Short communication

Phenological stages of a *Stevia rebaudiana* genebank according to BBCH scale

Estadios fenológicos de un banco de germoplasma de *Stevia rebaudiana* de acuerdo a la escala BBCH

Carlos Jorge Budeguer[1*]; Luis Ernesto Erazzú[2]

[1]Facultad de Agronomía, Zootecnia y Veterinaria, Universidad Nacional de Tucumán. Florentino Ameghino s.n., El Manantial (4104), Tucumán, Argentina.
[2]Estación Experimental Agropecuaria Famaillá, Instituto Nacional de Tecnología Agropecuaria (INTA). Ruta Prov. 301 - km 32, Famaillá (4132), Tucumán, Argentina.

*E-mail: carlos.budeguer@faz.unt.edu.ar

Abstract

To evaluate the phenological stages of Stevia rebaudiana growing in Tucumán, Argentina, the Biologische Bundesanstalt, Bundessortenamt and Chemical Industry (BBCH) scale was used. The method was first applied in S. rebaudiana in France in 2020. Phenological data were collected from seven genotypes of the S. rebaudiana Genebank of the Estación Experimental Agropecuaria Famaillá, INTA and the duration of each phase of development was calculated using the growing degree days (GDD) in 2021-2022 season. Stages 1 to 9 were clearly identified and acumulate 291 to 2498 GDD between November 3, 2021, and March 23, 2022. The knowledge of these phenological stages of S. rebaudiana at the local agroecological conditions will be useful for its agronomic management and breeding.

Key words: Agronomic Management; BBCH Scale; Reproductive Stage; Stevia; Vegetative Stage.

Resumen

Para evaluar los estadios fenológicos del cultivo de *Stevia rebaudiana* en Tucumán, Argentina, se utilizó la escala Biologische Bundesanstalt, Bundessortenamt and Chemical Industry (BBCH). El método se aplicó por primera vez en *S. rebaudiana* en Francia en 2020. Se recolectaron datos fenológicos de siete genotipos del Banco de germoplasma de *S. rebaudiana* de la Estación Experimental Agropecuaria Famaillá, INTA y se calculó la duración de cada fase de desarrollo utilizando los grados día de crecimiento (GDD) en la temporada 2021-2022. Las etapas 1 a 9 fueron claramente identificadas y acumularon de 291 a 2498 GDD entre el 3 de noviembre de 2021 y el 23 de marzo de 2022. El conocimiento de estas etapas fenológicas de *S. rebaudiana* en las condiciones agroecológicas locales será útil para su manejo agronómico y mejoramiento genético.

Palabras clave: Escala BBCH; Estadio Reproductivo; Estadio Vegetativo; Manejo Agronómico; Stevia.

Stevia rebaudiana (Asteraceae) (2n = 2x = 22) is native of Paraguay and is of agroindustrial interest because of the contents of steviol glycosides (SG) in its leaves (Yadav *et al*., 2011; Borgo *et al*., 2021). These non-caloric sugars have a sweetening capacity 200 to 300 times greater than sucrose, which are a good alternative to synthetic sweeteners such as saccharin, acesulfame-K, aspartame and sucralose (Kinghorn, 2001; Brandle and Telmer, 2007). In Europe and Argentina, health organizations recommend reducing daily sugar intake to limit obesity and diabetes (Carocho *et al*., 2017; Carrera Lanestosa *et al*., 2017).

The primary economic importance of cultivating this species is to isolate and extract SG for use as a natural dietary sweeteners, as their consumption does not add calories to the body (Kryvenki *et al*., 2007). Additionally, dried leaves of the plant can be used to sweeten foods in Argentina (Res. 3-2018 APN-SRYGS-MSYDS). *S. rebaudiana* extract has been shown to help normalize insulin and blood glucose levels in diabetics patients (Wölwer-Rieck, 2012). In addition to its sweetening capacity, other beneficial health properties have been described; for example, Shivanna *et al.* (2013) provided data showing relief of liver and kidney damage in STZ diabetic rats, in addition to its hypoglycemic effect. Leaf

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extracts containing polyphenols and flavonoids have been shown to protect cellular components from oxidative damage, which is associated with early tumor development. Additionally, studies have tested their effectiveness in preventing dental caries (Iatridis *et al.*, 2022; Dey *et al.*, 2013).

