To cut or not to cut? That is the question in first year harvest of *Stevia rebaudiana* Bertoni production

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Abstract:

Stevia rebaudiana (Bertoni) is a perennial crop from north Paraguay (humid subtropical climate), belonging to the *Asteraceae* family. Stevia is an emerging crop in Europe (mild climate), cultivated for its steviol glycosides (SG), natural sweeteners that are 300 times sweeter than sucrose which is the main agronomical and industrial interest of stevia. Recent studies showed that it is possible to cultivate stevia in mild climates as a perennial and economically viable crop. However, lack of knowledge on cropping system specific to perennial plants, the duration of cultivation, the overwintering and the impact of first-year crop establishment act as a disincentive to crop expansion. Harvest management through the impact of harvesting in the first year of establishment was investigated for agronomic traits over three years of production- for 15 stevia genotypes cultivated in the south-west of France. Two harvest modalities were compared: 2H when the plant is not harvested in the first year of establishment and 3H when the plant is harvested in the first year. The genotypes performance was assessed based on: regrowth rate after winter, SG yield (g/plant) through its two components of SG content (%w/w) and dry leaf biomass (g/plant), and the SG profile. Two cumulative variables, cumulated SG yield and cumulated dry leaf biomass, were also added to the study to obtain an overview of genotype performance during cultivation time and in both harvest modalities. The tested genotypes showed a wide range of response for overwintering, but with a significant decrease of 30% survival rate for plants harvested in first year (3H). SG yield and dry leaf biomass results presented high variability among the different genotypes. These traits were also significantly impacted by the harvest modality, and a decrease in SG yield and dry leaf biomass was identified for plants harvested in first year (3H). No clear tendency was revealed for SG content or SG profile. Cumulative variables confirmed previous results showing a better SG yield and leaf biomass production for plants non-harvested during the first year (2H), at crop-life scale. Our results, on a wide range of genotypes, shed light on the agronomic management of *Stevia rebaudiana* in temperate conditions. They suggest the interest of a first year without harvest, allowing a better establishment of the crop, a better overwintering and a better cumulative yield.

Key words: stevia, overwintering, steviol glycosides yield, harvest management, crop establishment

Abbreviations: SG: steviol glycosides; SGDD: Sum of Growing Degree Days; ST: stevioside; RebA: rebaudioside A; RebC: rebaudioside C; DulA: dulcoside A; RebF: rebaudioside F; Rub: rubudioside; RebD: rebaudioside D; RebM: rebaudioside M; RebB: Rebaudioside B; SB: steviolbioside

1. **Introduction**

Stevia rebaudiana Bertoni, a native plant from Paraguay, is a perennial *Asteraceae* whose leaves are traditionally used by the Guarani Indians as a natural sweetener (Soejarto et al., 1983). The sweet taste comes from steviol glycosides (SG), that accumulate in the leaves (Angelini et al., 2018). Stevioside (ST) was the first SG identified and are 250 to 300 times sweeter than sucrose (Bridel and Lavieille, 1931). Others SG more recently identified, presenting a sweetening power varying from 50 up to 400 sweeter than sucrose (Ceunen and Geuns, 2013a; Chaturvedula et al., 2011; Chaturvedula and Meneni, 2017; Ibrahim et al., 2016; Mao et al., 2017; Perera et al., 2019, 2017; Prakash et al., 2014; Prakash and Chaturvedula, 2014). These glycosylated diterpenes compounds have been consumed in Japan as a natural alternative to synthetic sweeteners since the 1970s. More recently, western consumers have also begun using these natural sweeteners, as illustrated by the increase in product launches, with more than 14,000 food products now sweetened with stevia, on the market (Mintel Global New Products Database, 2017). This industrial sector requires a large supply of stevia leaves. Today, China is the main stevia leaf supplier (Gantait et al., 2018), accounting for 80% of global production in 2016, corresponding to 50,000 – 60,000 tons of dry leaves a year (Sun, 2016). However production is growing in many other parts of the world, including Europe.

For 15 years now, numerous experiments have been carried out in Europe with the aim of validating the crop's establishment and implementing improvement strategies. European experiments in Portugal (Coelho et al., 2019; Lankes and Grosser, 2015; Reis et al., 2015), Germany (Lankes and Zabala, 2011; Munz et al., 2018; Woelwer-Rieck et al., 2010), Denmark (Grevsen et al., 2015), Italy (Andolfi et al., 2006; Tavarini and Angelini, 2013), Greece (Zachokostas, 2016), Spain (Labrador et al., 2014), Switzerland (Vouillamoz et al., 2016), France (Barbet-Massin, 2015; Hastoy et al., 2019), Poland (Libik-Konieczny et al., 2018), Hungary (Takács-Hájos et al., 2019), Bulgaria (Denev et al., 2017), have confirmed the possibility of *Stevia rebaudiana* cultivation as a perennial plant in mild climate conditions (Andolfi et al., 2006), with sufficient yield to make the production economically viable (Ferrazzano et al., 2016).

S. rebaudiana's yield can be characterized through different key indicators: total SG yield, expressed in kg/ha and t/ha for agronomic production, SG yield, expressed in g/plant for research purposes and SG profile. Total SG yield (kg/ha or t/ha) and SG yield (g/plant) are defined by two measures: the dry leaf biomass, weighted per plant for SG yield (g/plant) or weighted per hectare for total SG yield (kg/ha or t/ha), and leaf SG content (%w/w dry leaves). Those variables were measured on fifteen stevia gentoypes cultivated in an experimental field for two years in southwestern France (Hastoy, 2018). In the conditions of Hastoy's study the total leaf SG content explains 20% of SG yield variance whereas dry leaf biomass explains 75% of SG yield variance.

Stevia's SG profiles are characterized by the diversity of SG present in its leaves and the proportion of each SG from total SG. So far, 46 SG have been identified in *Stevia rebaudiana* (Ceunen and Geuns, 2013a; Chaturvedula and Meneni, 2017; Ibrahim et al., 2016; Mao et al., 2017; Perera et al., 2017). However, in most studies the number of SG analyzed is limited by analytical capacities, ten of these SG are often used to characterize SG profile accumulated in highest content in stevia leaves : stevioside (ST), rebaudioside A (RebA), rebaudioside M (RebM), rebaudioside D (RebD), rebaudioside C (RebC), dulcoside A (DulA), rebaudioside F (RebF), rubudioside (Rub), rebaudioside B (RebB) and steviolbioside (SB), such as in Barbet-Massin et al., (2016) and Hastoy et al., (2019) studies. Among these ten, the two SG accumulated in higher quantities in leaves are ST and RebA (Ceunen and Geuns, 2013b). Each SG has a specific flavour: RebA, RebM and RebD have a sweet taste while ST, RebC and DulA elicit a bitter aftertaste (Hellfritsch et al., 2012).

SG yield and SG profile are significantly dependent on the genotype cultivated (Hastoy et al., 2019; Parris et al., 2016). However, while the SG profiles are relatively stable per genotype with respect to production years and environment (Barbet-Massin et al., 2016; Hastoy, 2018), SG yield is not, and is particularly susceptible to environmental and growing conditions. This interplay is all the more important as *S. rebaudiana* is a perennial plant.

Stevia leaf biomass production, a key yield variability factor, can vary according to environmental conditions such as climate, cropping system, years of production, genetic diversity, and interaction with environmental factors. The trait presents high variability, and ranges from 37.6 to 190 g dry matter/plant in temperate climates depending on the genotype (Barbet-Massin et al., 2015). Foliar biomass production can be considered a key driver of growth in improving stevia yield.

Total leaf SG content depends on the genotypes' interaction with environmental factors. In the literature, a wide range of SG content has been described from 4.6 to 27.3 %w/w dry leaves according to genotype and environment (Barbet-Massin et al., 2016; Montoro et al., 2013). SG content increases significantly with years of production (Barbet-Massin, 2015; Hastoy, 2018).

