dry matter purposes, all leaves and the entire stem were collected. Samples were lyophilized, ground and stored at -80 °C prior to further analysis. Plants were watered every other day with 50 mL distilled water and once every 7 days with liquid fertilizer (REMO Nutrients, Grow 2-3-5 and Micro 3-0-1, 1:1000 (v/v) with 50mL per plant/pod) until the appearance of the first flower buds.

2.2. Chlorophyll quantification

Chlorophylls (Chlorophyll A and B) content was determined spectrophotometrically according to Lichtenthaler (1987). Initially, 8 mL of 80% (v/v) acetone were added to 0.1 g of each sample. Subsequently, the mixture was vortexed, filtered and the volume

adjusted to 10 mL with cold acetone. The absorbance of the extracts was read at 665.2, 652.4 and 470 nm.

2.3. DPPH radical scavenging assay and total phenolic content

The antioxidant activity was assessed by the 2,2-diphenyl-1-picryl-hydrazil (DPPH) radical scavenging assay as described by Batista et al. (2020), whereas total phenolic content was measured by using the Folin-Ciocalteu phenol reagent as described by Santos et al. (2018). Further details can be found at Supplementary Material and Methods.

2.4. Superoxide Dismutase (EC 1.15.1.1) activity and isoforms

For SOD activity purposes, protein extracts were prepared by macerating 0.1 g of each sample t with 1 mL of phosphate buffer (pH 7.4) in a dismembrator device (Sartorius, Mod. Mikro-Dismembrator S) for 1 min at 2.000 rpm. The evaluation SOD activity was assessed spectrophotometrically at 560 nm (Varian - Cary 50 MPR Microplate Reader) in a 96-well microplate as described by Gomes Neto et al., (2018). Further details can be found at Supplementary Material and Methods.

2.5. Antimicrobial activity

Antimicrobial activity of the extracts was evaluated using the successive microdilution assay in 96-well microplates as described by Ribeiro et al. (2011). The antimicrobial activity of the extracts was evaluated against the following microorganisms: *Bacillus cereus* Frankland & Frankland (nº CCT 0096), *Staphylococcus aureus* (ATCC 6538), and *Candida albicans* (ATCC 18804). Further details can be found at Supplementary Material and Methods.

2.6. Metabolite profiling analyses

2.6.1. Nuclear magnetic resonance (NMR) analysis

For metabolite profiling purposes, 0.1 g of each sample were mixed with 1 mL of potassium phosphate buffer (pH 7.4) (Brito et al., 2020). Then, the samples were

crushed and homogenized in a dismembrator device (Sartorius, Mod. Mikro-Dismembrator S) twice for 5 minutes at 2.000 rpm. Subsequently, samples were centrifuged three times at 15.000 rpm for 10 min each time (Ribeiro et al., 2014). The extracts were lyophilized and resuspended in a mixture of D_2O and CD_3OD (2:1 v/v), after which they were filtered and transferred to 5-mm NMR tubes. NMR spectra were measured at 25 °C (Bruker Avance III 9.4 T) a 400MHz.

2.7. Data processing and statistical analysis

MestreNova was used to process ^1H NMR spectra, which included correction of the baseline, alignment and integration of the peaks. Multivariate statistical analysis was performed with the aid of the MetaboAnalyst platform (https://www.metaboanalyst.ca) (Chong et al., 2019; Neto et al., 2020) and SISVAR 5.6. Analysis of variance was used to identify statistically significant differences between the samples ($p < 0.05$) followed by Tukey's multiple comparison tests. The results are presented as the mean of replicates ± standard deviations.

3. Results and discussion

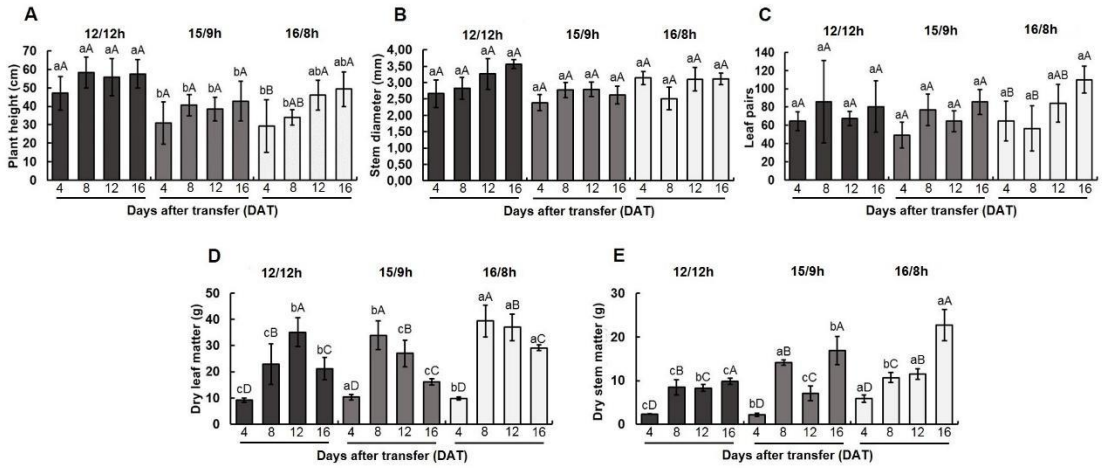
3.1. Cultivation of stevia under different photoperiods

Stevia cultivation has faced some pushbacks since this species is highly responsive to environmental factors, including light availability. It is worth mentioning that stevia is a short-day species with a critical photoperiod of 12 to 13h of light, below which plants enter an unwanted precocious reproductive stage, reducing the content of SGs in its leaves. For that reason, plants were initially grown for 60 days under continuous light and then they were transferred to different photoperiods, i.e. 12/12h – reproductive mode (control), and 15/9h, and 16/8h of light/dark – vegetative modes.

Plants grown under controlled photoperiod conditions (12, 15 and 16h of light) were phenotyped by measuring plant height, stem diameter, the number of leaf pairs per plant, and dry matter (Figure 1). These parameters were strongly affected by the photoperiod conditions and the length of exposure to these different photoperiods. By comparing sampling points within the same photoperiod, no differences in terms of plant height were observed for plants growing at 12/12h and 15/9h photoperiods (Figure 1A). However, plants growing at the 16/8h photoperiod that were collected at 12 and 16

DAT showed greater plant height than those plants collected at 4 and 8 DAT. When comparing the same sampling point, but in different photoperiod conditions, it seems that plants growing at 12/12h showed greater height as compared to those plants growing at 15/9h and 16/8h. No differences whatsoever were observed in terms of stem diameter and number of leaf pairs (Figure 1B). Plants collected at 4 DAT showed lower leaf and stem dry matter as compared to plants collected at 8, 12 and 16 DAT for all three photoperiods (Figure 1D and E). It seems, however, that leaf and stem dry matter showed a contrasting pattern for plants collected at 8, 12 and 16 DAT. For leaf samples, dry matter was reduced across plants collected at 8, 12 and 16 DAT, whereas for stem samples dry matter increased across plants collected at 8, 12 and 16 DAT. Taking into account that no differences whatsoever were observed in terms of stem diameter and number of leaf pairs, it seems that stevia plants induced biomass production in the stem rather than in the leaves as a response to different photoperiods (Figure 1B and C). This might compromise the yield of SGs, highlighting the relevance of determining the best sampling point to maximize SG yield.

Figure 1. Morphophysiological analysis of *Stevia rebaudiana* (Bert.) Bertoni under different photoperiods. The lower-case letters compare each photoperiod within each collection time and the upper-case letters compare the collections within each photoperiod according to the HSD de Tukey ($P < 0.05$).



Stevia plants seem to be readily responsive to light availability, since different photoperiods influence the plants phenotypic architecture, biomass allocation and photosynthetic activity (Yoneda et al., 2017a). Nevertheless, the extent and pattern of light availability influence on stevia plants are rather controversial. Plants growing at

short-day photoperiod conditions showed compromised vegetative growth in favor of the reproductive mode (Ceunen and Geuns, 2013a). In this study, however, it seems that short-day photoperiod conditions promoted plant elongation, but with compromised biomass allocation. It is also important to highlight that large-scale production of stevia for industrial purposes would have to take into account other variables (environmental factors). For example, nutrient fertilization might be a key factor for large-scale production of stevia as reported by Díaz-Gutiérrez et al. (2020), whereas Castañeda-Saucedo et al. (2020) observed that application of the plant hormone indole-3 butyric acid produced larger plants, with higher number of leaves, leaf dry matter, leaf area, and total biomass accumulation as compared to the control plants (untreated). Furthermore, stevia plants can respond adaptively to moderate levels of NaCl stress (30 mM), while increasing the content of SGs in its leaves (Aghighi Shahverdi et al., 2019). It is clear, however, that vegetative growth and subsequent SG accumulation in stevia plants involve complex multi-factor mechanisms that still need to be clarified. Nonetheless, compromised vegetative growth might have an effect not only on the physiology of the plant, but also on the levels of the SGs. Thus, it is pivotal to assess the effect of different photoperiod conditions on the SGs levels.

3.2. Chlorophyll quantification in stevia extracts

The content of photosynthetic pigments (chlorophyll A, B and total chlorophyll) in the leaf extracts obtained from plants growing at the 12/12h and 15/9h photoperiods did not vary in response to the increasing length of exposure to these different photoperiods. However, the leaf extracts of 16 DAT plants growing at the 16/8h photoperiod showed a significative decrease in the content of photosynthetic pigments (chlorophyll A, B and total chlorophyll) as compared to the other 3 sampling points (Table 1).

Table 1. Chlorophyll A, B, and total chlorophyll content of leaf extracts of *Stevia rebaudiana* (Bert.) Bertoni plants grown under different photoperiods and stages of development.

Photoperiod (hours light/dark)	Days after transfer (DAT)	Chlorophyll a [mg.g^{-1}FW]	Chlorophyll b [mg.g^{-1}FW]	Total chlorophyll [mg.g^{-1}FW]
12/12	4	2.00±0.12^{aA}	4.38±0.10^{aA}	6.37±0.16^{bA}
	8	2.20±0.15^{aA}	3.40±0.11^{bB}	5.60±0.03^{bB}
	12	2.07±0.15^{abA}	3.80±0.30^{bAB}	5.87±0.19^{aAB}
	16	1.90±0.09^{aA}	4.42±0.23^{aA}	6.32±0.15^{aA}
15/9	4	1.92±0.14^{aA}	4.57±0.08^{aA}	6.49±0.22^{aA}
	8	1.93±0.08^{aA}	4.45±0.06^{aA}	6.38±0.10^{aA}
	12	1.94±0.04^{bA}	4.53±0.46^{aA}	6.46±0.42^{aA}
	16	1.89±0.09^{aA}	4.59±0.04^{aA}	6.48±0.12^{aA}
16/8	4	1.92±0.18^{aBC}	4.40±0.39^{aA}	6.32±0.21^{aA}
	8	2.07±0.18^{aAB}	3.79±0.47^{abA}	5.87±0.31^{abA}
	12	2.34±0.05^{aA}	2.39±0.81^{cB}	4.73±0.78^{bB}
	16	1.70±0.26^{aC}	0.87±0.10^{bC}	2.57±0.15^{bC}

Chlorophyll B has the ability to promote greater efficiency in photosynthesis by capturing energy at specific wavelengths (blue light) and then transfer it to chlorophyll A (Cantabella et al., 2017; Ouzounis et al., 2015). Exposure to stress conditions generally results in a reduction of the chlorophyll content, which might be linked to a decrease in biosynthesis, to an increase in degradation, or even to the combined effect of both (Simlat et al., 2019). The observed reduction of chlorophyll B might suggest that the 16/8h photoperiod is being perceived by the plants as a stress condition. Consequently, plants growing at the 16/8h photoperiod might have triggered protective mechanisms that caused the reduction of light absorption, and consequently photosynthesis. This usually occurs through photoinhibition and photo-oxidation in order to protect the plants (Lima-Melo et al., 2019). Concomitantly, the 16/8h photoperiod may have caused an excessive reduction in the electron transport chain, causing photooxidation and altering the organism's redox balance (Gururani et al., 2015). Thus, the reduction in photosynthetic pigments levels can be seen as biochemical markers of the plant perception to stress, which might regulate plant growth, seed dormancy, embryo development and germination, cell division and flowering (Maoka, 2020).

3.3. Antioxidant activity and total phenolic content in stevia extracts

The antioxidant activity and total phenolic content of the extracts showed a close dependency on the tissue, photoperiod and length of exposure to the different photoperiods. The antioxidant activity (IC_{50}) of the leaf extracts varied from 11.13 ± 0.99 to 95.43 ± 7.61 $\mu g.mL^{-1}$, whereas for the stem extracts it varied from 20.31 ± 2.04 to 756.59 ± 60.11 $\mu g.mL^{-1}$ (Table 2). The antioxidant activity of the leaf extracts increased (lower IC_{50} values) with the increasing length of exposure to these different photoperiods. It seems, however, that leaf extracts of plants growing at the 16/8h photoperiod condition showed greater accumulation of antioxidant-like metabolites as compared to the other two photoperiod conditions, if we compare the same length of exposure. The same pattern was observed for the antioxidant activity of the stem extracts, however with more variance. Nevertheless, in general stem extracts of plants growing at the 16/8h photoperiod condition showed greater accumulation of antioxidant-like metabolites as compared to the other two photoperiods.

Table 2. Total phenolic compounds and antioxidant activity of leaf and stem extracts of *Stevia rebaudiana* (Bert.) Bertoni plants grown under different photoperiods and stages of development.