The plants initially used for cultivation were taken from their center of origin in Paraguay and distributed to several countries, such as Japan, India, the United States, and France (Liaudat, 2021). Currently, China is the largest producer with approximately 80,000 ha, followed by the United States, India, Paraguay, Brazil, and to a lesser extent, Argentina (Kryvenky *et al.*, 2007; Yadav *et al.*, 2011). In Argentina, around 200 ha are cultivated by small and medium-sized producers, constituting a productive alternative of great interest. In Tucumán, there is limited information on the agronomic management of *S. rebaudiana* under local agroecological conditions. Therefore, it is important to understand the phenological behavior of available genotypes. This will help to improve the cultivation of *S. rebaudiana* in the region.

As noted by Falasca and Ulberich (2003), Stevia rebaudiana exhibits a bioclimate defined by climatic indices that align with the temperature and precipitation conditions of its native habitat. They also indicate an agroclimate covering an area enclosed between Salta and Tucumán (provinces of northwestern Argentina), delimited by absolute minimum temperatures above -3.8 ºC. This delimitation allows Tucumán to be considered suitable for *S. rebaudiana* production. Although it is a short-day plant, it is advisable to harvest before flowering, as this is when the concentration of steviol glycosides is highest (Ceunen and Geuns, 2013).

Describing the phenological stages of a crop involves studying the specific growth and development stages in the annual cycle of a plant. Knowledge of these different stages is essential for researchers, plant breeders, and agronomists. Phenological scales have been developed to illustrate the morphological changes that occur during the vegetative cycle (Zadoks *et al.*, 1974; Le Bihan *et al.*, 2020).

As interest in the cultivation of *S. rebaudiana* in Argentina is recent, there is limited information available on phenological stages. Existing studies were conducted under temperate climate conditions using different methodological tools, leading to overlapping stages and complicating comparisons between regions (Moraes *et al.*, 2013; Tavarini and Angelini, 2013; Barbet-Massin *et al.*, 2016). In the experimental field of the Estación Experimental Agropecuaria Famaillá, INTA, Tucumán, Argentina, a genebank of *S. rebaudiana* from different fields of Argentina was developed in 2013 with the aim of breeding.

Carneiro (2007) published the first complete phenological scale for cultivated *S. rebaudiana* in Brazil, with codes and illustrations specifying each stage. Vegetative stages are described using the letter "V," and reproductive stages use the letter "R." Numbers are added to each letter to specify the developmental level of the phenological stages, representing the number of nodes or the percentage of floral apex. Additionally, with the objective to develop a specific phenological scale for *S. rebaudiana*, Le Bihan *et al.* (2020) provided the first detailed description of phenological stages using the Biologische Bundesanstalt, Bundessortenamt, and Chemical Industry (BBCH) scale in eight genotypes from a genebank in France. The BBCH scale was initially developed for cereals by Zadoks *et al.* (1974) and consists of a two-digit code to describe each phenological stage. The first digit corresponds to one of the 10 main growth stages numbered 0 to 9 according to the specific phenological stage observed in each crop. The second digit corresponds to secondary sub-stages, also coded from 0 to 9. The system has been adopted for different crops and allows for comparisons between different regions where *S. rebaudiana* grows (Le Bihan *et al.*, 2020; Zadoks *et al.*, 1974).

This study aimed to characterize each of the phenological stages of *S. rebaudiana* cultivation in Famaillá, Tucumán, as there are no existing references for this region of Argentina. Phenological data were collected from the *Stevia rebaudiana* Genebank (SRG) of the Estación Experimental Agropecuaria Famaillá, INTA. This region has a humid subtropical climate with dry season. Seven genotypes from the genebank were used: 1.8, 5.16, 9.18, 10.7, 4.18, 12.5, and 1.12. Five replicates were obtained for each genotype through the multiplication of apical cuttings with three nodes in November 2021. Planting of each cutting was carried out in speedling-type trays with 35 cells, using a substrate composed of peat (70%) and perlite (30%) (Growmix multipro®). Irrigation was performed by sub infiltration from the base of the tray and overhead spraying when necessary. Plants were maintained in 3 L pots in the field.