Among the various environmental factors that can play a key role in stevia performance, response to water availability and nutrients has been widely described (Angelini et al., 2018; Barbet-Massin et al., 2015; Lavini et al., 2008; Pordel et al., 2015). *S. rebaudiana* also responds sharply to photoperiod variations. A short photoperiod with 12h of light, leads to early flowering (Ceunen and Geuns, 2013b; Metivier and Viana, 1979). However a long-day photoperiod, with 16h of light, increases the SG content in leaves up to 30%, as it contributes to extending vegetative growth and increases biomass yield (Ceunen and Geuns, 2013b). In temperate growing conditions, flowering occurs at the beginning of autumn as the day length declines. Vegetative phenological stages of stevia are characterized by an increase in SG content, reaching a maximum at flower budding stage (Barbet-Massin, 2015; Ceunen and Geuns, 2013b) followed by a decrease in SG content after the beginning of flowering (Barbet-Massin et al., 2016). Another study, investigating the kinetics of SG accumulation on five stevia genotypes during vegetative growth, shows that maximum SG content was reached a month before the stage previously identified (Hastoy, 2018). These studies have improved the definition of the harvest period for this species grown in mild climate conditions.

Stevia yield, especially biomass production is also influenced by traits specific to perennial crops such as first-year crop establishment, age of the stevia plant on the field and overwintering. Plantation time in temperate climate conditions is between March and May as there is less risk of frost than in winter (Angelini et al., 2018; Serfaty et al., 2013). The harvested stevia plants' age impacts biomass production, with a major increase in yield from the first to the fifth year of cultivation (Andolfi et al., 2006). Under mild climate conditions, the number of potential harvests per year was tested, with the result that a single harvest at the end of cultivation time leads to a higher yield than two or three harvests performed over the same cultivation time (Moraes et al., 2013; Serfaty et al., 2013). In a context of agronomic production in southwestern France, farmers generally harvest stevia in the first year of production up until the time of reduced yield.

To cultivate stevia as a perennial crop under temperate conditions, we need to take susceptibility to overwintering into account. This is linked to stevia resistance to low temperatures and is studied through stevia post-winter regrowth rate. The crop can bear a temperature range from 0-2°C to 35°C (Sumida, 1980), but is susceptible to winter frost, with leaf injury below 0°C (Moraes et al., 2013). For stevia to survive low winter temperatures, a plastic or straw mulch provides a solution, making it possible to cultivate the plant as a perennial crop (Moraes et al., 2013). However, in Germany, winter temperatures are too low for a satisfactory post-winter regrowth rate (Lankes and Zabala, 2011).

Overall, most European environments and climate conditions suit stevia cultivation as a perennial crop, but its cropping system in such conditions has still not yet been fully elucidating. In Europe, studies investigated stevia production as perennial, it was either harvested in the first or the second year of production, but no study to date has investigated the impact of a first year harvest on stevia establishment, biomass production, SG content and yield over several years of production. However, in perennial crops, the impact of harvesting during crop establishment is known to potentially reduce future yield (Leyshon and Campbell, 1992; Strik and Buller, 2005).

The goal of this study is to investigate stevia harvest management in temperate conditions. Most studies on stevia have been conducted with a very limited number of genotypes, often misidentified, with generic names such as “Rebaudiana”, “Sugar Love”, “AX” and “Candy” (Libik-Konieczny et al., 2018; Munz et al., 2018; Parris et al., 2016). Our study was conducted on 15 genotypes of various origins that were listed and genetically characterized (Cosson et al., 2019). The impact of harvesting in the first year of production on agronomic traits over three years of production in south-west France was evaluated for these 15 stevia genotypes. Genotype performances is assessed based on usual stevia production agronomic traits: regrowth rate, SG yield through its two components, SG content and leaf biomass, and SG profile. Cumulative variables such as SG cumulated yield and cumulated dry leaf biomass have also been measured as indicators to study the impact of a first-year harvest on stevia genotype performance during cultivation.

2. Material & methods

2.1. Plant material:

Fifteen genotypes were selected from the INRAE collection to be planted in the field trial (Table 1). The genotypes, which originally come from Argentina, Paraguay, Spain and Israel, were obtained from different providers: EUSTAS gene bank (Hortilab, Telgte, Germany), SteviaStore (Paraguay) and Oviatis' collection (Lacaussade, France).

2.2. Experimental design:

Each genotype was produced as a clone through *in vitro* cuttings from the parental plant of each genetic resource. *In vitro* cuttings were produced and grown under regulated greenhouse conditions for seven weeks (22°C – 18°C). Acclimatization begun with 10 days with saturated hygrometry level, followed by two weeks of gradual aeration. Cuttings from 10 to 15 cm high (3 to 5 nodes) were transplanted into a Jiffy®7 pellet (42 mm diameter, Jiffy, France). The plantlets were planted on a private farm in Liposhtey (44°17'56.9''N 0°53'14.7''W). Plantation was performed at the end of June 2016 with plantlets aged 7 weeks old after acclimatization. The planting process and the conditions were previously described in Hastoy et al., (2019).

The field trial consisted of 4 randomized complete blocks design (RCBD). Each block was composed of 3 rows. In a block, plant spacing was 33 cm x 60 cm. Distance between two successive blocks was 1 m. The field trial density was 3.75 plants / m². The fifteen genotypes were planted in each block. It consisted of 21 clones per genotype (7 plants x 3 rows). 64 clones per genotypes were planted in total.

Each winter, all the plants were cut down at 5 cm above the soil in December and covered with a wintering veil (30 g/m²). In 2017 and 2018, this crop protection was removed in

March. In summer, irrigation of 1 L/h per drip was applied to each plant for 1 hour every day, while in autumn the water supply was limited to 30 minutes. Irrigation was the same over the 3 years of the study. The drop-by-drop irrigation system was used for fertilization in July 2017 with a supply of NovaTec® Solub 14-8-30 (Compo Expert), at 40 kg nitrate/ha, and in July 2018 a solution of UNIVERSOL® Blue 18-11-18-2,5 (N, P, K, MgO) (ICL Specialty Fertilizers) was applied. Weeds were removed by hand at the plant collar. In order to control Septoria Leaf Spot disease in 2016, 2017 and 2018, Score® 250EC or Ortiva®25SC fungicides (Syngenta) at 0.5 L/ha were applied 3 times (May, July, August). Harvesting was in September at phenological stage 50, corresponding to the emergence of inflorescence (Le Bihan et al., 2020). Every genotype was harvested at this phenological stage, corresponding to different date in September according to phenological precocity.

2.3. Measured plant traits

32 plants were selected per genotype, equally distributed in the four blocks, corresponding to 8 plants per genotype per block. In the first year of production on September 23, 2016, 20 clones were harvested from 32 per genotype. These plants come under the "Harvested in the first, second and third year of production" modality, hereafter called the 3H modality (Figure 1). The plants of the 3H modality were all located in the middle of the block side-by-side. Of the rest of the plants, 12 clones from 32 per genotype were cut down in December 2016. These plants are considered as the "Harvested in the second and third years of production" modality, hereafter called the 2H modality (Figure 1). The plants of the 2H modality were separated from 3H modality plants by border plants, not harvested in second and third years of production. In December 2016, plants of the 3H modality were cut back, in order to install the wintering veil on the all experimental field.

Genotype performance was studied over three years of production. For each year, plant regrowth was recorded for two months after the first signs of regrowth, generally occurring in

March. This involved scoring the presence (1) or the absence (0) of each plant planted in 2016.

To evaluate the stevia plant performance at harvest stage, dry leaf biomass (g/plant) and SG content (%w/w) were measured, providing the plant SG yield calculation according to the formula:

$$Plant\ SG\ yield\ (g/plant) = SG\ content\ \left(\%\ w/w_{DWleaf}\right) \times Dry\ leaf\ biomass\ (g/plant)$$

The sampling procedure to measure dry leaf biomass (g/plant) was similar to the protocol described by Hastoy et al,. 2019. Briefly, the whole plant was cut at 10 cm above the ground to collect aerial biomass. Plant samplings were dried at 40°C for 60 hours in a heat chamber (UF750 MEMMERT). The leaves were separated from the stems in order to weight the dry leaf biomass separately from the dry stem biomass.