Photoperiod (hours light/dark)	Days after transfer (DAT)	Antioxidant activity (IC_{50}, $\mu g.mL^{-1}$)		Total phenolics (mgGAE.g^{-1} of dry weight)	
		Leaf	Stem	Leaf	Stem
12/12	4	$95.43±7.61^{cD}$	$148.01±14.75^{bB}$	$44.46±1.54^{aB}$	$17.61±2.00^{bB}$
	8	$49.03±5.32^{bC}$	$756.59±60.11^{cC}$	$37.98±1.44^{bB}$	$21.74±3.42^{bB}$
	12	$33.38±0.40^{bB}$	$39.60±7.68^{aA}$	$83.85±4.53^{cA}$	$57.78±8.51^{bA}$
	16	$19.35±1.08^{aA}$	$143.36±14.70^{bB}$	$98.26±6.59^{cA}$	$58.29±7.05^{bA}$
15/9	4	$39.33±3.54^{aB}$	$31.15±0.63^{aA}$	$47.30±1.14^{aC}$	$52.98±2.97^{aB}$
	8	$34.55±1.26^{aB}$	$113.79±27.65^{bB}$	$75.62±7.65^{aB}$	$62.72±12.33^{aB}$
	12	$14.26±1.00^{aA}$	$30.24±2.95^{aA}$	$159.42±19.47^{bA}$	$103.46±7.83^{aA}$
	16	$12.56±0.60^{aA}$	$62.47±15.65^{aA}$	$158.29±8.79^{aA}$	$104.46±7.80^{aA}$
16/8	4	$57.79±9.99^{bC}$	$32.44±2.81^{aA}$	$47.96±1.18^{aD}$	$53.28±3.51^{aB}$
	8	$33.80±2.74^{aB}$	$57.57±11.55^{aA}$	$80.59±8.82^{aC}$	$30.87±2.97^{bC}$
	12	$11.13±0.99^{aA}$	$20.31±2.04^{aA}$	$178.31±11.41^{aA}$	$94.94±9.78^{aA}$
	16	$11.81±3.40^{aA}$	$24.11±3.95^{aA}$	$129.40±10.11^{bB}$	$19.33±1.99^{cC}$

Plants face dynamic environment changes imposed by abiotic and biotic stresses that can ultimately cause an increase in the production of reactive oxygen species (ROS).

One of the strategies used by plants to mitigate the possible damage to cellular structures caused by ROS is the activation of the antioxidant system, which confers enhanced tolerance to different stresses (Laxa et al., 2019). Thus, it is clear that plants growing at the 16/8h photoperiod showed greater accumulation of antioxidant-like metabolites than plants growing at the 12/12h and 15/9h photoperiods, which might suggest that long-day photoperiods might have induced the accumulation of higher amounts of reactive oxygen species. ROS formation in plant cells in response to light availability is somehow controversial. On one hand, it is widely accepted that increasing light availability may lead to higher ROS production and downstream signaling events. On the other hand, it seems that photoperiod-regulated ROS production depends on the number of hours per day that light is perceived by the plants (Krasensky-Wrzaczek and Kangasjärvi, 2018). Chloroplasts, mitochondria, peroxisomes, apoplast and plasma membranes are the primary sites for generating cellular ROS, but chloroplasts are the main sites for the production of ROS. In this sense, it is possible to establish a relationship between long-day photoperiods with greater generation of ROS and consequently greater activity of the plant's antioxidant system (Hasanuzzaman et al., 2020), which seems to be effective in promoting the accumulation of SGs at 16/8h condition. Under moderate light stress, plants scavenging system are able to maintain low levels of ROS, which in turn might trigger adaptative and tolerance responses. Severe light stress, however, affects the scavenging system compromising the effective elimination of ROS. It is believed that high light availability produces great amounts of singlet oxygen $^1O_2$, causing oxidative modification of PSII proteins. Additionally, greater accumulation of ROS due to high light availability may cause inhibition of *de novo* protein synthesis (Pospíšil, 2016)**.**

Total phenolic content of the leaf extracts varied from 37.98 ± 1.44 to 178.31 ± 11.41 mgGAE.g^{-1} of dry weight, whereas for the stem extracts it varied from 21.74 ± 3.42 to 104.46 ± 7.80 mgGAE.g^{-1} of dry weight (Table 2). Total phenolic content of the leaf extracts increased with the increasing length of exposure to the different photoperiods. It seems, however, that leaf extracts of plants growing at the 15/9h and 16/8h photoperiods showed greater total phenolic content after 12 days as compared to the other photoperiod condition and length of exposure. For the stem extracts, however, plants growing at the 15/9h photoperiod showed greater total phenolic content after 12 days as compared to the other photoperiod conditions and length of exposure. As a

matter of fact, the stem of plants growing at the 16/8h photoperiod condition showed very low total phenolic content in counterpart to higher proportion of contents in leaves (Table 2).

Phenolic compounds are secondary involved in several physiological processes including pollination, coloring for camouflage and defense against herbivores, antibacterial and antifungal activities, and the plant's antioxidant defense response system (de la Rosa et al., 2018; Edreva et al., 2008; Lin et al., 2016). Phenolic compounds are antioxidant-like molecules that can protect cellular structures against oxidative damage, thus playing great importance for the physiology of the plant (Lin et al., 2016). The biosynthesis and accumulation of phenolic compounds are vastly responsive to light. In soybean, ten phenolic compounds were highly correlated with the light spectra, culminating in the production of phenolic compound-enriched soybean microgreens with high nutritional quality (Zhang et al., 2019). In *Hypericum perforatum*, phenolic acids, flavonols, flavan-3-ols, and xanthones were accumulated in hairy roots upon light treatment as compared to hairy roots grown in the dark (Tusevski et al., 2013). Taken together, it seems that long-day photoperiods induce the generation of ROS to a higher extent in stevia plants as compared to short-day photoperiods, but their deleterious effect is mitigated by the enhanced production of phenolic compounds with antioxidant properties.

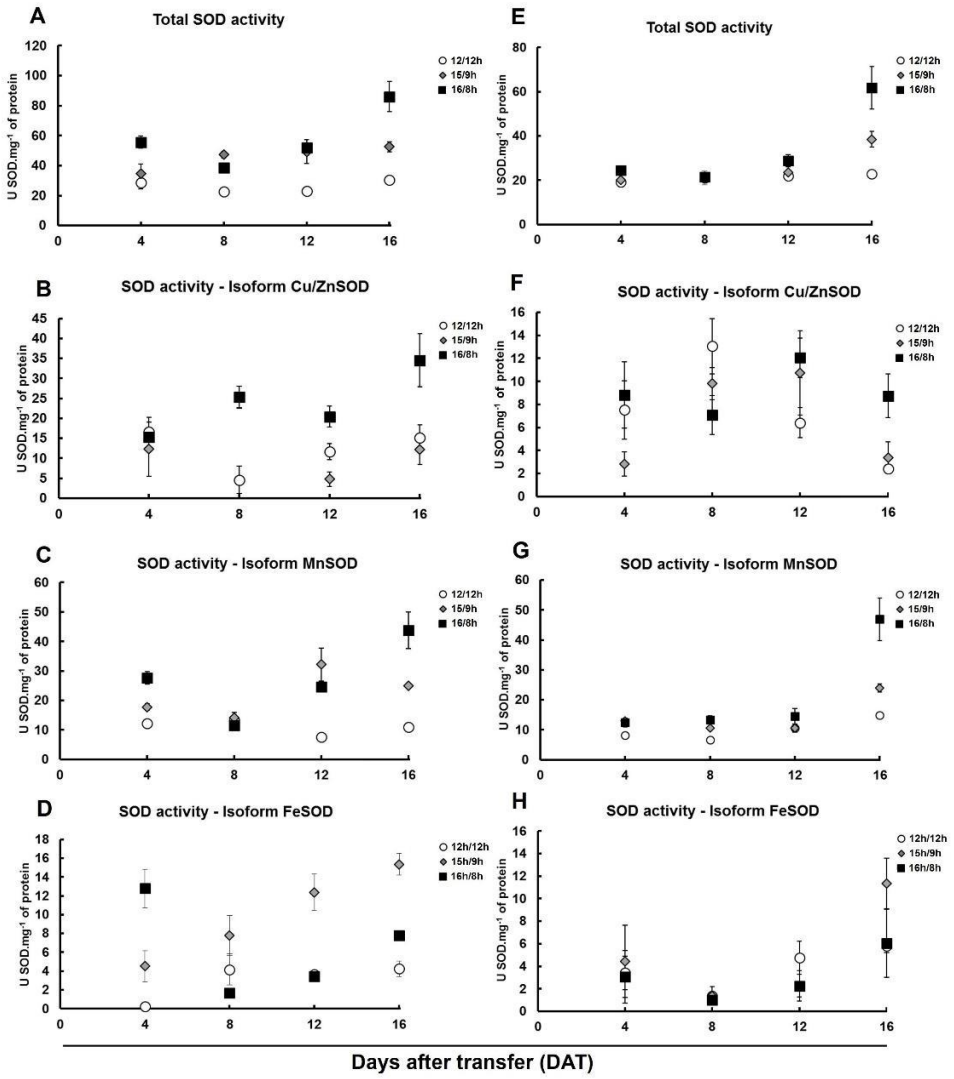
3.4. Superoxide dismutase (SOD) enzyme activity and their isoforms

Plants have an elaborate defense system against environmental adversities that can enhance the levels of reactive oxygen species (ROS) (Asensio et al., 2012; Feng et al., 2015; Pilon et al., 2011; Wang et al., 2004). Superoxide dismutase (SOD) is an enzyme that plays an important role in the defense against ROS-mediated toxicity, so it is suggested that SOD acts in the acquisition of stress tolerance by plants (Bela et al., 2017). The dismutation of the superoxide radical in hydrogen peroxide and oxygen catalyzed by SOD is the first barrier employed by plants to prevent damages of cellular structures. Therefore, the ability to eliminate superoxide is very important for plants to overcome challenging environmental conditions (Stephenie et al., 2020).

In this study, total SOD activity of the leaf extracts varied from 22.09 ± 1.14 to 86.05 ± 9.94 U SOD.mg^{-1} of protein, whereas for the stem extracts it varied from 19.22 ± 1.52

to 61.72 ± 9.64 U SOD.mg^{-1} of protein (Figure 2). Total SOD activity of the leaf and stem extracts showed a very peculiar pattern in response to the increasing length of exposure to these different photoperiods. Initially, plants growing at the 12/12h photoperiod showed basal and unresponsive SOD activity for both leaf and stem extracts. Thus, it seems that plants growing at the 12/12h photoperiod condition perceive it as a non-stress condition.

Figure 2. SOD activity and your isoforms in *Stevia rebaudiana* (Bert.) Bertoni extracts in response to different photoperiods and developmental stages. (A) Leaf; (B) Stem. The lower-case letters compare each photoperiod within each collection time and the upper-case letters compare the collections within each photoperiod according to the HSD de Tukey (P<0.05) (Supplementary tables 2 and 3).



Plants growing at the 15/9h and the 16/8h photoperiod conditions showed increased total SOD activity with the increasing length of exposure for both leaf and stem extracts (Figure 2). It seems, however, that leaves are faster to respond to different photoperiods than stem, since even at only four days after transfer, the leaf extract showed increased total SOD activity for 16/8h photoperiod, whereas for the stem extracts increased total SOD activity was only observed at least 12 days after transfer. Nevertheless, 16 DAT plants growing at the 16/8h photoperiod condition showed increased total SOD activity than plants growing at 15/9h, which in turn showed increased total SOD activity than plants growing at 12/12h. It seems that the main contributors for total SOD activity of plants growing at the 16/8h photoperiod condition were Cu/Zn and Mn SOD isoforms, whereas the main contributor for total SOD activity of plants growing at the 15/9h photoperiod condition was the Fe-SOD isoform. Abiotic stresses can cause changes in the regulation of the expression of SOD isoforms, mainly Fe-SOD and Cu/Zn-SOD (Pilon et al., 2011). As discussed in the previous section, abnormal generation of ROS in organisms may cause the interruption of homeostasis leading to stress oxidative. Thus, the antioxidant system is constantly acting to maintain balance. In addition to small antioxidant-like molecules, such as phenolic compounds, antioxidant enzymes are considered effective biochemical markers for the relationship between abiotic stresses and ROS generation (Sharma et al., 2012).

Stevia plants submitted to salinity stress showed increased activity of antioxidant enzymes such as ascorbate peroxidase (APX), phenol peroxidase (POX), catalase (CAT), and superoxide dismutase (SOD) after 16 days of exposure to the stress. Light availability and the type of light can have different effects on stevia plants, leading to the generation of ROS (Ye et al., 2017). The use of LED light, for example, can influence SOD activity in different ways. It was observed that red LED light can promote an increase in the enzymatic activity of SOD, whereas blue LED light decreased the activity of this enzyme (Simlat et al., 2016). As observed in our studies, the most responsive SOD isoforms were Cu/Zn and Mn-SOD, which could be related to their subcellular location. Mn-SOD isoform is mainly present in the peroxisome and mitochondria, Cu/Zn-SOD is mainly present in the cell wall, chloroplasts, peroxisomes and cytosol, whereas Fe-SOD is only found in the chloroplast (Arora and Bhatla, 2015). Thus, it seems that SOD isoforms act synergically with the phenolic compounds to prevent possible damages caused by ROS that are produced in plants growing at long-day photoperiods.

3.5. Antimicrobial activity

In general, leaf extracts showed more promising antimicrobial activity than the stem extracts (Table 3). Interestingly, the antimicrobial activity showed a strong specificity in response to different photoperiods and length of exposure to these different photoperiods. Plants growing at 15/9h photoperiod showed far greater antimicrobial activity than plants growing at the 12/12h and the 16/8h photoperiods. Leaf extracts from plants growing at the 15/9h photoperiod condition showed antimicrobial activity against *Bacillus cereus* at all sampling points, with MIC of 500 $\mu g.mL^{-1}$. They also showed antimicrobial activity against *Staphylococcus aureus* at all sampling points, with MIC varying from 250 to 500 $\mu g.mL^{-1}$, and antimicrobial activity against *Candida albicans* only after four and eight days after transfer, with MIC of 500 $\mu g.mL^{-1}$. None of the stem extracts obtained from plants growing at 15/9h photoperiod showed antimicrobial activity.