The locality of Famaillá have an average annual temperature of 19.2 ºC, a minimum annual temperature of 13.4 ºC and a maximum annual temperature of 25.5 ºC; the average annual rainfall is 1,301 mm. Along the time of the present work (November 2021 to March 2022), the minimum temperature ranged between 15.9 to 20.8 ºC and the maximum, between 21.2 to 33.6 °C. The rainfall ranged between 116.8 mm in November 2021 to 7.9 mm in March 2022 (Figure 1).

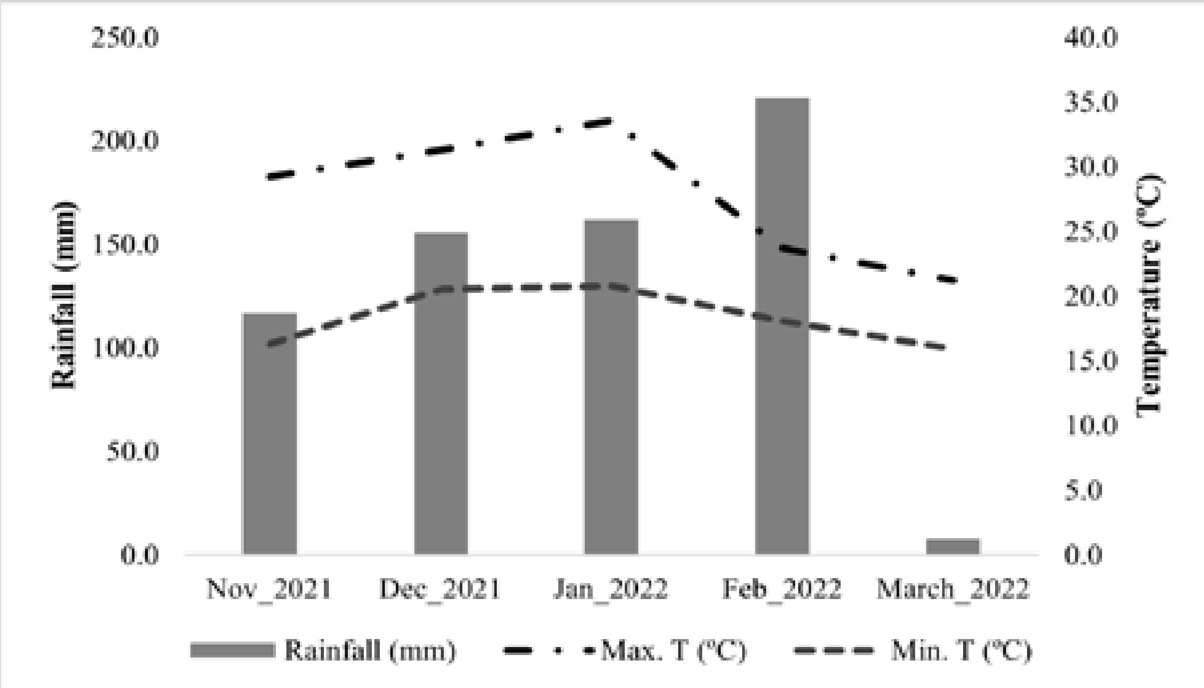

Figure 1. Climate data of Famaillá during the time of the experiment. Maximum, minimum temperature (ºC) and rainfall (mm).

Observations and measurements were performed every week on five replicates of each genotype at both vegetative and reproductive stages, from plantlets until senescence, resulting in 35 observations per week. Measurements were made according to the BBCH scale (Le Bihan *et al.*, 2020) from November 2021 to the end of March 2022. Pictures of different stages were taken with a Samsung SM-A515F camera with settings at 1/100 s, 4.60 mm, ISO 32. From the observations of the five replicates of each genotype, the most representative stage for each genotype was recorded, excluding any extreme values or atypical plants.