The SG extraction and quantification protocol was developed by Hastoy et al., (2019). In a few words, SG extraction is performed on 20 mg of dried leaves mixed in 2 mL of ultra-pure water. Samples were maintained at 80°C for 2h in water bath (Isotemp, GPD10, Fischer Scientific). 5µL of supernatant is filtered through a 0.45 µm pore size filter (Agilent), and injected for quantification into a C18 column (Agilent) with the guard column on a Reverse Phase High Performance Liquid Chromatography (RP-HPLC) system. SG elution and detection were identical to the one described in Hastoy et al., (2019), parameters of the quantification are presented Table A.1. This method detects 10 SG: RebD, RebM, ST, RebA, RebC, RebF, DulA, Rub, RebB, SB. The results were expressed as content per unit of dry leaf biomass (%w/$w_{dryleaf}$) for each SG and total SG, and as a proportion (%) of the content of each SG to total SG content.

To calculate total SG yield (kg/ha) corresponding to field production, the plant SG yield (g/plant) was multiplied by genotype density. The use of density takes the changes due to winter plant losses into account in both harvest modalities and for each genotype. This means that the total SG yield (kg/ha) will be measured by the post-winter regrowth rate.

$$Total\ SG\ yield\ (kg/ha) = plant\ SG\ yield\ (g/plant) \times\ density$$

Cumulated dry leaf biomass and cumulated total SG yield were also calculated to compare them for both harvest modalities. The mean per block was calculated for each genotype per harvest condition. 2016, 2017 and 2018 values were then calculated to obtain the final cumulated yield value.

2.4. Evaluation of environmental parameters

The environmental parameters recorded in this study are daily temperature, maximum and minimum value, and daily rainfall. These data were collected from plantation time in the first year of production until the end of the experiment using AquaFox Sentek equipment (Agralis). Temperature statements were used to calculate daily GDD (Growing Degree Day). Calculations began from regrowth time (achieved at 50% of regrowth of the plants at crop level), corresponding to 26 March 2017 and 16 April 2018. In 2016, it was calculated from plantation time, in other words, 24 June.

GDD was calculated daily according to the equation and first method presented by McMaster and Wilhelm, 1997:

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

With Tmax = Daily maximum air temperature

Tmin = Daily minimum air temperature

Tbase = Temperature below which vegetative growth is considered to stop. For stevia, we used 10°C as the Tbase according to Guerrero et al., (2015) and Munz et al., (2018).

To obtain SGDD (Sum Growing Degree Day), everyday GDDs were cumulated from the 50% regrowth day until harvest time.

2.5. Statistical analysis :

Statistical analysis was performed with R software version 1.1.463.0 (R Core Team, 2018).

Outliers were deleted from the complete final dataframe, using the “car” package (Fox and Weisberg, 2011).

Regrowth rate trait was analyzed through a generalized linear model, performing via the “glm” function from the “stats” package:

(1) $glm\ (y \sim A\ \times B + \frac{1}{C})$

With y= regrowth scoring; A = genotype factor; B = first year harvest factor; C = block random factor

Mixed linear models were performed on quantitative variables, performing by “lmer” function from the “lme4” package (Bates et al., 2014):

(2) $lm(y{\sim}A\ \times B \times C + \frac{1}{D})$

With y= quantitative variable studied; A = genotype fixed factor; B = first year harvest fixed factor; C = year fixed factor; D = block random factor

Variance analysis of Type II were performed on mixed linear models and generalized linear models using the “car” package, “Anova” function (Fox and Weisberg, 2011).

Marginal means and standard error on linear models with the “emmeans” function were calculated from the “emmeans” package (Russel, 2018). This package was also used to calculate significant differences between genotypes and first year harvest modality with Tuckey’s Honestly Significant Difference (HSD) test on marginal means. A heatmap on evaluated traits was created on the standardized marginal means with the “heatmap.2” function from the “gplots” package (Warnes et al., 2016).

Graphics were created using the “ggplot2” package (Wickham et al., 2016).

3. Results

3.1. Temperature accumulation is similar between the 3 years of monitoring according to the SGDD

Sum of Growing Degree Day (SGDD) expresses the accumulated thermal time received by plants during growing time. SGDD appeared to be lower in 2016 compared to 2017 and 2018, as it was calculated from plantation time (Figure 2A). The regrowth date was earlier in 2017, on 26 March, compared to 2018, on16 April, explaining the SGDD difference between the second and third year of production.

Linear regressions performed on SGDD evolution of each year for the study provided a comparison of SGDD evolution (Figure 2B). Monthly SGDD evolution follows the same regression slopes between years. This result was validated with the calculation of the regression slopes' director coefficient, which was not significantly different between years (Table A3). The increase in temperature during crop growth is similar each year, while significant differences can be shown between months and years (Figure A1 and Table A2). An SGDD calculation was also performed from the beginning of March, independent of the stevia regrowth rate, as represented in Figure A3. In this Figure, the 2016 and 2018 curves are very similar, while the 2017 curve presents a slightly higher increase. The difference between the 2016 and 2018 curves and the 2017 curve starts in March, indicating that the temperature was warmer at the beginning of 2017.

For rainfall, minimum rainfall occurred in April 2018, with just 8.2 mm, while maximum rainfall occured in January 2018, at 156.4 mm (Figure A2). A variance analysis enabled us to observe a major monthly effect (Table A2). However, as the crop was irrigated, we decided not to focus on this record.

3.2. Regrowth rate is significantly impacted by harvest modality

Regrowth rates (%) were recorded in the 2nd and 3rd year of production for 15 genetic resources of *S. rebaudiana* in order to show whether harvesting modalities (3H or 2H) and genotypes have an impact on winter survival capacity over 3 years (Figure 3).

The regrowth rate trait presents high variability among genotypes from 0% for the “Cult33_FRA” genotype to 100% for the “Cult51_FRA” genotype in the third year at 3H modality. Whatever the 2H or 3H modality, major plant loss occurred during the 2016-2017 winter. For 3H modality, 144 plants died from a total of 300, while a less significant decrease was observed during the 2017-2018 winter, with 33 plants dying from a total of 156 plants (Figure 3). For the 2H modality, 26 from a total of 180 plants died in winter 2016-2017, and 14 from a total of 154 plants died in winter 2017-2018.

Overall, the regrowth rate at 3H modality was lower than the regrowth rate at 2H modality, which represents a difference of 30% of the total planted population in 2018. At 2H modality, the lowest regrowth rate group ranged from 23 to 60.9% of planted stevia, while the highest regrowth rate group ranged from 85 to 99%. At 3H modality, the group with the lowest regrowth rate of 0% to 25% comprised of the four genotypes already found at 2H modality plus “Cult29_FRA”, “Cult12_CAN”, and “Cult31_FRA”, while the group with the highest regrowth rate ranging from 60 to 100% comprised of “Cult76_GER”, “Cult37_FRA”, “Cult103_SPA”, “Cult32_FRA”, and “Cult51_FRA” also included at 2H modality. These groups were confirmed by multiple comparison analyses (Table A4). For most genotypes, the same trend was observed with respect to harvest modality, where a genotype showing a low regrowth rate at 3H modality also showed a low regrowth rate at 2H modality, and respectively. A variance analysis between harvest modalities and genotypes reveals a significant harvest modality effect for most of the genotypes and a significant interaction between genotypes and the harvest modality factor for the trait regrowth rate (Table A4), confirming the previous observations. Furthermore, some genotypes (“Cult29_FRA”, “Cult31_FRA”, “Cult102_SPA”) present a high regrowth rate decrease between 2H modality and 3H modality, indicating that a first-year harvest strongly impacts their ability to regrow after the winter. However, three exceptions were identified: the “Cult37_FRA”, “Cult51_FRA” and “Cult103_SPA” genotypes presented a regrowth rate that was not significantly different in either modality.

3.3.SG content and dry leaf biomass are differentially impacted by harvest modality in the third year of production

To evaluate the most productive harvest management in stevia, dry leaf biomass (g/plant) and leaf SG content (%w/w) was followed during a 3-year period in order to measure plant SG yield (g/plant).

Depending on the harvest modality (2H or 3H), plant SG yield (g/plant) of the 15 genotypes is presented in Figure 4 for the third year of production, except for four genotypes ("Cult33_FRA", "Cult75_GER", "Cult12_CAN" and "Cult29_FRA") which presented a very high mortality rate making it impossible to evaluate them. For most genotypes, higher plant SG yield was observed for the 2H modality compared with the 3H modality, with a significant plant SG yield decrease for some genotypes ("Cult102_SPA", "Cult35_FRA"). However, contrasting groups can be identified for both harvest modalities. "Cult103_SPA" and "Cult36_FRA" are high SG producers in both modalities, while some other genotypes are specific to each modality, such as "Cult102_SPA" for the 2H modality and "Cult37_FRA" for the 3H modality. The genotypes with the lower plant SG yield are "Cult51_FRA" and "Cult32_FRA" in both modalities.