Table 3. Antimicrobial activity in extracts of leaves and stems of *Stevia rebaudiana* (Bert.) Bertoni plants grown under different photoperiods and stages of development.

Photoperiod (hours light/dark)	Days after transfer (DAT)	*Bacillus cereus* [$\mu g.mL^{-1}$]		*Staphylococcus aureus* [$\mu g.mL^{-1}$]		*Candida albicans* [$\mu g.mL^{-1}$]	
		Leaf	Stem	Leaf	Stem	Leaf	Stem
12/12	4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	8	n.a.	n.a.	n.a.	n.a.	500	n.a.
	12	n.a.	n.a.	n.a.	125	n.a.	n.a.
	16	500	n.a.	500	n.a.	500	n.a.
15/9	4	500	n.a.	500	n.a.	500	n.a.
	8	500	n.a.	250	n.a.	500	n.a.
	12	500	n.a.	500	n.a.	n.a.	n.a.
	16	500	n.a.	250	n.a.	n.a.	n.a.
16/8	4	500	n.a.	250	n.a.	n.a.	n.a.
	8	n.a.	n.a.	n.a.	n.a.	n.a.	500
	12	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	16	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Gentamicin		32.5	32.5	32.5	32.5	-	-
Ciclopirox olamine		-	-	-	-	75	75

Leaf extracts from 16 DAT plants growing at the 12/12h photoperiod showed antimicrobial activity against *B. cereus*, *S. aureus*, and *C. albicans*, with MIC of 500

µg.mL^{-1}, whereas 8 DAT plants showed antimicrobial activity against *C. albicans*, with MIC of 500 µg.mL^{-1}. Only stem extracts obtained from 12 DAT plants growing at the 12/12h photoperiod showed antimicrobial activity against *S. aureus*, with MIC of 125 µg.mL^{-1}.

Leaf extracts from 4 DAT plants growing at the 16/8h photoperiod only showed antimicrobial activity against *B. cereus*, with MIC of 500 µg.mL^{-1} and against *S. aureus* after 4 days of exposure, with MIC of 250 µg. mL^{-1}. Stem extracts from 8 DAT plants growing at the 16/8h photoperiod condition only showed antimicrobial activity against *C. albicans*, with MIC of 500 µg.mL^{-1}. All MIC values shown correspond to the bacteriostatic and fungistatic activity, since no single extract showed bactericidal or fungicide activity after subculture on nutrient agar.

Stevia extracts are well known for their antibacterial activity against *Bacillus subtilis*, *Vibrio mimicust*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Shigella dysenteriae* (Siddique et al., 2014). The antimicrobial activity observed for leaf samples can be justified by the presence that steviol glycosides, flavonoids and polyphenols (Puri and Sharma, 2011). Lemus-Mondaca et al. (2018) also observed the activity of stevia leaf extracts against the growth of *Listeria innocua*. However, the antimicrobial properties of stevia extracts acted beyond growth inhibition; alcoholic extracts were effective in inhibiting the growth of *Borrelia burgdoferi* biofilms (Theophilus et al., 2015). Our findings demonstrate the potential of stevia plants as a natural source of antimicrobials, expanding their industrial application spectrum. Taken together, our results suggest that plants growing at the 15/9h photoperiod condition showed the most promising antimicrobial activity. This could be of pivotal importance to optimize the production of functional bioactive compounds in stevia plants.

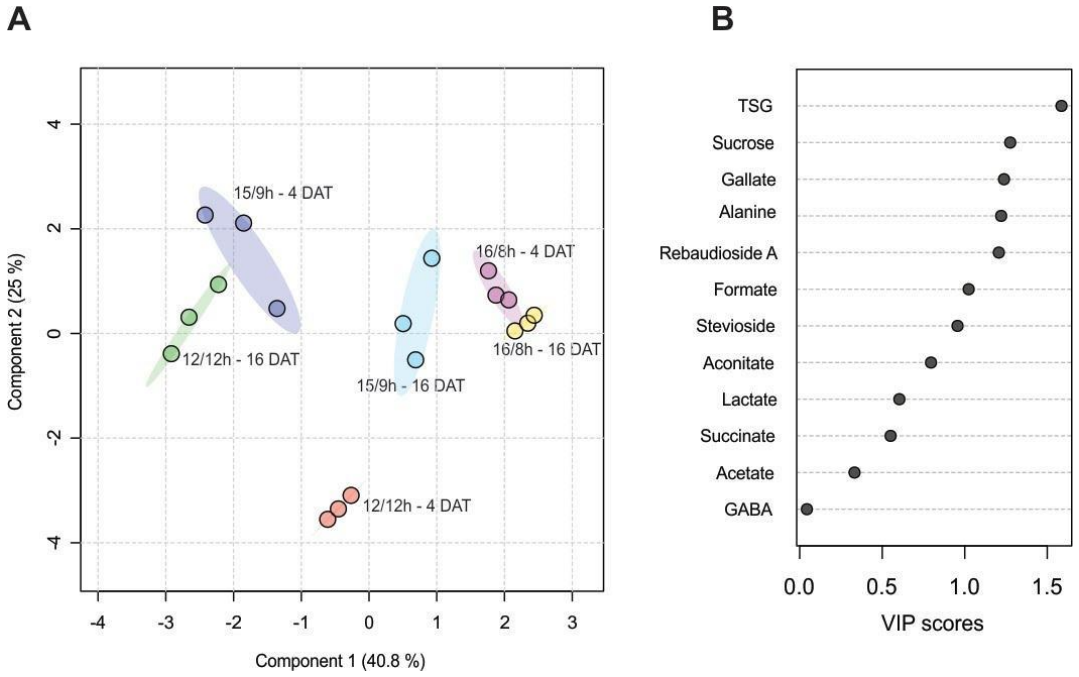
3.6. Metabolite profiling

Since the most remarkable biochemical (antioxidant, total phenolics and SOD activity) and physiological differences were observed after at least 8 days of exposure to different photoperiods, we selected 4 and 16 DAT plants for metabolite profiling purposes. Our hypothesis is that these two sampling points will maximize the differences in the stevia metabolome providing a clear response to the different photoperiods.

We used an NMR-based metabolite profiling approach to assess the chemical composition of stevia leaf and stem extracts, focusing on their change in response to different photoperiods and length of exposure to these different photoperiods. In total, 16 metabolites were identified amongst leaf and stem extracts, which encompassed three amino acids (alanine, aspartate, and valine), six organic acids (acetate, aconite, γ-aminobutyric acid, lactate, succinate, and formate), two SGs (stevioside, rebaudioside A), two phenolic compounds (gallate and kaempferol-3-Op-glucopyranosyl-7-Oa-ramnopyranoside), along with choline, glycerol, and sucrose (Supplementary table 1). Additionally, total content of SGs was also determined. Sucrose was exclusively identified in leaf extracts, whereas valine, aspartate, choline, glycerol and kaempferol-3-Op-glucopyranosyl-7-Oa-ramnopyranoside were exclusively identified in the stem extracts.

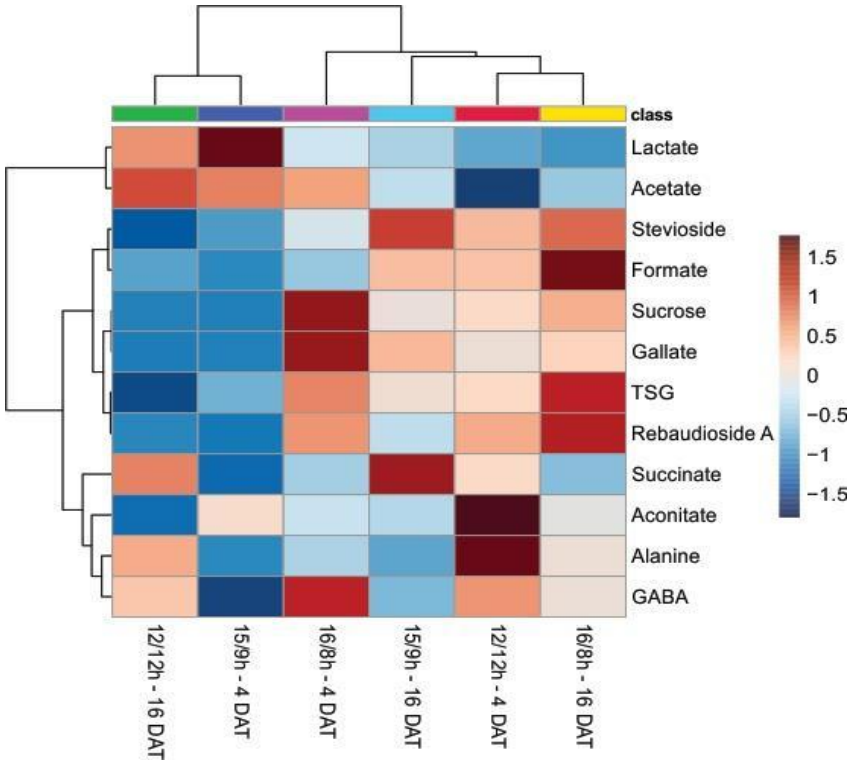
Initially, partial least squares discriminant analysis (PLS-DA) was applied to the whole dataset to evaluate changes in the leaf metabolome in response to different photoperiods and the length of exposure to these different photoperiods (Figure 3A). Principal component 1 (PC1) explained 40.8% of the total variation, whereas principal component 2 (PC2) explained 25.0%. The goodness of fit (R2 = 0.91) and the predictability of the model (Q2 = 0.85) values suggest that this is a PLS-DA model very strong predictive power that allowed us to extract metabolite changes from the dataset (Supplementary Figure 1). In the PLS-DA plot, we can use the concept of "*proximity means similarity*" to hypothesize that plants growing at the 16/8h and 12/12h photoperiod conditions showed the most differences in terms of metabolome. Interestingly, 4 DAT plants growing at the 12/12h photoperiod showed the most distinct metabolome, whereas 16 DAT plants growing at the 12/12h photoperiod seems to have a similar metabolome to 4 DAT plants growing at the 15/9h photoperiod. Similarly, 16 DAT plants growing at the 15/9h photoperiod seems to have a similar metabolome to those plants growing at the 16/8h photoperiod. Therefore, it seems that not only the different photoperiods are affecting the leaf metabolome, but also the length of exposure to these different photoperiods might also exert considerable influence on the leaf metabolome.

Figure 3. NMR and metabolite profile of leaf extracts from plants of *Stevia rebaudiana* (Bert.) Bertoni grown under different photoperiods and stage of development. (A) PLS-DA and (B) VIP scores.



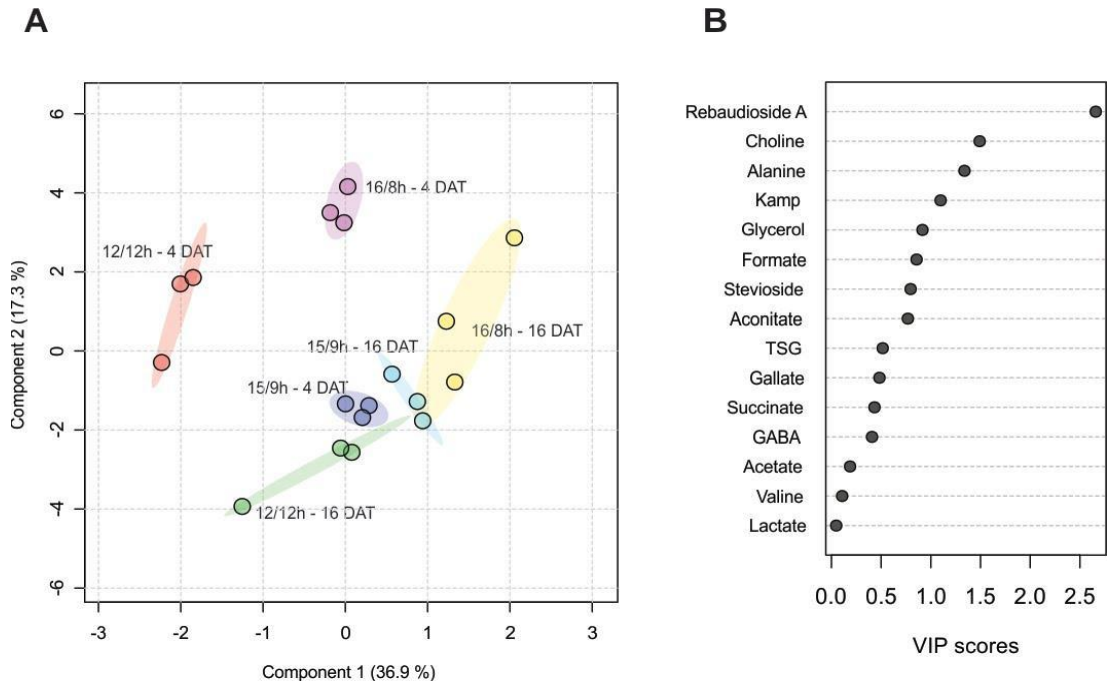
Variable Importance in Projection (VIP) scores were used to assess the contribution of each metabolite in the separation of the groups in the PLS-DA analysis. For that, we set the VIP threshold as one, and five metabolites reached this threshold (Figure 3B). That included formate, rebaudioside A, alanine, gallate, sucrose, along with total SG content. Formate showed the highest levels in 16 DAT plants growing at the 16/8h photoperiod condition, alanine showed the highest levels in 4 DAT plants growing at the 12/12h photoperiod condition, whereas gallate and sucrose showed the highest levels in 4 DAT plants growing at the 16/8h photoperiod condition. The highest content of rebaudioside A and total SG were observed at plants growing at the 16/8h photoperiod condition, after both 4 or 16 days of exposure (Figure 4).