The duration of each phase of development was calculated using accumulated thermal time (ºC/day). The growing degree days (GDD) was calculated according to the McMaster and Wilhelm equation (1997):

$$GDD = \left[\frac{Tmax - Tmin}{2}\right] - Tbase$$

where Tmax is the daily maximum temperature, Tmin is the daily minimum temperature, and Tbase is the base temperature of 10 ºC for *S. rebaudiana*, as the plant resumes its growth activity above this temperature (Guerrero *et al.*, 2015). Daily temperatures were obtained from the INTA Agrometeorological Information and Management System (http://siga.inta.gob.ar). The thermal time in accumulated degree days was determined from the beginning of the vegetative stage to plant senescence.

The duration of the phenological stages of the BBCH scale in accumulated thermal time is shown in Figure 2. There is an overlap between stages 4 and 5, while no such overlap exists between the remaining stages. Stage 4 corresponds to the development of the harvestable vegetative part, and stage 5 represents the emergence of the inflorescence. Le Bihan *et al.* (2020) reported that some stages are concomitant with *S. rebaudiana* growing in temperate climate in France.

The results were compared with those obtained by Le Bihan *et al.* (2020) at Bordeaux (France) and Guerrero *et al.* (2015) at Misiones (Argentina), who worked with different genotypes than those

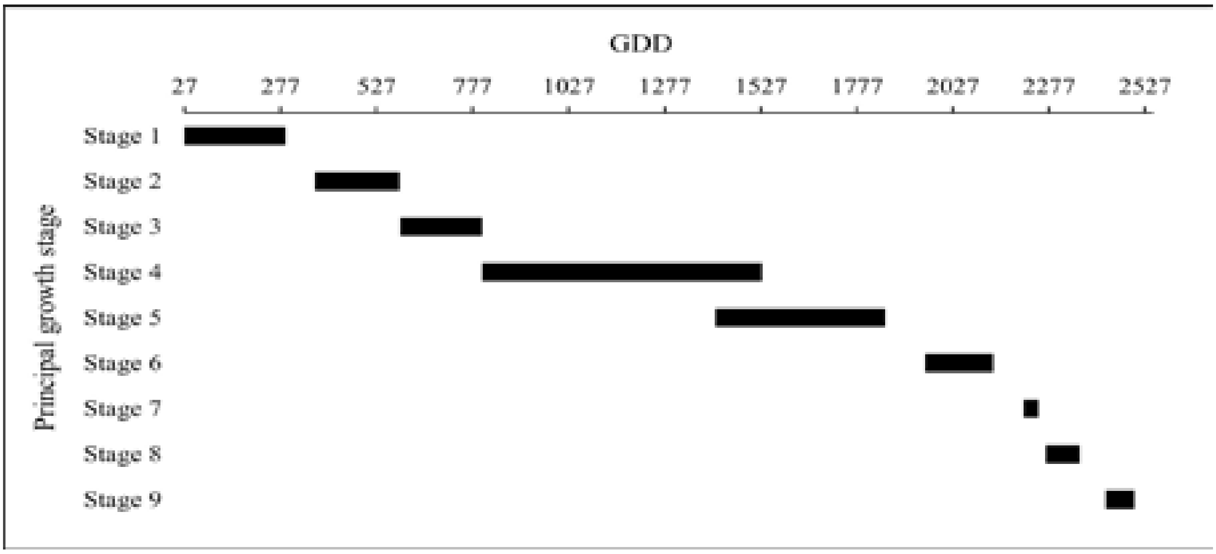

Figure 2. Duration and chronology of principal growth stages (described by the BBCH scale) of *Stevia rebaudiana* in Famaillá, Tucumán, according to the growing degree days (GDD). The black rectangle represents the variability in the duration of each stage of the phenotypes observed, from the the most precocious to the latest.

analyzed in this study. Differences found among the different trials may be attributed to the genotypes used, edaphoclimatic conditions, and the age of the evaluated plants. The discussion is centred on the authors mentioned earlier, with a distinction made between two stages: vegetative and reproductive.