For dry leaf biomass traits, extreme behaviours were identified among our tested genotypes, showing a wide variability range (Figure A4.A). Globally, genotypes at 2H modality produced more leaf biomass than genotypes at 3H modality. In the third year of production, 2H and 3H modalities both beget some of the best leaf biomass producers ("Cult103_SPA", "Cult31_FRA", "Cult36_FRA") while some genotypes pertain to the 2H modality, or only to the 3H modality as shown on the heatmap (Figure 5). Regarding the poorest leaf biomass producers among the genotypes tested, each modality shows different genotypes apart from "Cult51_FRA" which is common to the 2H modality and the 3H modality (Figure 5). Only

"Cult102_SPA" and "Cult35_FRA" have completely different performances, depending on whether they were harvested in the first year of production or not.

On the other hand, the SG content trait (%w/w) does not show significant variability compared to previously presented traits (Figure A4.B). The higher SG accumulators at 3H modality are the same at 2H modality, apart from"Cult35_FRA" (Figure 5). Genotypes with low SG content (%w/w) are "Cult63_GER", "Cult31_FRA" and "Cult51_FRA" in both modalities with "Cult32_FRA" and "Cult33_FRA" at 3H and 2H modality, respectively. However, the observation of this trait does not indicate a clear trend in harvest modality, and most genotypes have a similar SG content in both modalities. Some exceptions were identified however: "Cult102_SPA", "Cult36_FRA", "Cult35_FRA" and "Cult51_FRA" presented a clearly higher SG content (%w/w) at 2H modality (Figure A4.B).

Therefore, classification of the fifteen genotypes performed with the heatmap (Figure 5) reveals three groups. Group 1 includes genotypes with higher SG yield and regrowth rate performances than the agronomic performances of the rest of the evaluated genotypes, as shown through the predominance of a warm color. Group 2 includes genotypes with performances close to the mean performances of the evaluated genotypes. Finally, group 3 includes genotypes displaying the lowest performances among this genetic collection, identified on the heatmap by the cold color.

3.4. Dry leaf biomass is impacted by harvest modality over the three years of production

Production traits, according to the harvest modality, are presented in Figure 6.

For most genotypes tested, plant SG yield is lower at 3H than at 2H. Most low performance genotypes at 2H are identified in 3H as well, and respectively for high performance genotypes. However, for a few genotypes, such as "Cult102_SPA" (Figure 6 – A,B), the 3H modality appears to have considerable impact showing as significant decrease in plant SG yield compared to the 2H modality. A significant effect of genotypes was found, with variance analysis appearing to be the most important effect, followed closely by the harvest modality effect (Table A5).

An increase in plant SG yield was also observed between the year of production, whatever the harvest condition considered (Figure 6 – A,B). Indeed, a significant year effect was found in variance analysis, but this effect explains variability less than genotype or harvest modality (Table A5).

Plant SG yield ranges from 2.9 g/plant for the "Cult63_GER" genotype at 3H modality in the second year of production, to 16.7 g/plant for the "Cult102_SPA" genotype at 2H modality in the third year of production. Maximum plant SG yield is five times higher than minimum plant SG yield (Figure 6 – A,B).

Based on plant SG yield results over the 3-year period, the genotypes studied can be classified in different pool of genotypes. The first pool consists of genotypes that are ill-adapted to temperate production conditions as their regrowth rate is equal to zero at 3H condition ("Cult33_FRA", "Cult75_GER", "Cult12_CAN" and "Cult29_FRA"). The second pool of genotypes ("Cult51_FRA", "Cult32_FRA", "Cult63_GER", "Cult37_FRA" and "Cult31_FRA") are adapted to both harvest modalities, and no plant SG yield difference is observed between the 3H and 2H modalities. The last pool presents a higher plant SG yield at

2H than at 3H (“Cult34_FRA”, “Cult35_FRA”, “Cult76_GER”, “Cult103_SPA”, “Cult36_FRA” and “Cult102_SPA”). These genotypes give a better performance in 2H condition.

Dry leaf biomass also presents high variability. It varies between 11 g/plant for the “Cult34_FRA” genotype in the second year of production for the 3H modality to 124.7 g/plant in the third year of production for the “Cult29_FRA” genotype (Figure 6 – C,D). For most genotypes, a dry leaf weight increase was measured between the year of production, the older the plant is, the more leaf biomass it produces. Significant effects of years, harvest modality and genotype on dry leaf weight were detected by variance analyses (Table A5).

The variability of SG content (%w/w) among tested genotypes is also critical. The lowest SG content was 4.5%w/w for the “Cult63_FRA” genotype in the second year of production at 2H modality, while the maximum content reached 18%w/w for the “Cult102_SPA” genotype in the second year of production for 3H modality (Figure 6 – E,F). For most genotypes, a year effect is observed. At 2H modality, there is a global SG content increase between the second and third year with the exception of the “Cult102_SPA”, “Cult36_FRA” and “Cult35_FRA” genotypes, while there is no clear trend for SG content variation for 3H (Figure 6 – E,F). Variance analysis validates these observations, showing a significant and major effect of harvest modality on SG content (%w/w) as well as year of production and genotype effects (Table A5).

No impact of harvest treatment was observed in the first year of production on SG composition between tested genotypes and years (Table A6).

3.5.Negative impact on yield for the 3H modality until 3 years after plantation revealed by cumulated yield

Total SG yield (kg/ha), corresponding to field production, is obtained by multiplying plant SG yield (g/plant) by genotype density, which takes into account winter losses for each genotype and first-year harvest conditions (Table A7).

Comparing the different genotypes, the variability range of cumulative SG yield (230 kg/ha to 1039 kg/ha) is very large, as observed in the individual year study but with an increase in the variability range caused by density balancing. At 2H modality, cumulated total SG yield ranged from 242 kg/ha for “Cult12_CAN” to 1039 kg/ha for “Cult29_FRA”, whereas at 3H modality, cumulated SG yield variability started at 230 kg/ha for “Cult12_CAN” and ended at 602 kg/ha for “Cult103_SPA” (Figure 7 – A). A significant effect of harvest modality on this trait is shown in Table A8. Cumulated dry leaf yield ranged from 2482 kg/ha for “Cult12_CAN” to 7591 kg/ha for “Cult29_FRA” at 2H modality, while it ranged from 1940 kg/ha for “Cult75_GER” to 5486 kg/ha for “Cult103_SPA” at 3H modality (Figure 7 – B). Therefore, a significant effect of harvest modality on dry leaf biomass was also validated (Table A8).

Multiple comparison analyses allowed us to determine some significant differences for harvest modality among the genotypes studied. This can be observed a few genotypes that are the best SG producers, namely “Cult31_FRA”, “Cult102_SPA”, “Cult103_SPA” and “Cult29_FRA”, which all have a better SG yield at 2H condition than at 3H. These genotypes are characterized by a high regrowth rate at 2H but not at 3H, which could explain the significant difference. For cumulated dry leaf weight, we observed the same situation for the same genotypes with the exception of “Cult37_FRA” genotype which has a better cumulated dry leaf weight at 3H modality. This genotype regrowth was not impacted by first-year harvest modality, explaining why the cumulated yield over three years is higher than in two years of production. For some other genotypes, gaps between the modalities can be visually identified, but are not confirmed by statistical analyses. For eleven genotypes, cumulated yield at the 3H modality is lower than at the 2H modality. This allows us to consider each genotype for its economic potential and performance as summarized in Figure 8.

4. Discussion

Under mild weather conditions, as observed in Europe, stevia cultivation as perennial crop is submitted to specific constraints. Indeed, cultivation over several years is possible if the crop can tolerate low winter temperatures that are less frequently encountered in its native environment in Paraguay (Soejarto, 2002). Moreover, little is known about stevia behaviour under temperate conditions, and nothing is known about favorable cropping system. This study therefore examined, the impact on the main yield-related traits of a harvest in the first year of production, evaluated through fifteen genotypes planted in southwest France over three years.