Figure 4. Hierarchical cluster analysis. Representation of the heat map of the correlations between the metabolites identified in samples of *Stevia rebaudiana* (Bert.) Bertoni leaves in response to different photoperiods and stages of development. Correlation coefficients were calculated based on Pearson's correlation.



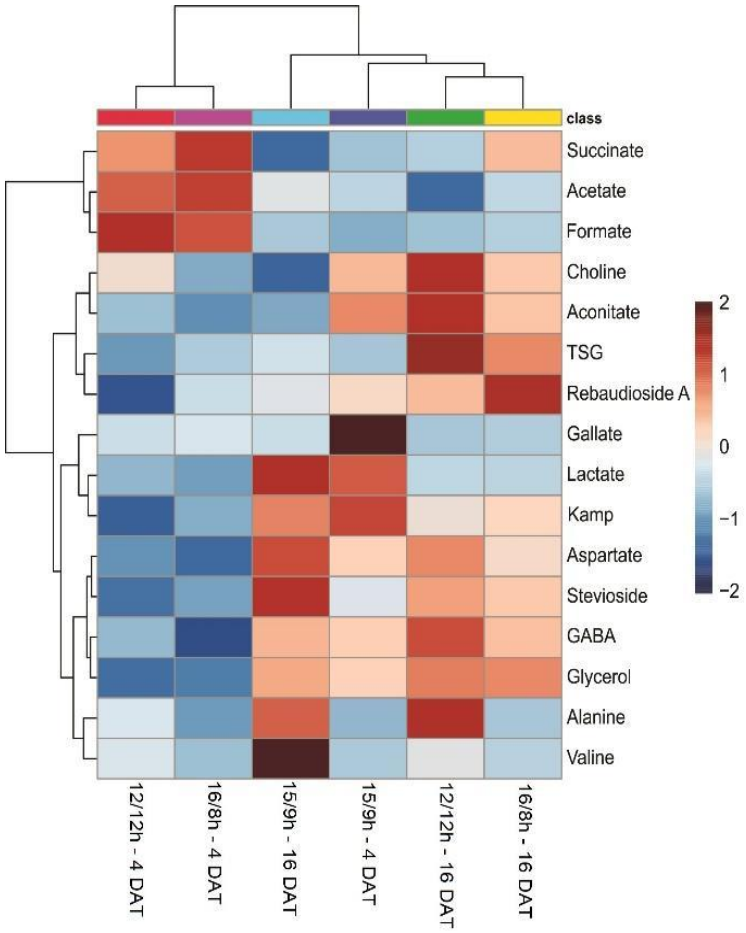
Subsequently, partial least squares discriminant analysis (PLS-DA) was applied to the whole dataset to evaluate changes in the stem metabolome in response to different photoperiods and length of exposure to these different photoperiods (Figure 5A). Principal component 1 (PC1) explained 36.9% of the total variation, whereas principal component 2 (PC2) explained 17.3%. The goodness of fit (R2 = 0.86) and the predictability of the model (Q2 = 0.70) values suggest that this is a PLS-DA model with very strong predictive power that allowed us to extract metabolite changes from the dataset (Supplementary Figure 2). However, a less clear phenotype is present for the stem extracts than for the leaf extracts. It seems that 16 DAT plants growing at all three photoperiod conditions converged to a more uniform metabolome, whereas 4 DAT plants growing at the 16/8h and 12/12h photoperiods showed a more distinct metabolome than the other samples. Therefore, it seems that the length of exposure to these different photoperiods is the main influence on the stem metabolome.

Figure 5. NMR and metabolite profile of stem extracts from plants of *Stevia rebaudiana* (Bert.) Bertoni grown under different photoperiods and stages of development. (A) PLS-DA; (B) VIP scores.



Variable Importance in Projection (VIP) scores indicates that four metabolites are the main responsible for the separation in the PLS-DA plot (Figure 5B). This included rebaudioside A, choline, alanine, and kaempferol-3-Op-glucopyranosyl-7-Oa-ramnopyranoside. Rebaudioside A and choline showed the highest levels in 16 DAT plants growing at the 12/12h and 16/8h photoperiods, alanine showed the highest levels in 16 DAT plants growing at all three photoperiods, whereas kaempferol-3-Op-glucopyranosyl-7-Oa-ramnopyranoside showed the highest levels in 4 and 16 DAT plants growing at the 15/9h photoperiod. Similarly, to the leaf extracts, rebaudioside A showed the highest levels in plants growing at the 16/8h photoperiod condition (Figure 6). It seems, therefore, that this is the best photoperiod condition to maximize rebaudioside A and total SG content, which make this extremely relevant considering that most of the industrial interest on stevia is on the content of SGs.

Figure 6. Hierarchical cluster analysis. Representation of the heat map of the correlations between the metabolites identified in samples of *Stevia rebaudiana* (Bert.) Bertoni stem in response to different photoperiods and stages of development. Correlation coefficients were calculated based on Pearson's correlationon.



The carbon skeleton used for the biosynthesis of amino acids in plants is derived from organic acids produced in glycolysis, from the oxidative pentose phosphate pathway and from the TCA and Calvin cycles (Batista-Silva et al., 2018). In general, the content of free amino acids in plants increases considerably when subjected to abiotic stress conditions. In response to specific stresses, they can act as energy donors through their catabolism in the TCA cycle to support protein synthesis and as precursors of multiple secondary metabolites (Batista-Silva et al., 2018; Galili et al., 2014).

Branched-chain amino acids (BCAAs), such as valine, play important roles in the plant’s growth and defense systems, which may justify the high values observed for stem samples in different photoperiods (Xing and Last, 2017). Additionally, degradation

of BCAA provides energy for plants during periods of prolonged darkness, early stages of germination and final stages of senescence (Gipson et al., 2018).

Alanine plays important roles in plant physiology and metabolism as a defense compound that allows plants to resist various stresses such as hypoxia, flooding and drought, and indirectly as a precursor to the compounds pantothenate and CoA, which are involved in a variety of functions. In addition, alanine is converted to beta-alanine betaine, which has additional protective functions related to salt tolerance, whereas it can also be converted to homoglutathione, which can be critical for nitrogen fixation (Gipson et al., 2018).

In plants, organic acids are associated with various functions, such as energy production, carbon storage, stomatal conductance, and amino acid biosynthesis, which allow plants to deal with excess cations, changes in osmotic conditions and low nutrient content (Vallarino and Osorio, 2019). Formate is generated as a product of reduction of CO_2 by a single pair of electrons, making it one of the simplest organic acids which can provide carbon and reducing power to the cells. It is a compound involved in the glyoxylate cycle, where condensation of two formate molecules produce glyoxylate, which is later converted to glycine and serine (Igamberdiev et al., 1999; Igamberdiev and Eprintsev, 2016; Vallarino and Osorio, 2019). In stevia plants, high levels of formate might be a strategy of the plant to fixate carbon under unfavorable conditions. However, further studies are still necessary to unravel formate role in stevia response to different photoperiods.

Choline is a vital metabolite in plants, since it is an essential element in the constitution of phosphatidylcholine, a membrane phospholipid (McNeil et al., 2001). Additionally, it is involved in the maintenance of plants' homeostasis under abiotic stress (Zhang et al., 2010), by maintaining structural integrity of cell membranes (Zeisel, 2006). Choline is also one of the main precursors during glycine betaine biosynthesis, a compound that is related to the preservation of macromolecule activity and the maintaince of the integrity of membranes against stress and eliminating ROS (Annunziata et al., 2019). Taken together, it seems that alanine, formate, and choline are synergically acting towards maintaining stevia homeostasis under long-day photoperiod conditions.

Steviol glycosides have great industrial and commercial interest due to their sweetening properties, which confers them high economic value in the healthy food market (Brandle et al., 1998b; Brandle and Rosa, 1992; Ceunen and Geuns, 2013b; Lemus-

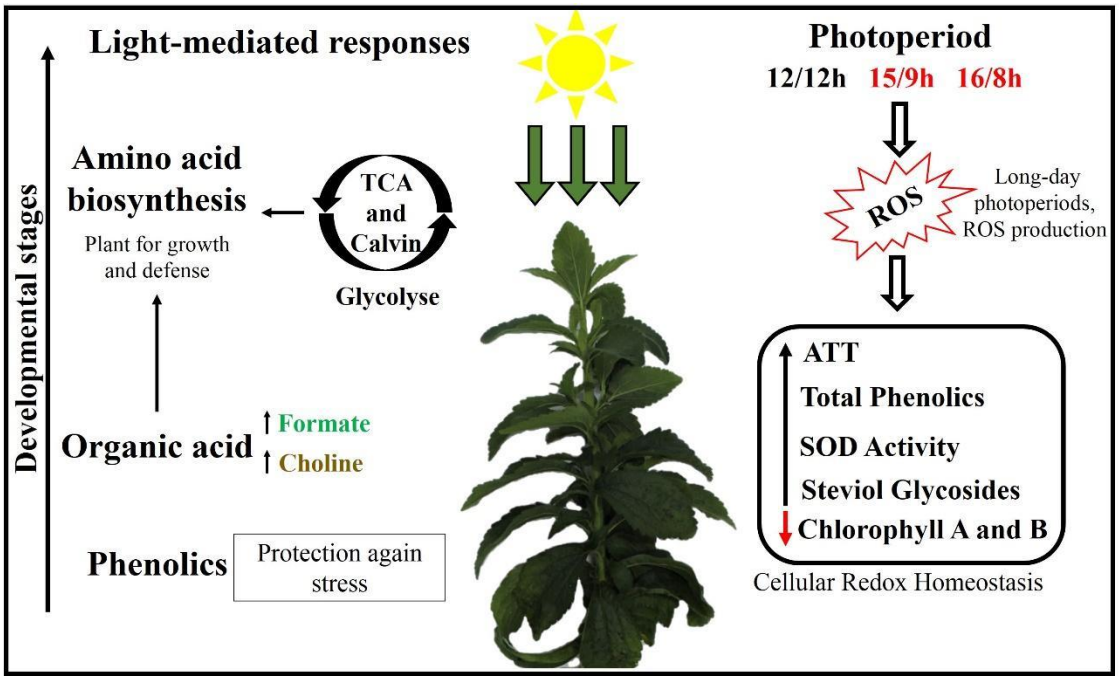
Mondaca et al., 2012; Soejarto et al., 2019). The content of compounds with sweetening characteristics were higher in leaf (stevioside, TSC and sucrose) and stem (Rebaudioside A) extracts obtained from plants growing at the 16/8h photoperiod. Steviol glycosides are also reported to exhibit the ability to act as potent ROS scavengers, where their role in the cellular antioxidant network is primarily related to the diterpene skeleton common to all glycosides (Ceunen and Geuns, 2013a; Gardana et al., 2010; Lemus-Mondaca et al., 2012; Metivier and Viana, 1979). Herein, we hypothesize that in long-day photoperiods stevia plants enhance SG biosynthesis, partially to produce GA, since they share the same biosynthetic pathway (Brandle and Rosa, 1992; Ceunen and Geuns, 2013a, 2013b; Metivier and Viana, 1979).

Phenolic compounds are present in virtually all plant tissues, such as fruits, seeds, leaves, stems, and roots. Flavonoids and simple phenolic compounds are the main responsible for the antioxidant responses of plants. Flavonoids are important secondary metabolites that protect plants against ultraviolet radiation and against insects, fungi, viruses and bacteria attacks (de la Rosa et al., 2018; Georgiev et al., 2014). Kaempferol-3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside and gallic acid are mainly known for their antioxidant properties, protecting cells against oxidative damages (de la Rosa et al., 2018). Furthermore, gallic acid has shown relevant antifungal and antiviral properties (Maruszewska and Tarasiuk, 2019). It seems that kaempferol-3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside and gallic acid are the main phenolic compounds produced in stevia plants in response to long-day photoperiods. It is important to keep in mind, however, that an HPLC-MS approach would allow the identification and quantification of other polyphenols in the extracts. In this manuscript, we chose to focus our attention on the steviol glycosides (SG), since *S. rebaudiana* industrial importance relies on the natural occurrence of high amounts of SG in its leaves. Although other polyphenols might contribute, multivariate statistical analysis of the NMR data suggest that kaempferol-3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside and gallic acid are responsible for the strong antioxidant activity of the leaf extracts from stevia plants growing at the 16/8h photoperiod condition. Taken together, it seems that alanine, formate, choline, kaempferol-3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside, and gallic acid are acting synergically towards maintaining stevia homeostasis under long-day photoperiods.

4. Conclusions

We have conducted an in-depth characterization of *Stevia rebaudiana* (Bert.) Bertoni leaf and stem physiological and biochemical responses to different photoperiods. Since stevia is a short-day species with a critical photoperiod of 12 to 13h of light, the 12/12h photoperiod favored reproductive mode (control), whereas the 15/9h and 16/8h photoperiods favored vegetative modes. Photoperiods with less than 12h of light induces unwanted precocious reproductive mode reducing the content of SGs in its leaves. Long-day photoperiods (vegetative mode) enhanced ROS production, which could be inferred by the fact that plants growing at the 16/8h photoperiod showed greater accumulation of antioxidant-like metabolites and SOD activity than plants growing at the 12/12h and 15/9h photoperiod conditions (Figure 7).

Figure 7. Proposed schematic representation of *Stevia rebaudiana* (Bert.) Bertoni physiological and biochemical changes in response to different photoperiods.



Antioxidant-like metabolites were characterized by NMR and it was shown that alanine, formate, choline, kaempferol-3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside, and gallic acid are acting synergically to mitigate possible damages caused by ROS that are produced in plants growing at long-day photoperiods. Our study also demonstrates that different photoperiods might induce the biosynthesis of metabolites with industrial

interest. Plants growing at the 15/9h photoperiod condition showed the most promising antimicrobial activity, whereas the 16/8h photoperiod seems to be the best condition to maximize rebaudioside A and total SG content (Figure 7). These findings could be of pivotal importance to optimize the production of bioactive compounds in stevia plants, considering the fact that most of the industrial interest on stevia lays on the content of SGs.