The adaptation of the BBCH scale for *S. rebaudiana*, developed by Le Bihan *et al.* (2020), is appropriate and practical to use in the SRG. Four main stages could be identified throughout the plant cycle: vegetative (stages 1 to 4), flowering (stages 5 to 6), seed (stages 7 and 8) and senescence (stage 9). Figure 3 shows each of the stages in the studied genotypes. The stages can be further divided into sub-stages to give a more precise phenological description. In this work, only some of the stages proposed in the BBCH scale were presented, as the aim is to emphasize those of greatest agronomic importance. Stage 0 of the BBCH scale was not taken into account, as it focuses on the seed and germination, stages not contemplated in this study. Each of these stages is described below.

A) Stage 1: *Leaf development on the main stem.* This stage begins with the first pair of leaves fully developed on the main stem. This leaf development

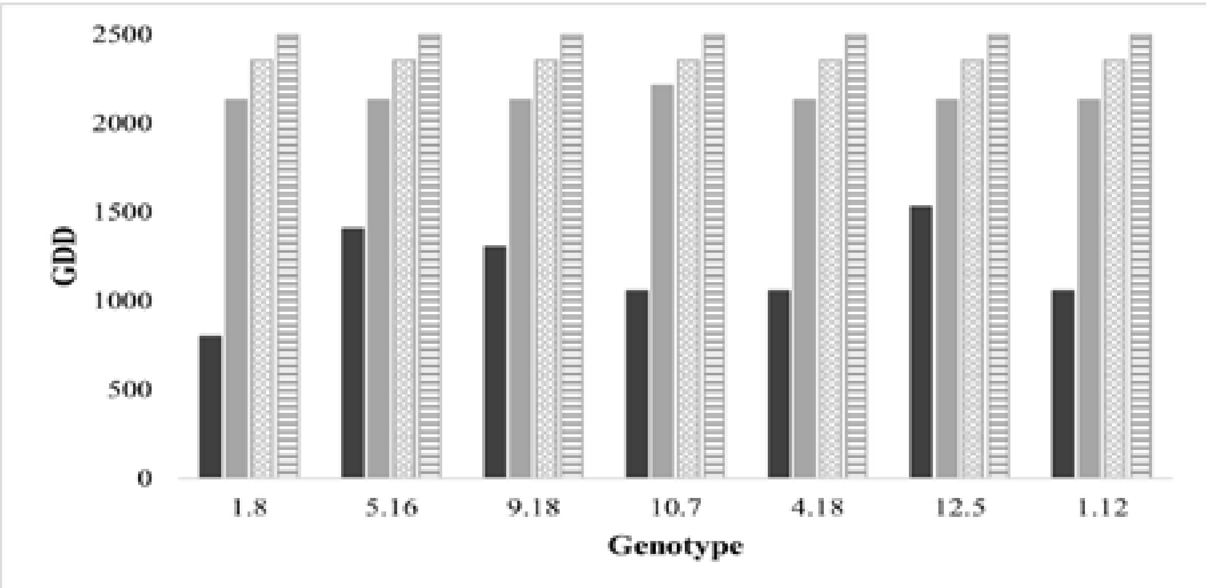

Figure 3. Growing degree days (GDD) reached by each genotype of five stages of *S. rebaudiana* in Famaillá, Tucumán. The type of bar indicates a stage. Black bar: vegetative, grey bar: flowering, dotted bar: seed stage, striped bar: senescence.

continues throughout the vegetative growth cycle of the crop. The number of main stems can vary according to genotype, production year and climatic conditions, starting from one in the first year of production and increasing year by year. This stage occurred between November 3 to 24, 2021, totaling 291.45 GDD, except for genotype 12.5 with 367.85 GDD (Figure 4 A and B).

Figure 4. Stage 1: **A.** second pair of leaves fully developed. **B.** third pair of leaves fully developed.