The fifteen genotypes tested in our study belong to the 145 genotypes, including cultivars and landraces studied for their genetic diversity in Cosson et al., (2019). The 15 genotypes belong to one of the 3 genetic clusters defined in this study. As shown in the 2019 study, these 3 genetic clusters each reveal a very high variability in the SG composition and content trait. This is also observed in our harvest management study. For each of the genetic clusters, a very high variability of response is also observed for traits related to post-winter regrowth, leaf biomass, SG content and SG yield.

In addition to genetic diversity, as we can conclude from our results, the year of cultivation is a key factor that can explain the variability of SG yield. In both harvest modalities, an increase over time in SG yield (g/plant) and leaf biomass is observed between the years of production. This finding is supported by previous studies (Andolfi et al., 2006). In the latter study, stevia biomass production from two genotypes over 8 years in Italy showed an increase in leaf biomass and SG yield up to 5 or 6 years. For the SG content trait, no clear trend was identified for genotypes tested. In a previous study performed over two years (Barbet-Massin et al., 2016), SG content (%w/w) in stevia leaves increased with one additional year of production. In our study, the stability of SG profiles and content was confirmed. The increase in SG yield (g/plant) observed over the years of production is mainly linked to the increase in biomass production. This result confirms the importance of foliar biomass in SG yield, as the variability of foliar biomass explains up to 75% of SG yield in stevia.

A wide range of regrowth rates is also observed, with winter tolerant genotypes presenting a regrowth rate ranging from 85% to 99% in the third year of production, to more susceptible ones presenting a regrowth rate from 0 to 25% in the third year of production. However, cold tolerant genotypes at 3H modality had a lower regrowth rate compared to the 2H modality. A few studies in the literature have presented findings on the regrowth trait. A 17% regrowth rate on a Criola population of 96 individuals, without winter coverage was observed by Barbet-Massin et al., (2016). An evaluation of winter hardiness was performed on stevia plants from Ritchers Herbs (Canada) by Moraes et al., (2013) without showing any effect. A Chinese study, conducted in the Hebei region (northeastern China), indicated a regrowth rate of 80% in field conditions with a mulch during the winter period, without specifying the genotypes evaluated (Qingfu and Aihua, 1998).

The wide variability between genotypes is also observed in yield component traits. In particular, significant differences are observed for the fifteen genotypes with respect to SG yield components between the harvest modalities. In the same year, leaf biomass yield and SG yield (g/plant) are higher at 2H treatment. This is particularly visible in the third year of production with a SG yield significantly higher in 2H treatment compared to 3H treatment. It is possible to link plant loss due to winter hardiness with climate between the first and the second year. Indeed, temperatures were colder in the first winter (December 2016 and January 2017) compared to the following winter (Figure A1) and could explain the plant loss rate. However, annual climate variability does not explain differences between the two harvest modalities. Indeed, harvesting in the first year of production had a significant impact on the plants' capacity to support winter temperatures for most of the genotypes tested, leading to a decrease in the regrowth rate in the 2nd and 3rd year of production compared to plants not harvested in the first year (Figure 3).

One explanation could be linked to stevia's capacity to build up the dormancy period. In general, plants can adapt their physiology by endodormancy before winter when the photoperiod and temperatures decrease (Lang et al., 1987). These plants are characterized by arresteed bud development, as well as an increase in ABA and ethylene leading to plant senescence (Fedoroff, 2002). In winter, plants that are in ecodormancy display growth inhibition through temporary unfavorable environmental conditions (Horvath et al., 2003). For perennials, like stevia, which do not retain the aerial part of the plant in winter, storage molecules can be relocated in the roots. These molecules vary depending on the plant: e.g., sucrose, amino acids, soluble proteins (Volenec et al., 1996) with increasing content as temperatures decrease (Shen et al., 2017). To date, no study has fully defined the nature of the storage molecules in stevia. In stevia, SG are accumulated in leaves with up to 12% of the DW in leaves and up to 0.25% in roots (Ceunen and Geuns, 2013b). The physiological role of SG production in plants is still under discussion. However their role as storage for SG molecules has been investigated in earlier research (Bondarev et al., 2003; Ceunen and Geuns, 2013c; De Guzman et al., 2018). The hypothesis of a short-term storage molecule seems unlikely. Indeed, SG content remains stable both day and night while sucrose and glucose fluctuated (Ceunen and Geuns, 2013c; De Guzman, 2010). However, SG may act as long-term storage molecules and seem to be involved in flowering and seed ripening, with a decrease of up to 35% in SG content in leaves, but an increase in roots at flower budding from 0.05 to 0.35% w/w (Ceunen and Geuns, 2013b). In the first year of production in our study, flowering only occurs at 2H modality, probably leading to higher SG content in roots.

Therefore, the post-winter regrowth rate of stevia could depend on the mobilization of storage molecules from the roots to the newly formed shoots as in all perennial plants (Cooke et al., 2012). In alfalfa, the genetic resources accumulating more vegetative storage proteins in the roots have enhanced winter hardiness (Cunningham et al., 2001). These storage components can also be used by plants for regrowth after harvest (Hendershot and Volenec, 1993). Even a partial harvest can impact the sink/source relationship in plants. Indeed, in forage plants, a defoliation event modifies the plant's carbon allocation, which is driven to the leaves rather than the roots in order to reestablish leaf biomass (Cullen et al., 2006). In a study on the blueberry, Strik and Buller, (2005), identified a negative impact of harvesting during establishment on the following year's production compared to no harvest modality, leading to a reduction in vegetative growth, aerial and root biomass production, as well as a decrease in fruit yield the following year (from between 19% and 44% depending on the genotypes). Identically, for asparagus (Wilson et al., 1999), a harvest performed just before winter led to a decrease in yield the following years, with fewer and lighter of spears. This seems due to the low amount of stored soluble carbohydrate.

At 3H modality, following the first year harvest, stevia carbon fixation is limited by the small remaining canopy and the small amount of fixed carbon is devoted to new shoot development, with no possibility of reallocating carbon to the roots. On the other hand, the 2H modality plants can pursue photosynthesis activity and the accumulation of storage molecules in roots until canopy senescence in late fall. 3H modality plants should present a lower storage molecule content in the roots than 2H modality plants, leading to higher winter mortality.

In mild growth conditions, this crop may need a complete first year of production to generate a fully functional plant ready to face winter conditions. To our knowledge, no study to date has looked at stevia's establishment duration or conditions. Our findings suggest that a four-month establishment duration (from June to September) is too short for some stevia genotypes. A precocious harvest could create stress in the potential non-mature plant, leading to lower tolerance to cold winter temperatures. A precocious harvest could also prevent regular stevia root development and impact the roots' resistance to frost tolerance. In plants, fine roots are less tolerant than lignified roots (Ambroise et al., 2020). Stevia roots are described as fibrous, filiform and perennial by Angelini et al., (2018) with a dense root system remaining in the superficial soil layers. Another study showed that at regrowth time, shoots appear on the previous year's stems where roots are attached (Moraes et al., 2013). The root architecture of the different genotypes and the capacity to survive through winter could explain the difference in regrowth rates observed between our two harvest modalities. Root architecture and its role in maintaining culture overtime are still largely unknown in stevia.

To evaluate the most productive itinerary, cumulated SG yield (kg/ha) offers an interesting study avenue. It allows us to compare production in both treatments over three years of production. This variable provides information closely linked to the agronomic and economic point of view. The evaluation of this trait clearly allows us to conclude that for most of the genotypes studied, a harvest in the first year has a negative impact that is not compensated in the following production years. Harvesting in the first year of production, which is the year of the crop's establishment, has a negative consequence on plant yield performance in the following years.