5. Conflict of interest

The authors declare no conflict of interest.

6. Acknowledgement

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Supplementary Material

Material and Methods

DPPH radical scavenging assay and total phenolic content

The extracts were prepared from 0.01 g of the samples that were macerated with methanol PA by using a Dismembrator device (Sartorius, Mod. Mikro-Dismembrator S) for 5 min at 2.000 rpm. The extract was centrifuged and the supernatant collected for further analysis. To assess the antioxidant activity, 500 µL of a DPPH solution (120 mmol L^{-1}; ethanol) was added to 500 µL of each extract to provide 1 mL of the reaction mixture with final extract concentrations ranging from 3 to 1102.5 µg mL^{-1}. After 30 minutes of reaction, absorbance was measured at 515 nm. Ethanol was used as a blank solution and DPPH (1.0 mL, 120 mmol L^{-1}) plus ethanol (1.0 mL) was used as a negative control (without expected DPPH consumption). Experiments were performed in triplicate and the results were expressed as IC_{50}.

To assess total phenolic content, an aliquot of 50 µL of the extract was mixed with 800 µL of water, with the subsequent addition of 50 µL of Folin-Ciocalteu phenol reagent. After one minute, 100 µL of sodium carbonate solution (Na_2CO_3, 7.5% w/v) was added to the mixture to a final volume of 1 mL. The reaction mixture was kept in the dark for 120 min. After that time, the absorbance of each reaction was read at 725 nm (Varian Cary 50 Microplate Reader). Gallic acid was used in the construction of a standard curve to calculate the total phenolic content in the extracts and the results were expressed in mgGAE.g^{-1} of dry extract.

Superoxide Dismutase (EC 1.15.1.1) activity and isoforms

The detection SOD activity is based on the quantification of the nitro-tetrazolium blue (NBT) reduction product, a formazan. Initially, 185 µL of the reaction mixture (50 mM PPB (pH 7.8), 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA) was mixed with 15 µL of enzymatic extract from leaf and stem samples. The reaction was initiated by placing samples under a 27 W fluorescent lamp at 25 °C for 12 min. The

enzymatic activity was expressed in units of SOD activity (U) per mg of total protein. One unit of SOD activity is expressed as the amount of enzyme needed to cause 50% inhibition of NBT reduction under experimental conditions.

The activity of different SOD isoforms (Mn-SOD, Cu/Zn-SOD and Fe-SOD) was determined spectrophotometrically at 560 nm (Varian Microplate Reader - Cary 50 MPR) following the prerogatives mentioned for the total SOD activity. However, the extracts were incubated for 30 min at 4º C in 5 mM H_2O_2, to inhibit the Cu/Zn-SOD and Fe-SOD isoforms, and in 2mM NaCN to inhibit Cu/Zn-SOD (Ribeiro et al., 2015).

Antimicrobial activity

Nutrient broth (Acumedia, USA) and malt extract (Acumedia, USA) were used as a culture medium for bacterial and fungal growth, respectively. Chloramphenicol (0.19–25 $\mu g.mL^{-1}$), gentamicin (0.039 - 5 $\mu g.mL^{-1}$) and cyclopyroxolamine (0.39–50 $\mu g.mL^{-1}$) were used as positive controls. The extracts were dissolved in 20% DMSO (v/v). DMSO was also used as a negative control. After serial dilution, the concentration of the extracts ranged from 3.90 to 500 $\mu g.mL^{-1}$. The 96-well microplates were incubated at 36° C (24h) and 26° C (72h) for bacterial and fungal growth, respectively. The evaluation of the minimum inhibitory concentration (MIC) was determined by the visualization of turbidity in the wells. The bioactive activity of the extracts was evaluated against the following microorganisms *Bacillus cereus* Frankland & Frankland (nº CCT 0096), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 94863), *Pseudomonas aeruginosa* (nº CCT 0090; ATCC 27853), *Candida albicans* (ATCC 18804) and *Fusarium oxysporum*. All analyzes were performed in triplicate. The MIC was determined by the absence of turbidity in the wells and the extracts were considered active when they inhibited microbial growth at concentrations below or equal to 500 $\mu g.mL^{-1}$. Of the wells that showed no turbidity, 10 µL of the contents were inoculated into broth. solid nutrient or malt extract to assess whether the observed activity was microbiostatic or microbiocidal.

Supplementary Table 1. Metabolites found in samples of leaf and stem of *S. rebaudiana*, with identification of signal, multiplicity, and value of J.

Metabolite	δH (J in Hz)	Referência
Valine	1.07 (d, 7.2)	(d, 7.0 Hz) Brito et al. (2020); (d, 6.8 Hz) Kim, Choi e Verpoorte (2010).
Lactate	1.34 (d, 6.4)	(d, 6.4 Hz) Brito et al. (2020); (d, 6.90) Boffo et al. (2012).
Alanine	1.48 (d, 7.2)	(d, 7.2 Hz) Brito et al. (2020); (d, 7.2 Hz) Kim, Choi e Verpoorte (2010).
Acetate	1.90 (s)	Brito et al. (2020);
Succinate	2.40 (s)	Brito et al. (2020); Augustijnet al. (2016);
Aspartate	2.77 (dd, 5.6, 5.6)	Augustijn et al., (2016)
Acid γ aminobutíric (GABA)	3.00 (t, 7.6)	(t,7.4 e 7.2 Hz) Kim, Choi e Verpoorte (2010)
Choline	3.03 (s)	Augustijn et al. (2016); Kim, Choi e Verpoorte (2010)
Glycerol	3.67 (dd, 4.4, 6.0)	
TSC	4.61 (d, 8)	(d, 8.0) Boffo et al. (2012); (d, 7.8 Hz) Kim, Choi e Verpoorte (2010).
Stevioside	5.20 (d, 3.6); 5.60 (t, 2.0)	Pieri et al. (2011); Tada et al. (2012)
Sucrose	5.24 (d, 3.6)	(d, 3.6 Hz) Brito et al. (2020)
Rebaudioside A	5.40 (d, 3.6)	Pieri et al., (2011); Tada et al., (2012)
Aconitate	6.52 (s)	Yuan et al., (2014)
Kaempferol 3-O-b-D-glucopyranoside-7-O-α-L-rhamnopyranoside	6.88 (d, 8.0)	(d, 8.0 Hz) Souza et al. (2014)
Galatte	7.02 (s)	Yuan et al. (2014)
Formate	8.46 (s)	Brito et al. (2020)

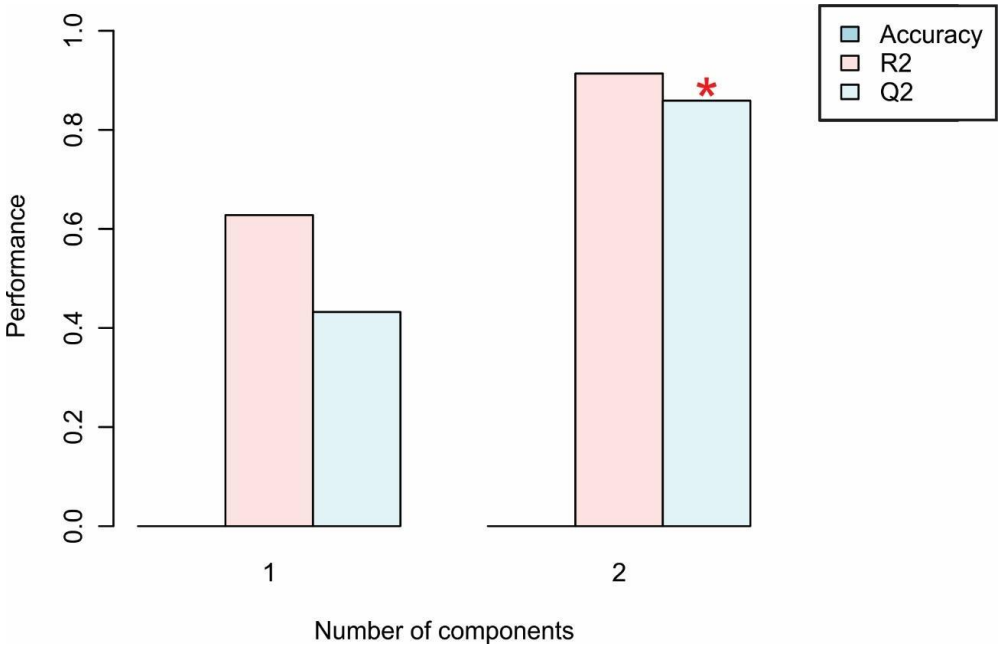
Supplementary Table 2. SOD activity in *Stevia rebaudiana* (Bert.) Bertoni leaf extract grown under different photoperiodic regimes.

Photoperiod (hours light/dark)	Days after transfer (DAT)	Total SOD [U SOD.mg^{-1} of protein]	Cu/Zn-SOD [U SOD.mg^{-1} of protein]	Mn-SOD [U SOD.mg^{-1} of protein]	Fe-SOD [U SOD.mg^{-1} of protein]
12/12	4	29.06±4.20^{bA}	16.58±3.69^{aA}	12.26±0.67^{cA}	0.22±0.08^{cA}
	8	22.09±1.14^{bA}	4.58±3.39^{bB}	13.33±0.95^{aA}	4.17±1.64^{bA}
	12	22.84±1.78^{bA}	11.65±2.05^{abAB}	7.51±0.69^{cA}	3.68±0.18^{bA}
	16	30.42±2.46^{cA}	15.18±3.14^{bA}	11.00±0.27^{cA}	4.24±0.83^{cA}
15/9	4	34.38±6.44^{bB}	12.16±6.72^{aB}	17.71±1.15^{bC}	4.51±1.67^{bC}
	8	47.29±2.29^{aA}	25.40±2.67^{aA}	14.10±1.87^{aC}	7.80±2.11^{aC}
	12	49.30±8.07^{aA}	4.75±1.77^{bB}	32.16±5.55^{aA}	12.39±1.95^{aA}
	16	52.54±3.38^{bA}	12.24±3.76^{bB}	24.95±0.63^{bB}	15.35±1.16^{aB}
16/8	4	55.66±4.06^{aB}	15.28±0.27^{aC}	27.59±2.14^{aB}	12.79±2.04^{aB}
	8	38.50±2.02^{aC}	25.32±2.77^{aB}	11.51±0.87^{aC}	1.67±0.24^{bC}
	12	51.84±3.13^{aB}	12.87±1.92^{aC}	24.59±1.11^{bB}	14.38±3.35^{aB}
	16	86.05±9.94^{aA}	34.54±6.65^{aA}	43.74±6.21^{aA}	7.77±0.30^{bA}

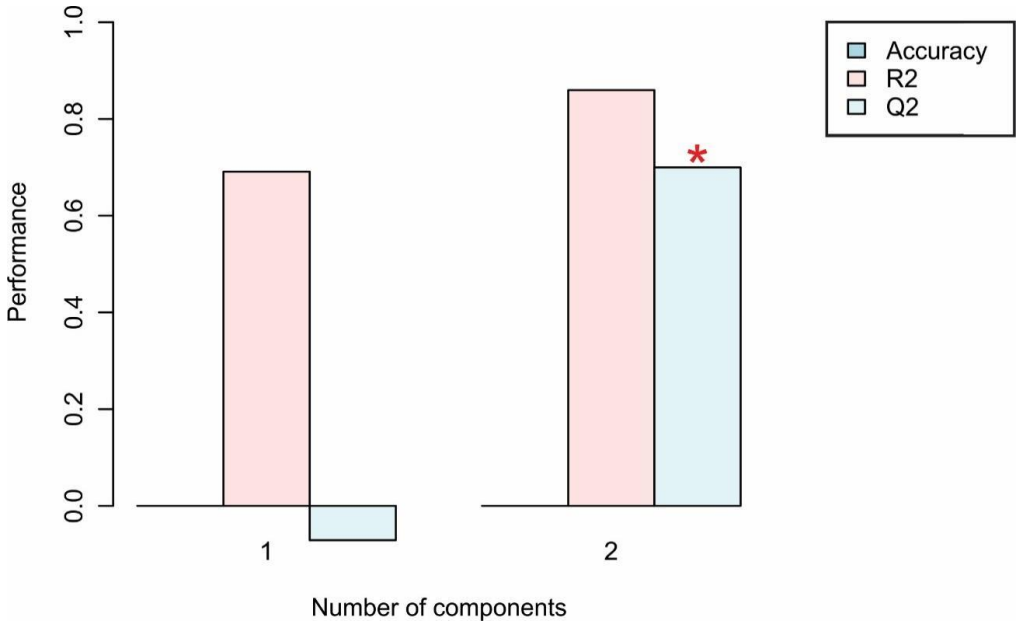
Supplementary Table 3. SOD activity in *Stevia rebaudiana* (Bert.) Bertoni stem extract grown under different photoperiodic regimes.