B) Stage 2: *Side shoot development.* This stage starts with the formation of side shoots, from the appearance of the primordium under the axil of a pair of leaves, until the complete formation of the last pairs of secondary stems. This stage reveals the architecture that the plant will have by the development of lateral stems. For the seven genotypes, this stage covers from November 29 to December 6, 2021, except for genotype 12.5 which showed a longer period between November 29 to December 14, around 471.7 and 589.4 GDD respectively (Figure 5 A and B).

Figure 5. Stage 2: **A.** appearance of lateral primordia on the main stem. **B.** development of 30% of the lateral stems.

C) Stage 3: *Stem elongation.* This occurs throughout the vegetative development of *S. rebaudiana* and allows an increase in leaf biomass. It implies an increase in the number of nodes, which leads to an increase in the number of leaves. For the seven genotypes analyzed, it covers a period from December 14 to 20, 2021, except for genotypes 10.7, 4.18 and 12.5 which showed a longer period from December 14 to 27, 2021; with 684 GDD and 801 GDD, respectively.

D) Stage 4: *Development of the harvestable vegetative part.* This stage implies an increase in harvestable biomass during vegetative development until the apex begins to differentiate into flower buds. In our conditions, climate conditions, this stage was observed in spring and early summer. This stage showed greater variability between all the genotypes, varying from 1057 GDD in December to 1530 GDD in January (Figure 6).

Figure 6. Stage 4: maximum development of harvestable biomass.

E) Stage 5: *Inflorescence emergence.* This stage comprises from the first differentiated bud development until 90% of the differentiated apex. No variation was observed among the seven genotypes studied, as the first flower buds started to appear around 1408 GDD and up to 1851 GDD. This phase started on January 18 and ended on March 2, 2022 (Figure 7 A and B).

F) Stage 6: *Flowering.* This stage starts with the opening of the first flowers on the inflorescence until all flowers fully open. All seven genotypes showed white petals and flower opening was clearly identifiable, starting from 1956 GDD to 2131 GDD. This phase occurred in the period between March 9 to 23, 2022 (Figure 8 A, B and C).

G) Stage 7: *Fruit development.* The fruit development corresponds to the development of achenes that have a hairy Crown, known as pappus. This stage begins with the production of the first visible fruits and culminates when most of the fruits have reached their maximum size and the presence of the pappus can be appreciated. In the genotypes analyzed, the stage started at 2212 GDD and finished at 2250 GDD between March 31 to April 4, 2022.

Figure 7. Stage 5: **A.** emergence of inflorescences. **B.** 30% of the stem apices are differentiated and present inflorescences, but the flowers remain closed.

H) Stage 8: *Seed ripening.* Seed ripening is highly visible and begins with the fading of inflorescences and appearance of brownish stems. Seeds can be at different stages of maturation on the multiple inflorescences of the plant and can be dispersed at different times. At this stage, no difference in degree days was observed in any of the seven genotypes analyzed, starting with 2269 GDD and finishing the phase with 2357 GDD between April 6 to 13, 2022 (Figure 9).

I) Stage 9: *Senescence.* This stage begins when the vegetative and reproductive organs are turn brownish due to wilting of the flowers and drying of the plant. The leaves colour turns from green to brownish, and the oldest leaves fall. At the end of this stage the plant is observed to be completely brown and dry. The plant enter in winter dormancy. There were no differences between the seven genotypes, culminating the stage with 2498 GDD on April 27, 2022 (Figure 10).