5. Conclusion

The results obtained in the field conditions evaluation of two cropping systems for fifteen stevia genotypes over a three-year period show that harvesting in the first year of production negatively impacted genotype performances. Our study indicates that the regrowth rate, plant SG yield and leaf biomass yield decrease in the situation of harvesting in the first year of production for most of the genotypes evaluated. No pronounced tendency was observed for SG content. This study allowed us to identify the genotypes adapted to the southwestern environment in France, namely, “Cult103_SPA” and “Cult36_FRA” which present the best SG yield in both cropping systems. This information could be directly integrated into a breeding program. In the future, it would be interesting to perform further investigations on the long-term impact on stevia yield of an early harvest, such as 6 to 7 years. It would also be very interesting to study the impact of a partial harvest of the upper third of the crop, allowing both an income for the producer and the maintenance of the reserve capacity for the plant before winter. This study highlighted the lack of knowledges on the stevia root development mechanisms and stevia overwintering strategies in temperate conditions. Indeed, studies on the development of roots after plantation, the sink-source relationships between aerial and ground biomass during the life cycle of stevia, and the nature of stevia storage molecules would help to understand the tendencies observed between the two harvest modalities. More broadly, this study provides information on possible cropping system strategies for the development of stevia production in mild climate zones.

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Tables:

Table 1: List of the 15 genetic resources of *Stevia rebaudiana* in field conditions in the south-western of France (Cosson et al., 2019)

Name	Providers	Country
Cult75_GER	EUSTAS gene bank	Germany
Cult76_GER	EUSTAS gene bank	Germany
Cult63_GER	EUSTAS gene bank	Germany
Cult33_FRA	Oviatis	France
Cult34_FRA	Oviatis	France
Cult102_SPA	Oviatis	France
Cult103_SPA	Oviatis	France
Cutl35_FRA	Oviatis	France
Cult29_FRA	Oviatis	France
Cult12_CAN	Oviatis	France
Cult36_FRA	Oviatis	France
Cult37_FRA	Oviatis	France
Cult31_FRA	Oviatis	France
Cult32_FRA	Oviatis	France
Cult51_FRA	Stevia store	Paraguay

Figure captions:

Figure 1: Experimental design performed in a field trial in Liposthey (40). For every 15 genotypes planted in the field trial, 20 plants were harvested in the first, second and third years of production and correspond to the 3H modality, and 12 plants were cut down in December 2016 and harvested in the second and third years of production, corresponding to the 2H modality.

Figure 2: Evolution of Sum of Growing Degree Days (SGDD) for three years of production 2016, 2018 and 2017 in the experimental field in Liposthey (40). (A), SGDD was calculated from the plantation date for 2016 and from the regrowth point for 2017 and 2018. (B) represents linear regression between SGDD and Julian days.

Figure 3: Regrowth rate (%) in the 2nd and 3rd year of production for 15 genetic resources of *S. rebaudiana* planted in the experimental field located in Liposthey (40), south-west France, depending on the harvest modality in the first year of production (harvested or not harvested). The results show the regrowth rate measured on July 11’ 2017 and June 6, 2018. Genetic resources are classified according to the regrowth rate in the first-year harvest modality. The results of the Tuckey HSD test for a specific genotype according to its regrowth rate in both modalities are shown by the asterisk next to the genotype’s names. Genotypes represented with an asterisk have significantly different regrowth rate according to the harvesting modality, p=0.05.

Figure 4: Representation of SG yield (g/plant) for 15 genetic resources of *Stevia rebaudiana* planted in a field trial in Liposthey (40), south-west France, for the third year of production according to the harvest modality. The 3H modality corresponds to plants harvested in the first, second and third years of production, while the 2H modality corresponds to plants harvested only in the second and third year of production. The barplots represent the Least-Squares Mean of 4 randomized blocks, corresponding to a total of 3 to 20 plants per genetic resource, with standard deviation.

Figure 5: Classification of 15 *S. rebaudiana* genetic resources according to the stevia production traits: SG yield components and winter survival rate, according to the harvest modality. Heatmap was built based on a standardized matrix calculated on marginal means of the third-year of measure. For each variable in column, the color gradient represents the result for genetic resources in row: the cold colors (green and blue) indicates the lowest performances compared to the rest of genotypes while the warm color (orange and red) represents the highest performances compared to the rest of genotypes. 3H modality corresponds to plants harvested in first, second and third years of production while 2H modality corresponds to plants harvested only in the second and third year of production.

Figure 6: Representation of yield components of *S. rebaudiana* : SG yield (A-B), dry leaf biomass (C – D), SG content (E – F) of genetic resources planted in a field trial in Liposthey (40), south-west France, for three years of production according to the harvest modality. The 3H modality corresponds to plants harvested in the first, second and third years of production, while the 2H modality corresponds to plants harvested only in the second and third year of production. The barplots represent the Least-Squares Mean of 4 randomized blocks, corresponding to a total of 3 to 20 plants per genetic resource, with standard deviation.

Figure 7: Representation of *Stevia rebaudiana* cumulated yield components over three years of production: SG yield (A), dry leaf biomass (B) of genetic resources planted in a field trial in Liposthey (40), south-west France, according to the harvest modality. The 3H modality corresponds to plants harvested in the first, second and third years of production, while the 2H modality corresponds to plants harvested only in the second and third year of production. The barplots represent the Least-Squares Mean of 4 randomized blocks, corresponding to a total of 3 to 20 plants per genetic resource, with standard deviation. The results of multiple comparisons by Tukey's Honestly Significantly Difference are indicated by an asterisk, which shows a significant difference at p = 0.05 level.

Figure 8: Classification of 15 *S. rebaudiana* genetic resources on their cumulated SG yield (kg/ha) according to the harvest modality.

3H modality corresponds to plants harvested in the first, second and third years of production while 2H modality corresponds to plants harvested only in the second and third year of production.

For each variable in column, the color gradient represents the result for genetic resources in row: the green color indicates a lower performances compared to the other modality, while the red color represents a higher performance compared to the other modality. The color orange indicates that genotype performance is similar in both harvesting modality.

References:

Ambroise, V., Legay, S., Guerriero, G., Hausman, J.-F., Cuypers, A., Sergeant, K., 2020. The Roots of Plant Frost Hardiness and Tolerance. Plant Cell Physiol 61, 3–20. https://doi.org/10.1093/pcp/pcz196

Andolfi, L., Macchia, M., Ceccarini, L., 2006. Agronomic-productive characteristics of two genotype of Stevia rebaudiana in central Italy. Ital. J. of Agron. 1, 257–262.

Angelini, L.G., Martini, A., Passera, B., Tavarini, S., 2018. Cultivation of Stevia rebaudiana Bertoni and Associated Challenges, in: Mérillon, J.-M., Ramawat, K.G. (Eds.), Sweeteners. Springer International Publishing, Cham, pp. 35–85. https://doi.org/10.1007/978-3-319-27027-2_8

Barbet-Massin, C., 2015. Sélectionner et cultiver Stevia rebaudiana Bertoni en milieu tempéré : exploration de la variabilité de la teneur et de la composition en glycosides de steviol (PhD).

Barbet-Massin, C., Giuliano, S., Alletto, L., Daydé, J., Berger, M., 2016. Towards a semi-perennial culture of Stevia rebaudiana (Bertoni) Bertoni under temperate climate: effects of genotype, environment and plant age on steviol glycoside content and composition. Genet Resour Crop Evol 63, 685–694. https://doi.org/10.1007/s10722-015-0276-9

Barbet-Massin, C., Giuliano, S., Alletto, L., Daydé, J., Berger, M., 2015. Nitrogen Limitation Alters Biomass Production but Enhances Steviol Glycoside Concentration in Stevia rebaudiana Bertoni. PLoS ONE 10, e0133067. https://doi.org/10.1371/journal.pone.0133067

Bates, D., Mächler, M., Bolker, B., Walker, S., 2014. Fitting Linear Mixed-Effects Models using lme4. arXiv:1406.5823 [stat].

Bondarev, N.I., Sukhanova, M.A., Reshetnyak, O.V., Nosov, A.M., 2003. Steviol Glycoside Content in Different Organs of Stevia rebaudiana and Its Dynamics during Ontogeny. Biologia Plantarum 47, 261–264. https://doi.org/10.1023/B:BIOP.0000022261.35259.4f

Bridel, M., Lavieille, R., 1931. The sweet principle in Kaa-he-e (Stevia rebaudiana. Bertoni). II. Hydrolysis of stevioside by enzymes. III. Steviol by enzymic hydrolysis and isosteviol by acid hydrolysis. Bull. Soc. Chim. Biol 13, 781–96.