Photoperiod (hours light/dark)	Days after transfer (DAT)	Total SOD [U SOD.mg^{-1} of protein]	Cu/Zn-SOD [U SOD.mg^{-1} of protein]	Mn-SOD [U SOD.mg^{-1} of protein]	Fe-SOD [U SOD.mg^{-1} of protein]
12/12	4	19.22±1.52^{aA}	7.51±2.56^{aB}	8.18±0.45^{aB}	3.52±1.45^{aAB}
	8	21.02±3.04^{aA}	13.05±2.41^{aA}	6.61±0.77^{bB}	1.35±0.79^{aB}
	12	21.90±0.98^{aA}	6.41±1.30^{bBC}	10.60±0.94^{aAB}	4.89±1.46a^{AB}
	16	22.85±1.15^{cA}	2.39±0.39^{bC}	14.79±0.66^{cA}	5.67±0.57^{bA}
15/9	4	20.18±3.39^{aB}	2.81±1.05^{bB}	13.03±0.52^{aB}	4.34±3.12^{aB}
	8	21.74±1.06^{aB}	9.81±1.40^{abA}	10.61±0.29^{abB}	1.32±0.20^{aB}
	12	23.50±2.60^{aB}	10.74±3.66^{aA}	10.54±1.35^{aB}	2.22±0.99^{aB}
	16	38.41±3.55^{bA}	3.38±1.34^{bB}	23.95±1.42^{bA}	11.08±2.22^{aA}
16/8	4	24.38±1.85^{aB}	8.81±2.88^{aAB}	12.43±1.46^{aB}	3.14±2.20^{aAB}
	8	21.44±1.90^{aB}	7.07±1.70^{bB}	13.40±1.26^{aB}	0.97±0.25^{aB}
	12	28.78±2.85^{aB}	12.06±1.72^{aA}	14.52±2.61^{aB}	2.20±1.31^{aAB}
	16	61.72±9.64^{aA}	8.74±1.88^{aAB}	46.93±7.15^{aA}	6.05±3.05^{bA}

Supplementary Figure 1. Cross-validation based on NMR and metabolite profile of leaf extracts from plants of *Stevia rebaudiana* (Bert.) Bertoni grown under different photoperiods and stages of development.



Supplementary Figure 2. Cross-validation based on NMR and metabolite profile of stem extracts from plants of *Stevia rebaudiana* (Bert.) Bertoni grown under different photoperiods and stages of development



CAPÍTULO 3

O estágio de desenvolvimento e o fotoperíodo influenciam o conteúdo de StGlys na estévia

Developmental stage and photoperiod influence StGlys content in stevia

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Abstract

Stevia rebaudiana leaf extracts contain two important metabolites, stevioside and rebaudioside A, found in high concentrations and used as natural sweeteners because they are up to 300 times sweeter than sucrose. *S. rebaudiana* is a short-day (SD) perennial, and some studies claim that long-day (LD) treatment affects StGly accumulation. However, the optimal and detailed light regime needed to increase the total StGlys content remains debatable. We analyzed the effect of different photoperiods for *S. rebaudiana* plants at different developmental levels, to determine the optimal treatment for StGlys accumulation in leaf and stem samples. The method was standardized to achieve high efficiency, simplicity, versatility in the isolation and quantification of the main sweetening compounds. We performed the separation of the main StGlys with a total run time of 25 min. Eluents without buffer were used in the separations and only 10 mL (3.3 mL of CH3CN; 6.68 mL of H_2O and 0.2 mL of ultra-pure water) of solvent were required per analysis. A C18 stationary phase metal column (250 mm × 4.6 mm - SUPELCO Analytical, Col: 159475-06) was employed with a mobile phase composed of CH_3CN:H_2O for the excellent separation of steviol glycosides. The mobile phase flow rate was 0.4 $mL.min^{-1}$, column temperature of 22° C, with an injection volume of 10 µL. The glycosides were separated at a time of 11.604 minutes for rebaudioside A and 12.293 minutes for stevioside, the first with undesirable bitterness and the second with greater sweetening capacity. Concomitantly with the standardization of the method, we performed their quantification, where the quantification of the StGly of the samples varied among the studied botanical organs. The most abundant glycoside for the cultivar studied was stevioside for both botanical parts. For the leaf, the quantification of the main StGly, the stevioside, values that varied from 155.27 ± 7.57 to 471.78 ± 20.80 $µg.mL^{-1}$ of extract, while for samples of 22.05 ± 1 .22 to 116.04 ± 11.88 $µg.mL^{-1}$. For rebaudioside A, the values ranged from 49.30 ± 1.61 to 201.01 ± 6.73 $µg.mL^{-1}$ and from 17.88 ± 6.12 to 166.65 ± 17.00 $µg.mL^{-1}$ for leaf and stem respectively. The use of different exposures to light can favor the increase in the expression of genes related to the synthesis of StGly, being a tool of great value for the large-scale cultivation of the species.

Keywords: Luminosity, Metabolomics, Sweetening compounds, Chromatography.

1. Introduction

Stevia (*Stevia rebaudiana* Bertoni.) it's a notable plant for synthesizing, mainly in leaves, bioadoculant metabolites, steviol glycosides (StGly), which has aroused industrial interest in the species. *S. rebaudiana* is a plant belonging to the Asteraceae family, native to the Amambay region, on the border between Brazil and Paraguay. The genus Stevia includes more than 100 species found in America, however, only *S. rebaudiana* produces significant amounts StGly, which represents approximately 4 to 20% of the dry weight of the leaf and can be up to 300 times sweeter than sucrose (MYINT et al., 2020; PANDE; GUPTA, 2013).

A feature that leveraged its expansion across several countries is being characterized as an easy-to-grow plant. However, its growth and production of different glycosides can be influenced by several factors. When it comes to the production of steviol glycosides, the photoperiod is a crucial factor for the cultivation of the species because it can promote a greater production of the main metabolites of interest to the species (ANDRADE et al., 2021; CEUNEN; GEUNS, 2013a).

The identification and quantification of StGlys in stevia is an important weapon in the search for varieties with higher levels of StGlys and associated with this greater proportions of Rebaudioside A, composed with a less pronounced aftertaste (AMEER et al., 2017; MERT OZUPEK; CAVAS, 2021). When it comes to food products that contain stevia in its composition, identification and quantification becomes important to comply with the legislation in force which recommends the use of the isolated product of high purity (≥ 95%) (JECFA, 2017). It is believed that consumption of the isolated stevia product is expected to reach 10,254.93 metric tons by 2027, with annual consumption growth of around 7% and 8% (RD REPORTERS AND DATA, 2020). The expansion of the use of steviosides is mainly due to the beverage industry (PUTNIK et al., 2020).

Currently, the greatest requirement on the stevia market is the development of new varieties with characteristics suitable for obtaining good quality raw materials and consequently high levels of importance sweeteners. An important part of obtaining StGlys is their extraction and purification of plant material in a way that results in a high purity of unchanged compounds (ABDEL-AAL et al., 2021; CASTRO-MUÑOZ et

al., 2020; PERICHE et al., 2015). The extraction and quantification process involve several steps that will allow for its high performance. Dehydration or drying of plant material is necessary to prevent the growth of microorganisms and changes in biochemical characteristics. Several technologies have been applied for the extraction of StGlys, including maceration and extraction of heat, high temperature and high pressure, electrical voltage, radiation, ultrasound, and chromatographic techniques. However, such techniques are still relatively ineffective, considering some variables such as the solvent and the technique used (CASTRO-MUÑOZ et al., 2020). These low percentages and large amounts of solvents that need to be removed later in the purification process make it difficult to apply these technologies in large-scale production (CASTRO-MUÑOZ et al., 2020).

The most varied techniques can be used to identify the range of StGlys present in extracts of *S. rebaudiana*. These methods vary in their sensitivity, precision, and accuracy. These include enzymatic hydrolysis, HPTLC, capillary electrophoresis, near-infrared spectroscopy, HPLC with UV detection, UHPLC evaporative light scattering, LC-fluorimeter, MS desorption electrospray ionization, UHPLC-MS, LC-MS /MS, 1HNMR and LC in combination with hybrid quadrupole time-of-flight MS (ANDRADE et al., 2021; ARANDA-GONZÁLEZ; MOGUEL-ORDOÑEZ; BETANCUR-ANCONA, 2015; GALLO et al., 2017; GARDANA; SIMONETTI, 2018; MERT OZUPEK; CAVAS, 2021; PIERI et al., 2011; SOUFI et al., 2016; WALD; MORLOCK, 2017). However, according to FAO/WHO JECFA, the use of HPLC is considered the most effective method to estimate the steviol glycoside content (ZIMMERMANN, 2018).

The present study presents optimization data based on multilinear gradient retention times. The focus is to investigate how the retention time of stevioside and rebaudioside A is affected by extraction parameters using different solvents and by gradient elution. Thus, after optimizing the RP-HPLC-UV conditions for the identification of Stevioside and Rebaudioside A, we performed the quantification of these secondary metabolites present in extracts of stevia plants grown under different photoperiod conditions, the main area of interest for large-scale cultivation of the species.

2. Material and Methods

2.1. Plant growth and sampling

In this study, we used the cultivar IAN/VC-142/Eirete (Stevia Eirete 2) that was developed by the Instituto Paraguayo de Tecnología Agraria - IPTA. It is listed as an agricultural species in Brazil by the National Cultivar Registry (nº 35531, 04/29/2016). Plant was grown in a plant growth chamber (Panasonic/Sanyo, MLR-351H) equipped with 15 white fluorescent lamps (Mitsubishi/Osram, FL40SS-W/37) and adjusted to constant 25°C and 70% RH. Selected fertile seeds (darkish achene fruits) (GANTAIT et al., 2018) were previously germinated over germination paper moistened with 8 mL distilled water on plastic germination boxes (10 x 10 cm). These plastic germination boxes were incubated in the growth chamber for 10 days, after which the most vigorous seedlings were transplanted to plastic pots (700 mL volume, 13.7 cm high x 10 cm in diameter) filled with commercial growth substrate (Carolina Soil, pH 5.5, electrical conductivity 0.7 $mS.cm^{-2}$, maximum humidity 60% $m.m^{-1}$, dry density 130 $kg.m^{3}$, water holding capacity 350 % $m.m^{-1}$), as observed in the work of Andrade et al. (2021).

Young seedlings were initially cultivated for 60 days under constant light to allow adequate initial vegetative growth under similar conditions, ie, as the leaves are the main tissue for storing StGlys. After this period, the plants were transferred to different photoperiod conditions (12/12h, 15/9h and 16/8h of light / dark), thus allowing for greater growth and development until the appearance of the first floral buds in the plants submitted to the photoperiod inducing flowering, being these characteristics chosen as a phenotypic marker for the collection of the analyzed material (CEUNEN; GEUNS, 2013a; YADAV et al., 2011). Leaves and stems from 5 individual plants were sampled in triplicate at 4, 8, 12 and 16 days after transfer (DAT) for the different photoperiods (12/12h, 15/9h and 16/8h). All plants/individual pots were watered every other day with 50 mL of distilled water and once every 7 days with liquid fertilizer (REMO Nutrients, Grow 2-3-5 and Micro 3-0-1, 1:1000 (v/v) with 50 mL per plant / pod) until the appearance of the first flower buds.

2.2. Sample preparation

The concentrations of the main steviol glycosides (stevioside and rebaudioside A) were determined from leaves and stems collected from *S. rebaudiana* plants grown under different photoperiods (12/12h, 15/9h and 16/8h, light / dark) after 4, 8, 12 and 16 DAT.

Leaf and stem samples collected were lyophilized, macerated and frozen at -80°C for further evaluation. The extracts used were prepared from the weighing in analytical balance (Shimadzu, Model AUX 220) from 0.100 g of leaf and stem samples from different cultivation conditions, placed in microtubes (2 mL) together with a solution of methanol and ultrapure water in the proportion of 3:1 v/v (CH_3OH:H_2O). Then, the samples were ground and homogenized in the Dismembrator apparatus (Sartorius, Mod. Mikro-Dismembrator S) twice for 5 minutes at 2.000 rpm. Subsequently, successive centrifugations were performed (between 4 and 5 times) at 15.000 rpm, for 10 minutes, to separate the mixture. The supernatant was collected, the samples were then dried in a desiccator to eliminate all solvent (CH_3OH) and lyophilized to remove the water. After this step, a crystallized mass of the samples was obtained, from which the stevioside and rebaudioside A content was analyzed.

For further analysis, 0.001 g of the crystallized extract obtained from each sample was weighed on an analytical balance (SHIMADZU, Model AUX 220), which were solubilized in 1 mL of hydromethanolic solution, in the proportion of 3:1 v/v (CH_3OH:H_2O). For all different cultivation conditions samples were produced in biological triplicates.

2.3. Fractionation of steviol glycosides compounds

The main steviol glycosides analyzed, stevioside and Rebaudioside A, were analyzed with the aid of a High performance liquid chromatography (HPLC) system (SHIMADZU) was used, coupled to a DAD diode detector (Diode Array Detector) (SPD-M20A), binary pump (LC-20AD), degasser (DGU-20A3), thermostat (CTA-20A), Rheodyne Injector (SPD-M20A) and C18 steel column (250 mm × 4.6 mm) filled with stationary phase (dp = 5 μM) (SUPELCO Analytical, Col: 159475-06). Column

temperature was maintained at 22° C and UV detection was set at 202 nm. Each chromatographic run was performed for 25 minutes in a flow of 0.4 $mL.min^{-1}$.

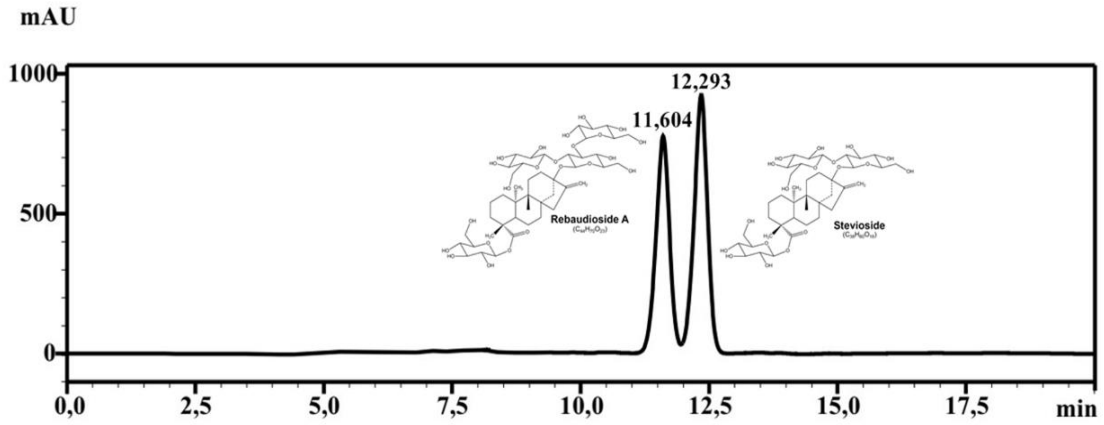
The mobile phase was composed of HPLC grade acetonitrile and ultra-pure water and formic acid (33:66.8:0.2 v/v). Quercetin was used as an internal standard, in the proportion of 1: 100 µL for each sample (Supplementary Figure 1). For the chromatographic evaluation of the samples, the solubilized extracts were filtered through syringe filters (PTFE Membrane, 0.45 µm, 4 mm - PHENEX). All analyzes were performed in triplicate.