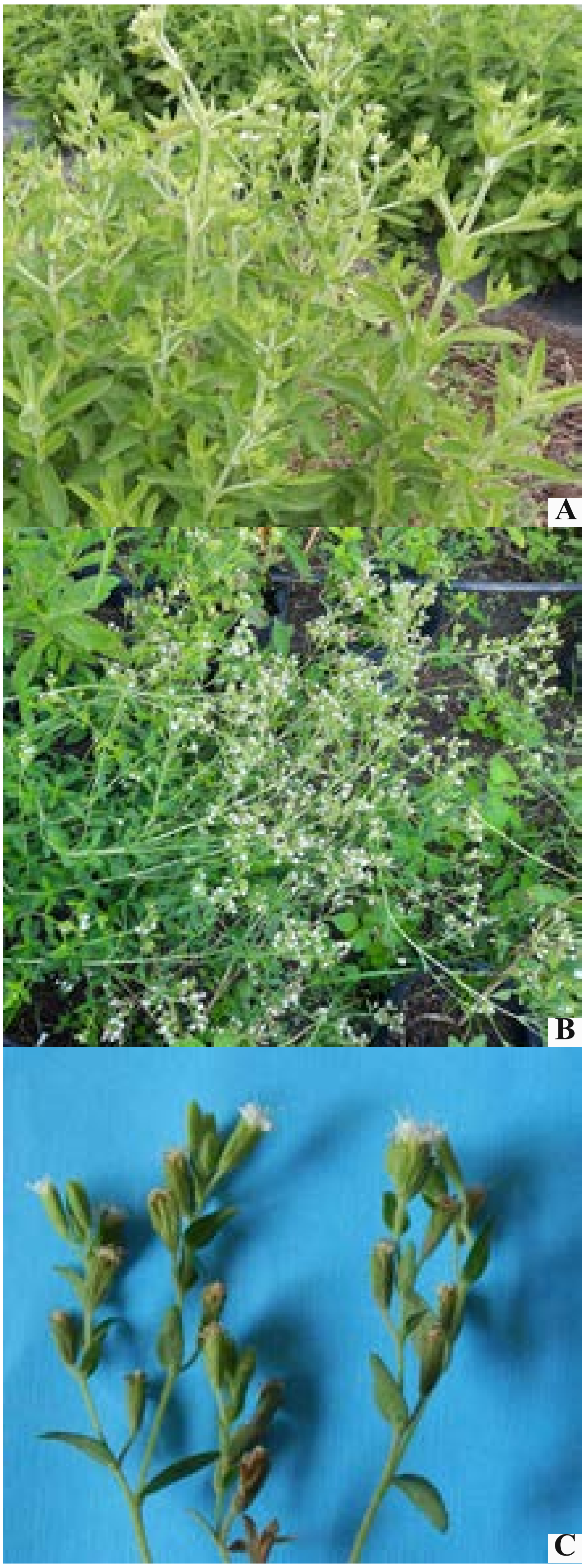

Figure 8. Stage 6: **A.** 20% of the inflorescences with open flowers. **B.** 50 % or more of the inflorescences with open flowers. **C.** flowers start fading, most of flowers' petals are drying out.

Figure 9. Stage 8: 90% of seeds are ripened, a large percentage of mature seeds are already dispersed by wind.

Figure 10. Stage 9: The entire plant is dry.

Two main stages can be clearly observed in the life cycle of *S. rebaudiana* over a season: vegetative and reproductive stage. According to Le Bihan *et al.* (2020), the vegetative stage ends at a cumulative degree day sum of 1450 GDD, while for Guerrero *et al*. (2015), this phase finished between 1800 and 2000 GDD. In the present study, the end of this phase was between 1305 to 1530 GDD, showing a behavior similar to that obtained by Le Bihan *et al.* (2020). In this crop, the vegetative period is crucial because it is the time when harvestable biomass develops. In Famaillá, this stage finished between late January and early February for the analyzed genotypes. Regarding the reproductive stage, for Le Bihan *et al.* (2020) it lasted 850 GDD, while for Guerrero *et al.* (2015), lasted approximately 1200 to 1400 GDD. In the present study, the duration of this stage was 968 GDD, taking intermediate values compared to the other works. In Famaillá, this stage ended in mid-April. It is important to consider this stage in the planning of targeted crossings for breeding purposes, as well as for seed collection, as it coincides with rainy season in Tucumán.

For agronomic management of *S. rebaudiana*, knowledge of these phenological stages in the local agroecological conditions will help to improve cropping management and breeding.

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ORCID iDs

C.J. Budeguer 0000-0002-4649-956X

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