Ceunen, Geuns, 2013a. Steviol Glycosides: Chemical Diversity, Metabolism, and Function. Journal of Natural Products 76, 1201–1228. https://doi.org/10.1021/np400203b

Ceunen, Geuns, 2013b. Influence of photoperiodism on the spatio-temporal accumulation of steviol glycosides in Stevia rebaudiana (Bertoni). Plant Science 198, 72–82. https://doi.org/10.1016/j.plantsci.2012.10.003

Ceunen, Geuns, 2013c. Glucose, sucrose, and steviol glycoside accumulation in Stevia rebaudiana grown under different photoperiods. Biol Plant 57, 390–394. https://doi.org/10.1007/s10535-012-0289-6

Chaturvedula, V.S.P., Meneni, S.R., 2017. A New Penta β-D-Glucopyranosyl Diterpene from Stevia rebaudiana. International Journal of Organic Chemistry 07, 91–98. https://doi.org/10.4236/ijoc.2017.72008

Chaturvedula, V.S.P., Upreti, M., Prakash, I., 2011. Structures of the novel α-glucosyl linked diterpene glycosides from Stevia rebaudiana. Carbohydrate Research 346, 2034–2038. https://doi.org/10.1016/j.carres.2011.06.023

Coelho, L., Osório, J., Beltrão, J., Reis, M., 2019. Efeito da aplicação de compostos orgânicos no controlo de infestantes na cultura de Stevia rebaudiana e nas propriedades do um solo na região do Mediterrâneo. Revista de Ciências Agrárias 42, 111–120. https://doi.org/10.19084/RCA18281

Cooke, J.E.K., Eriksson, M.E., Junttila, O., 2012. The dynamic nature of bud dormancy in trees: environmental control and molecular mechanisms. Plant, Cell & Environment 35, 1707–1728. https://doi.org/10.1111/j.1365-3040.2012.02552.x

Cosson, P., Hastoy, C., Errazzu, L.E., Budeguer, C.J., Boutié, P., Rolin, D., Schurdi-Levraud, V., 2019. Genetic diversity and population structure of the sweet leaf herb, Stevia rebaudiana B., cultivated and landraces germplasm assessed by EST-SSRs genotyping and steviol glycosides phenotyping. BMC Plant Biol 19, 436. https://doi.org/10.1186/s12870-019-2061-y

Cullen, B.R., Chapman, D.F., Quigley, P.E., 2006. Comparative defoliation tolerance of temperate perennial grasses. Grass and Forage Science 61, 405–412. https://doi.org/10.1111/j.1365-2494.2006.00548.x

Cunningham, S.M., Gana, J.A., Volenec, J.J., Teuber, L.R., 2001. Winter Hardiness, Root Physiology, and Gene Expression in Successive Fall Dormancy Selections from ‘Mesilla’ and ‘CUF 101’ Alfalfa. Crop Science 41, 1091–1098. https://doi.org/10.2135/cropsci2001.4141091x

De Guzman, R., 2010. Autoecological roleof steviol glycosides in Stevia Rebaudiana Bertoni. Faculty of Sciences, Engineering and Health CQUniversity Australia.

De Guzman, R., Midmore, D.J., Walsh, K.B., 2018. Do Steviol Glycosides Act Either as a Carbon Storage Pool or in Osmoregulation within Leaves of *Stevia rebaudiana* ? J. Nat. Prod. 81, 2357–2363. https://doi.org/10.1021/acs.jnatprod.8b00196

Denev, P.N., Uchkunov, I., Uchkunov, V., Kratchanova, M.G., 2017. Content of steviol glycosides in stevia (Stevia Rebaudiana B .) genotypes cultivated in Bulgaria. Bulgarian Chemical Communications 49, 121–125.

Fedoroff, N.V., 2002. Cross-Talk in Abscisic Acid Signaling. Sci. STKE 2002, re10–re10. https://doi.org/10.1126/stke.2002.140.re10

Ferrazzano, G.F., Cantile, T., Alcidi, B., Coda, M., Ingenito, A., Zarrelli, A., Di Fabio, G., Pollio, A., 2016. Is Stevia rebaudiana Bertoni a Non Cariogenic Sweetener? A Review. Molecules 21, 38. https://doi.org/10.3390/molecules21010038

Fox, J., Weisberg, S., 2011. Multivariate Linear Models in R 31.

Gantait, S., Das, A., Banerjee, J., 2018. Geographical Distribution, Botanical Description and Self-Incompatibility Mechanism of Genus Stevia. Sugar Tech 20, 1–10. https://doi.org/10.1007/s12355-017-0563-1

Grevsen, K., Sorensen, J.N., Clausen, M.R., Henriksen, M.B., Jensen, I., Kyed, S., 2015. A Danish research project for the development of ’Green Stevia’-a natural sweetener for organic food products. Proceedings of the 8th Stevia Symposium.

Guerrero, D.R., Bär, A., Kryvenki, M., Domínguez, M., 2015. Phenological and morphological evaluation of 10 clones of Stevia (Stevia rebaudiana) as the thermal time. RIA, Revista de Investigaciones Agropecuarias 41, 317–324.

Hastoy, C., 2018. Caractérisation de la variabilité phénotypique de ressources génétiques de Stevia rebaudiana (Bertoni) : analyse des composantes du rendement et critères de sélection en condition de production (thesis). Bordeaux.

Hastoy, C., Cosson, P., Cavaignac, S., Boutié, P., Waffo-Teguo, P., Rolin, D., Schurdi-Levraud, V., 2019. Deciphering performances of fifteen genotypes of Stevia rebaudiana in southwestern France through dry biomass and steviol glycoside evaluation. Industrial Crops and Products 128, 607–619. https://doi.org/10.1016/j.indcrop.2018.09.053

Hellfritsch, C., Brockhoff, A., Stähler, F., Meyerhof, W., Hofmann, T., 2012. Human Psychometric and Taste Receptor Responses to Steviol Glycosides. J. Agric. Food Chem. 60, 6782–6793. https://doi.org/10.1021/jf301297n

Hendershot, K.L., Volenec, J.J., 1993. Taproot nitrogen accumulation and use in overwintering alfalfa (Medicago sativa L.). Journal of Plant Physiology 141, 68–74. https://doi.org/10.1016/S0176-1617(11)80853-9

Horvath, D.P., Anderson, J.V., Chao, W.S., Foley, M.E., 2003. Knowing when to grow: signals regulating bud dormancy. Trends in Plant Science 8, 534–540. https://doi.org/10.1016/j.tplants.2003.09.013

Ibrahim, M.A., Rodenburg, D.L., Alves, K., Perera, W.H., Fronczek, F.R., Bowling, J., McChesney, J.D., 2016. Rebaudiosides R and S, Minor Diterpene Glycosides from the Leaves of Stevia rebaudiana. J. Nat. Prod. 79, 1468–1472. https://doi.org/10.1021/acs.jnatprod.6b00048

Labrador, J., Colmenares, R., Sánchez, E., Creus, J., García, N., Blázquez, J., Moreno, M.M., 2014. Soil response to biodynamic farming practices in estevia -Stevia Rebaudiana- (Extremadura, Spain) 16, 1118.

Lang, G.A., Early, J.D., Martin, G.C., Darnell, R.L., 1987. Endo-, para-, and ecodormancy: physiological terminology and classification for dormancy research. HortScience 22, 371–377.

Lankes, G., Grosser, P., 2015. Evaluation of Stevia rebaudiana genotypes at a location in the Alentejo region in Portugal. Proceedings of the 8th Stevia Symposium 167–176.

Lankes, G., Zabala, U.M., 2011. Evaluation of Stevia rebaudiana genotypes. Proceedings of the 5th Stevia Symposium 75–87.

Lavini, A., Riccardi, M., Pulvento, C., Luca, S.D., Scamosci, M., d’Andria, R., 2008. Yield, Quality and Water Consumption of Stevia rebaudiana Bertoni Grown under Different Irrigation Regimes in Southern Italy. Italian Journal of Agronomy 3, 135–143.