2.4. Method validation

To identify the peaks of interest in the extracts it was necessary to use pure standards. The standard working solutions, containing stevioside (≥ 98% purity) and rebaudioside A (≥ 96% purity), were prepared by diluting 1 mg of the standard stock (Sigma - S3572 and Sigma - 01432, respectively) in 1 mL of mobile phase. Standard solutions were vortexed and filtered through syringe filters (PTFE Membrane, 0.45 µm, 4 mm - PHENEX) before use (HPLC injection) to ensure maximum solubilization of each compound in the mixture. The injection volume was set to 10 µL at a flow rate of 0.4 $mL.min^{-1}$.

From the identification of their respective retention times, 11,604 minutes for Rebaudioside A and 12,293 minutes for stevioside (Figure 1), it was possible to identify these compounds in the analyzed samples.

Figure 1. Chromatogram of the main steviol glycosides identified in *Stevia rebaudiana* (Bert.) Bertoni.



The peaks of the sample chromatograms were identified and compared with the retention time and the area of the standards, for the quantification of the compounds, for which they were obtained using three independent replicates.

2.5. Data processing and statistical analysis

LabSolutions software was used to process the chromatograms obtained via HPLC, including the wavelength, alignment, identification, and integration of peaks.

Multivariate statistical analysis was performed using the SISVAR 5.6 software. Analysis of variance was used to identify statistically significant differences between samples ($p < 0.05$) followed by Tukey's multiple comparison tests. Results are presented as mean of replicates ± standard deviations.

3. Results and Discussion

3.1. Identification of steviol glycosides (StGlys)

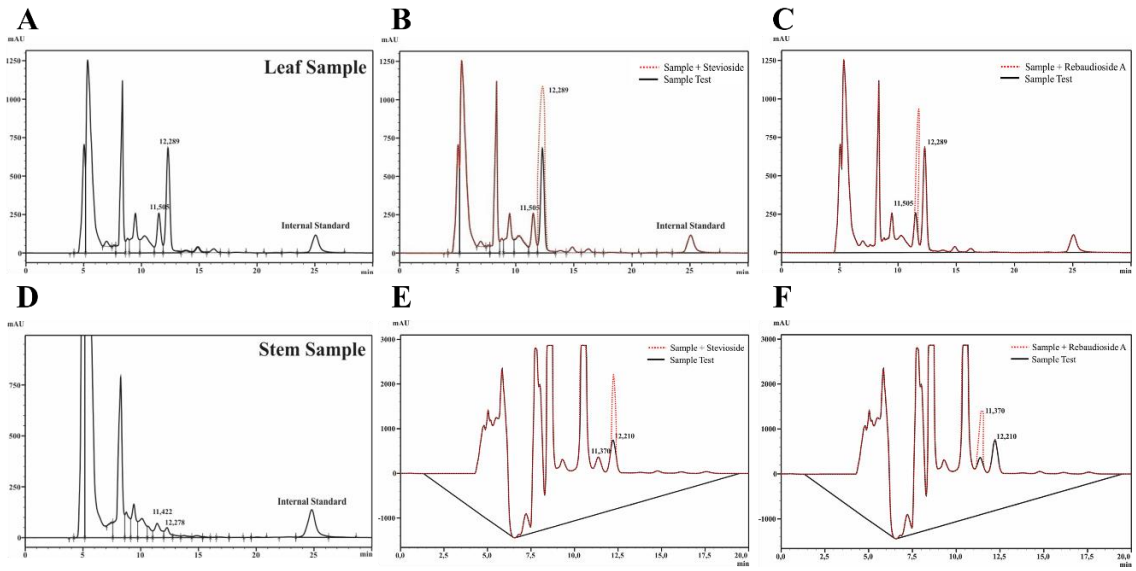
For the identification of glycosides (Stevioside and Rebaudioside A), we initially performed the standardization of pure standards, with variation in the volume of each injection and flow to obtain the best definition of the peaks referring to each substance

and their retention time. Furthermore, from these results we associated the pure patterns to the peaks identified in the chromatograms of the leaf and stem samples in the different photoperiods and cultivation time (Figure 2 A and D).

The chromatograms of each sample were compared with the standards to determine the peaks of interest. The best way to identify them was comparing the retention time of the peaks obtained for the samples, with the retention time of stevioside and rebaudioside A of the standards. Subsequently, to confirm the data, the leaf and stem samples were enriched with pure standards of Stevioside and Rebaudioside A, respectively, to validate the identified peaks (Figure 2 B - C for leaf and E - F for stem).

As can be seen in Figure 2, it was possible to obtain confirmation of the peaks of interest for the stevia samples. With the superposition of different chromatograms, pure samples and samples with the complementation of different standards, we can observe the increase in signal intensity for the different peaks corresponding to Stevioside and Rebaudioside A in the leaf and stem samples.

Figure 2. Chromatograms representing the identification of the main steviol glycosides present in *Stevia rebaudiana* (Bert.) Bertoni plants grown under different photoperiods. (A-D) Leaf and stem samples; (B- E) and (C-F) samples enriched with stevioside and Rebaudioside A standards respectively.



3.2. Quantification of steviol glycosides (StGlys)

The StGly present in stevia are a group of secondary metabolites derived from the mono-, di- and tetra-terpene biosynthetic pathway, the same pathway as gibberellic acid (CEUNEN; GEUNS, 2013b). Their use as a high-potency sweetener has made them an object of significant scientific and commercial interest, thus obtaining a high financial potential for the species. Plants and mainly stevia leaves accumulate a variety of these glycosides, whose concentration varies according to the genotype and cultivation conditions used, making their quantification relevant (BRANDLE; ROSA, 1992; BRANDLE; STARRATT; GIJZEN, 1998; RICHMAN et al., 1999; SOEJARTO; ADDO; KINGHORN, 2019). It is estimated that there are more than 60 StGly detected in stevia (DÍAZ-GUTIÉRREZ et al., 2020; JECFA, 2018). However, substantially measurable amounts, the main ones are Stevioside, Rebaudiosides A, B, C, D, E, F, Dulcoside A and Steviolbioside (STARRATT et al., 2002; TAVARINI et al., 2016; WÖLWER-RIECK, 2012), of which only rebaudioside A is considered the most economically important glycoside due to its flavor characteristics (CEUNEN; WERBROUCK; GEUNS, 2012; SAMUEL et al., 2018; WÖLWER-RIECK, 2012).

The quantification of stevioside and rebaudioside A content was calculated using the following formula:

$$\text{ST or Reb A}_{(\text{Sample})} = [(A_s/A_{\text{Querc}})*A_{\text{ST or Reb A}}]/W$$

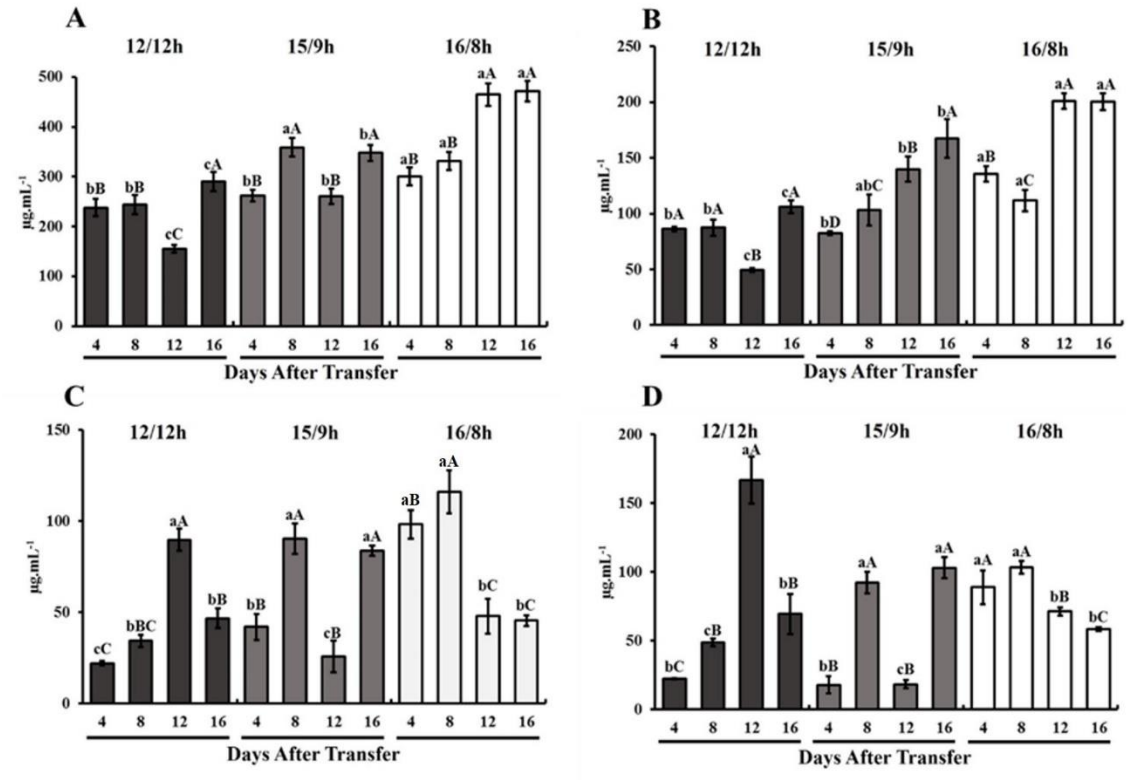
Where:

A_s - Peak area of the analyzed glycoside present in the samples; A_{Querc} - Quercetin peak area; $A_{\text{ST or}}$ Reb A - Peak area of the stevioside or rebaudioside A pattern; W – Weight.

The quantification of the StGly of the samples varied between the botanical parts studied. For the leaf, material of greatest interest for the stevia culture, the quantification of the main StGly presented higher values for stevioside, values ranged from 155.27 ±

7.57 to 471.78 ± 20.80 µg.mL^{-1} of extract (Figure 3A), and for the stem 22.05 ± 1.22 to 116.04 ± 11.88 µg.mL^{-1} (Figure 3C). While for rebaudioside A, the values varied between 49.30 ± 1.61 to 201.01 ± 6.73 µg.mL^{-1} (Figure 3B) and from 17.88 ± 6.12 to 166.65 ± 17.00 µg.mL^{-1} for leaf and stem respectively (Figure 3D). The leaf tends to produce greater amounts of glycosides when compared to the stem, in other words, the stevia leaf is the commercial raw material to produce sweeteners. However, the stem can become a representative source of this resource. For the analyzed stem samples, expressive amounts of StGly were observed both for the plants under 12/12h of light, 12 DAT and at 16/8h, 4 DAT.

Figure 3. Quantification of the main steviol glycosides identified in *Stevia rebaudiana* (Bert.) Bertoni. (A-B) Leaf and (C-D) stem samples under different photoperiods. The lower-case letters compare each photoperiod within each collection time and the upper-case letters compare the collections within each photoperiod according to the HSD de Tukey ($P < 0.05$); Supplementary Table 1.



The 16/8h photoperiod was the one with the highest values of quantification of glycosides for both leaf and stem. Concomitant to this, plants with longer cultivation

time showed better results, for the stevioside reaching 471.78 ± 20.80 µg.mL^{-1} for plants with 16 DAT of cultivation and for rebaudioside A, 201.01 ± 6.73 µg.mL^{-1} for plants with 12 DAT.

Several studies denote the influence of the photoperiod and the biosynthesis of sweetening compounds in stevia, in our study it was possible to observe that the amount of these glycosides tends to increase with a greater exposure of plants to light (CEUNEN; GEUNS, 2013b; YONEDA et al., 2017). Since these high yields may be related to higher leaf biomass of plants acquired with the highest exposure to light, above 13h is considered the critical photoperiod for the species (METIVIER; VIANA, 1979; YONEDA et al., 2017).

There is a correlation between flowering and the levels of StGly present, with a drop in their yield with the beginning of this period (CEUNEN; GEUNS, 2013b; KANG; LEE, 1981; RICHMAN et al., 1999). However, such a decrease may have other correlated factors such as the presence of dry senescent leaves, a decrease in leaf biomass due to reproductive development, the displacement of StGly to reproductive organs participating in their formation, or a catabolism attributed to these compounds (SERFATY et al., 2013).

For plants grown at 12/12h this drop in yield can be observed between 8 and 12 DAT of cultivation, but normalizing for the last period, for plants under 15/9h and 16/8h this characteristic was not observed. Thus, our observations demonstrate that the collection of stevia can be extended during flowering in plants grown above the critical photoperiod of the species as observed by Ceunen and Geuns (2013a), a period in which higher concentrations of StGly can be observed. The use of cultivars with certain morphological characteristics such as a large number of lateral branches during reproductive development will probably be more suitable for harvesting after opening the flowers, due to their higher leaf yield (CEUNEN; GEUNS, 2013a).

In addition to the length of the day, the quality or type of light to which the plant is subjected may have an influence on the StGly. Working with the length of days to which stevia plants are subjected, Rivera-Avilez et al. (2021) promoted the nocturnal interruption, where they observed an increase in net photosynthesis, internode length, leaf area, delay in the flowering phase and in the amount of the main glycosides.

Yoneda et al. (2017) concluded that the treatment with red and blue light, for example, had an effect in the amount of StGly per leaf and especially under treatment with blue light, the plants showed short stems and small leaf areas, however a more concentrated StGly content than in other light treatments.

StGly, besides being influenced by light, different types of stress can change their balance in plants. Saline stress, for example, can alter the levels of StGly in stevia, and low levels of concentration can increase these biocomposites, suggesting that StGly play the role of osmoprotective molecules under stress conditions and osmotic adjustment in the stevia plant under saline stress (AGHIGHI SHAHVERDI et al., 2019; CANTABELLA et al., 2017; CEUNEN; GEUNS, 2013a). Thus, the nutrition offered also favors the production of StGly by stevia, even though it is not a plant with high nutritional requirements (DÍAZ-GUTIÉRREZ et al., 2020).

Among the sweetening compounds with more abundance in stevia, rebaudioside A stands out for being sweeter and presenting a lower residual bitterness that can cause discomfort when consumed, the correlation between these two compounds being a characteristic sought by the market in the search for promising cultivars (SINGLA; JAITAK, 2016). Because rebaudioside A has greater economic value than stevioside as an alternative sweetener, the relationship between these metabolites mainly in leaves is an important measure to validate cultivars for commercial extraction (CEUNEN; GEUNS, 2013b). Alternatives to improve this proportion have been studied, such as the expression of related genes, enzymatic transglycosylation and the biotransformation of components of the biosynthetic pathway of steviol glycosides (DEVLAMYNCK et al., 2019; GERWIG et al., 2017; TE POELE et al., 2018).

4. Conclusions

We conducted the growth and quantification of the main steviol glycosides of commercial interest in leaf and stem samples of *Stevia rebaudiana* (Bert.) Bertoni at different photoperiods. Characterized as a short-day species with a critical photoperiod of 12 to 13h of light, with the growth of plants in the photoperiod of 12/12h it was possible to observe mainly the activation of the reproductive mode of the plants, while the photoperiods of 15/9h and 16/8h favored the vegetative mode.

Concomitantly, we noticed that the long photoperiods favored higher values for the quantification of steviol glycosides. According to our results, the synthesis of StGly can be affected by light, a factor that can also play an important role in the physiological development of plants. Therefore, the results obtained may be commercially interesting, as they indicate the importance of the controlled photoperiod for the cultivation of Stevia as a culture to produce StGly.

5. Acknowledgement

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6. Conflict of interest

The authors declare no conflict of interest.

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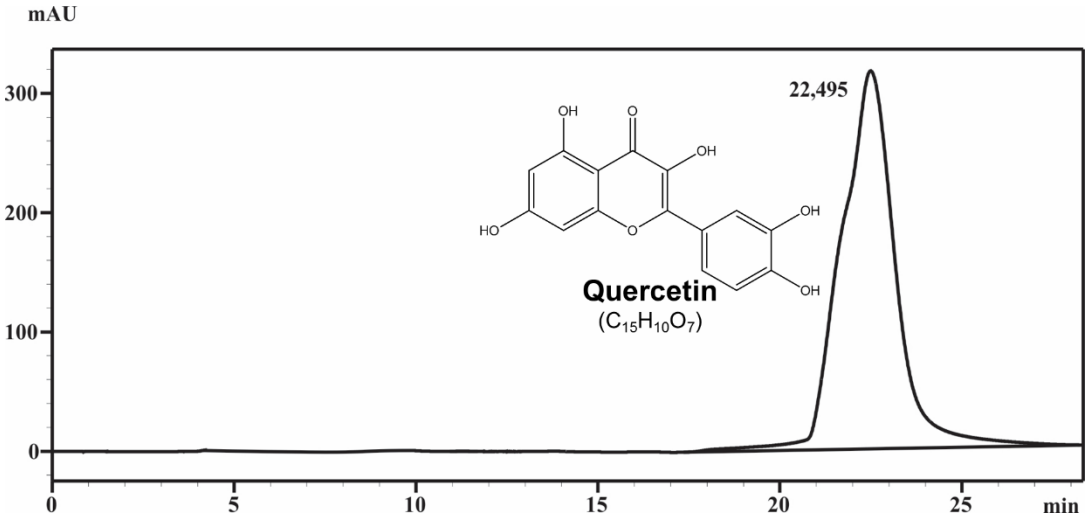
Supplementary Material

Supplementary Figure 1. Quercetin chromatogram. Substance used as an internal standard for chromatographic runs on *Stevia rebaudiana* (Bert.) Bertoni leaf and stem samples.



Supplementary Table 1. Quantification of the main steviol glycosides (StGlys) identified in *Stevia rebaudiana* (Bert.) Bertoni leaf and stem samples under different photoperiods. The lower-case letters compare each photoperiod within each collection time and the upper-case letters compare the collections within each photoperiod according to the HSD de Tukey ($P < 0.05$).

Photoperiod (hours light/dark)	Days after transfer (DAT)	Stevioside ($\mu g.mL^{-1}$)		Rebaudioside A ($\mu g.mL^{-1}$)	
		Leaf	Stem	Leaf	Stem
12/12	4	238,09±16,86^{bB}	22,05±1,22^{cC}	86,17±2,22^{bA}	22,27±0,64^{bC}
	8	243,94±18,81^{bB}	34,37±3,39^{bBC}	87,36±7,10^{bA}	48,70±2,60^{cB}
	12	155,27±7,57^{cC}	89,79±6,01^{aA}	49,30±1,^{61cB}	166,65±17,00^{aA}
	16	290,06±19,02^{cA}	46,74±5,37^{bB}	106,01±5,62^{cA}	69,47±14,59^{bB}
15/9	4	261,94±11,83^{bB}	42,04±7,03^{bB}	82,46±1,78^{bD}	17,88±6,12^{bB}
	8	359,08±19,07^{aA}	90,44±8,21^{aA}	103,12±13,73^{abC}	92,27±7,84^{aA}
	12	260,45±15,64^{bB}	25,68±8,69^{cB}	139,78±11,40^{bB}	18,36±3,05^{cB}
	16	347,65±16,48^{bA}	83,87±2,70^{aA}	167,52±17,31^{bA}	103,00±7,68^{aA}
16/8	4	300,71±17,53^{aB}	98,23±7,69^{aB}	135,68±6,80^{aB}	88,75±12,28^{aA}
	8	331,24±17,94^{aB}	116,04±11,88^{aA}	111,70±9,65^{aC}	103,17±4,60^{aA}
	12	464,82±22,78^{aA}	47,93±9,48^{bC}	201,01±6,73^{aA}	71,15±2,85^{bB}
	16	471,78±20,80^{aA}	45,56±2,94^{bC}	200,31±7,67^{aA}	58,40±1,46^{bC}

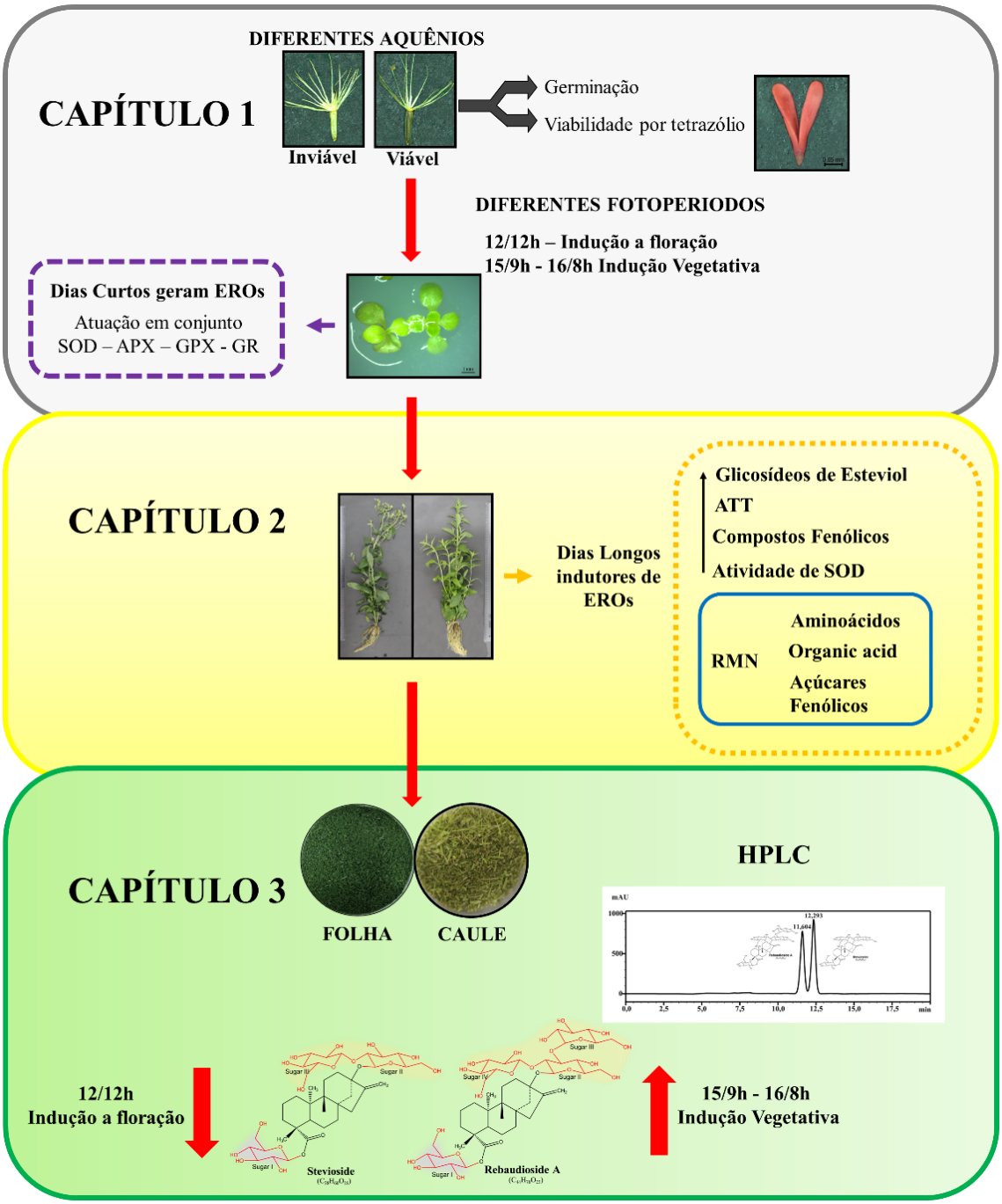
CONSIDERAÇÕES GERAIS

A estévia é uma planta que apresenta diversas peculiaridades, dentre as quais podemos destacar a produção de diferentes aquênios produzidos em uma mesma planta, o que pode ser um parâmetro para seleção entre sementes viáveis e inviáveis. Em outra linha, podemos destacar a sensibilidade da espécie a diferentes exposições a fotoperíodos (LIBIK-KONIECZNY et al., 2021).

Em nosso trabalho buscamos realizar um estudo dessas características e assim oferecer uma visão mais aprofundada quanto a espécie (Figura 1). Inicialmente, com os diferentes aquênios podemos descrever tais características que envolve a produção de aquênios viáveis e inviáveis, a partir de uma observação fenotípica e histológica dos mesmos. A seguir observamos que mudas (Plântulas) de estévia apresentam uma alta sensibilidade há diferentes condições de fotoperíodo, assim como plantas adultas. Entretanto em mudas, diferentes fotoperíodos podem ser geradores de EROs, sendo uma informação deveras importante para o estabelecimento de mudas de qualidade direcionadas ao plantio e cultivo em larga escala. Condições de maior tempo de exposição à luz, dias longos, podem favorecer o estabelecimento de mudas mais saudáveis, visto que foi observado em nosso estudo que o fotoperíodo de 12/12h, como uma condição indutora de floração precoce para a espécie, sendo assim considerada uma condição desfavorável ao seu cultivo inicial, provocando a ativação de mecanismo envolvidos com a floração da espécie, sendo os dados da atividade das enzimas antioxidantes analisadas um marcador bioquímico importante.

Em outra linha, num estudo realizado com o cultivo de plantas adultas conseguimos observar que o uso de um cultivo baseado em dias longos (16/8h) favorece uma série de características importantes à cultura. Maiores teores de glicosídeos de esteviol poderão ser observados, característica de grande importância para a espécie. Além disso, observamos também que fotoperíodos de dias longos proporcionaram maiores teores de compostos fenólicos e atividade antioxidantes tanto em extratos de folha como de caule. Para a análise enzimática da superóxido dismutase, foi possível observar características bem contrastantes quando comparado às análises de plântula com os extratos de folha e caule.

Figura 1. Proposta de representação esquemática do estudo realizado com a *Stevia rebaudiana* (Bert.) Bertoni, envolvendo a caracterização abrangente do ciclo de vida, desde as sementes até a planta adulta, sob diferentes condições de fotoperíodo.



A estévia uma planta característica de dias curtos originalmente (CEUNEN; GEUNS, 2013; YADAV et al., 2011), observamos que para a análise da SOD, plantas sob 12/12h de fotoperíodo não sentem essa condição como algo estressante, visto que a indução de uma fase vegetativa prolongada o oposto pôde ser observado, principalmente para o

fotoperíodo de 16/8h. Nosso estudo também demonstrou que diferentes fotoperíodos podem induzir a biossíntese de metabólitos com interesse industrial como aminoácidos e fenólicos por exemplo.

Nesse sentido, o nosso trabalho apresenta e reafirma a sensibilidade apresentada pela estévia a diferentes condições de fotoperíodo, além de demonstrar o valor comercial da espécie para além de suas propriedades adoçantes. No entanto, ainda se torna necessário estudos mais abrangentes a respeito dos fatores relacionados à inviabilidade das sementes (Aquênios) apresentados pela espécie o que pode alavancar seu cultivo em larga escala.

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