Le Bihan, Z., Cosson, P., Rolin, D., Schurdi-Levraud, V., 2020. Phenological growth stages of stevia (Stevia rebaudiana Bertoni) according to the Biologische Bundesanstalt Bundessortenamt and Chemical Industry (BBCH) scale. Annals of Applied Biology n/a. https://doi.org/10.1111/aab.12626

Leyshon, A.J., Campbell, C.A., 1992. Effect of timing and intensity of first defoliation on subsequent production of 4 pasture species. Rangeland Ecology & Management / Journal of Range Management Archives 45, 379–384.

Libik-Konieczny, M., Capecka, E., Kąkol, E., Dziurka, M., Grabowska-Joachimiak, A., Sliwinska, E., Pistelli, L., 2018. Growth, development and steviol glycosides content in the relation to the photosynthetic activity of several Stevia rebaudiana Bertoni strains cultivated under temperate climate conditions. Scientia Horticulturae 234, 10–18. https://doi.org/10.1016/j.scienta.2018.02.015

Mao, G., Chaturvedula, V.S.P., Vick, J.E., Yu, O., 2017. Enzymatic Synthesis and Structural Characterization of Rebaudioside D3, a Minor Steviol Glycoside of <i>Stevia rebaudiana</i> Bertoni. American Journal of Plant Sciences 08, 441–450. https://doi.org/10.4236/ajps.2017.83030

McMaster, G., Wilhelm, W., 1997. Growing degree-days: one equation, two interpretations. Publications from USDA-ARS / UNL Faculty.

Metivier, J., Viana, A.M., 1979. The Effect of Long and Short Day Length upon the Growth of Whole Plants and the Level of Soluble Proteins, Sugars, and Stevioside in Leaves of *Stevia rebaudiana* Bert. Journal of Experimental Botany 30, 1211–1222. https://doi.org/10.1093/jxb/30.6.1211

Mintel Global New Products Database, 2017. Global Food and Beverage Products with Stevia: 2011–2016 Data. (2017).

Montoro, P., Molfetta, I., Maldini, M., Ceccarini, L., Piacente, S., Pizza, C., Macchia, M., 2013. Determination of six steviol glycosides of Stevia rebaudiana (Bertoni) from different geographical origin by LC–ESI–MS/MS. Food Chemistry 141, 745–753. https://doi.org/10.1016/j.foodchem.2013.03.041

Moraes, R.M., Donega, M.A., Cantrell, C.L., Mello, S.C., McChesney, J.D., 2013. Effect of harvest timing on leaf production and yield of diterpene glycosides in Stevia rebaudiana Bert: A specialty perennial crop for Mississippi. Industrial Crops and Products 51, 385–389. https://doi.org/10.1016/j.indcrop.2013.09.025

Munz, S., Präger, A., Merkt, N., Claupein, W., Graeff-Hönninger, S., 2018. Leaf area index, light interception, growth and steviol glycoside formation of Stevia rebaudiana Bertoni under field conditions in southwestern Germany. Industrial Crops and Products 111, 520–528. https://doi.org/10.1016/j.indcrop.2017.11.021

Parris, C.A., Shock, C.C., Qian, M., 2016. Dry Leaf and Steviol Glycoside Productivity of Stevia rebaudiana in the Western United States. horts 51, 1220–1227. https://doi.org/10.21273/HORTSCI11149-16

Perera, W.H., Ghiviriga, I., Rodenburg, D.L., Alves, K., Bowling, J.J., Avula, B., Khan, I.A., McChesney, J.D., 2017. Rebaudiosides T and U, minor C-19 xylopyranosyl and arabinopyranosyl steviol glycoside derivatives from Stevia rebaudiana (Bertoni) Bertoni. Phytochemistry 135, 106–114. https://doi.org/10.1016/j.phytochem.2016.12.001

Perera, W.H., Ramsaroop, T., Carvalho, R., Rodenburg, D.L., McChesney, J.D., 2019. A silica gel orthogonal high-performance liquid chromatography method for the analyses of steviol glycosides: novel tetra-glucopyranosyl steviol. Natural Product Research 33, 1876–1884. https://doi.org/10.1080/14786419.2018.1478826

Pordel, R., Esfahani, M., Kafi, M., Nezami, A., 2015. Response of Stevia rebaudiana Bertoni root system to waterlogging and terminal drought stress. J. Bio & Env. Sci. 6, 238–247.

Prakash, I., Chaturvedula, V.S.P., 2014. Structures of Some Novel α-Glucosyl Diterpene Glycosides from the Glycosylation of Steviol Glycosides. Molecules 19, 20280–20294. https://doi.org/10.3390/molecules191220280

Prakash, I., Markosyan, A., Bunders, C., 2014. Development of Next Generation Stevia Sweetener: Rebaudioside M. Foods 3, 162–175. https://doi.org/10.3390/foods3010162

Qingfu, M., Aihua, M., 1998. Studies on overwintering of Stevia rebaudiana Bertoni perennial root. Journal of Hebei Agricultural Sciences 01.

Reis, M., Coelho, L., Santos, G., Kienle, U., Beltrão, J., 2015. Yield response of stevia (Stevia rebaudiana Bertoni) to the salinity of irrigation water. Agricultural Water Management 152, 217–221. https://doi.org/10.1016/j.agwat.2015.01.017

Russel, L., 2018. Estimated Marginal Means, aka Least-Squares Means.

Serfaty, M., Ibdah, M., Fischer, R., Chaimovitsh, D., Saranga, Y., Dudai, N., 2013. Dynamics of yield components and stevioside production in Stevia rebaudiana grown under different planting times, plant stands and harvest regime. Industrial Crops and Products 50, 731–736. https://doi.org/10.1016/j.indcrop.2013.08.063

Shen, X., Li, J., Feng, P., Jiang, C., Li, R., Zhang, H., Jia, H., Zheng, H., Zhu, B., 2017. Root physiological traits and cold hardiness of alfalfa grown alone or mix-sowed with meadow fescue. Acta Agriculturae Scandinavica, Section B — Soil & Plant Science 67, 235–244. https://doi.org/10.1080/09064710.2016.1249940

Soejarto, D., 2002. Botany of Stevia and Stevia rebaudiana, in: Stevia : The Genus Stevia, Medicinal and Aromatic Plants - Industrial Profiles. CRC Press, pp. 18–39. https://doi.org/10.1201/9780203165942.ch2

Soejarto, D.D., Compadre, C.M., Medon, P.J., Kamath, S.K., Kinghorn, A.D., 1983. Potential sweetening agents of plant origin. II. field search for sweet-tastingStevia species. Econ Bot 37, 71–79. https://doi.org/10.1007/BF02859308

Strik, B., Buller, G., 2005. The Impact of Early Cropping on Subsequent Growth and Yield of Highbush Blueberry in the Establishment Years at Two Planting Densities is Cultivar Dependant. HortScience 40, 1998–2001. https://doi.org/10.21273/HORTSCI.40.7.1998

Sumida, T., 1980. Studies on Stevia rebaudiana Bertoni as a new possible crop for sweetening resource in Japan. Kenkyu hokoku. = Journal of the Central Agricultural Experiment Station-Japan, Nogyo Shikenjo, Konosu.

Sun, J., 2016. Development of stevia and steviol glycosides industry in China, in: Proceedings of the 9th Stevia Symposium 2016. Presented at the From field to fork, Jan M.C. Geuns, Gothenburg, Sweden, pp. 145–150.

Takács-Hájos, M., Rubóczki, T., Szabó, F.M., Kiss, A., 2019. Effect of Environmentally Friendly Nutrition Supply on Stevia (Stevia rebaudiana B.) Production. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 47, 201–206. https://doi.org/10.15835/nbha47111232

Tavarini, S., Angelini, L.G., 2013. Stevia rebaudiana Bertoni as a source of bioactive compounds: the effect of harvest time, experimental site and crop age on steviol glycoside content and antioxidant properties. Journal of the Science of Food and Agriculture 93, 2121–2129. https://doi.org/10.1002/jsfa.6016

Volenec, J.J., Ourry, A., Joern, B.C., 1996. A role for nitrogen reserves in forage regrowth and stress tolerance. Physiologia Plantarum 97, 185–193. https://doi.org/10.1111/j.1399-3054.1996.tb00496.x

Vouillamoz, J.F., Wolfram-Schilling, E., Carron, C.A., Baroffio, C.A., 2016. Agronomical and phytochemical evaluation of Stevia rebaudiana genotypes. Julius-Kühn-Archiv 86–88.

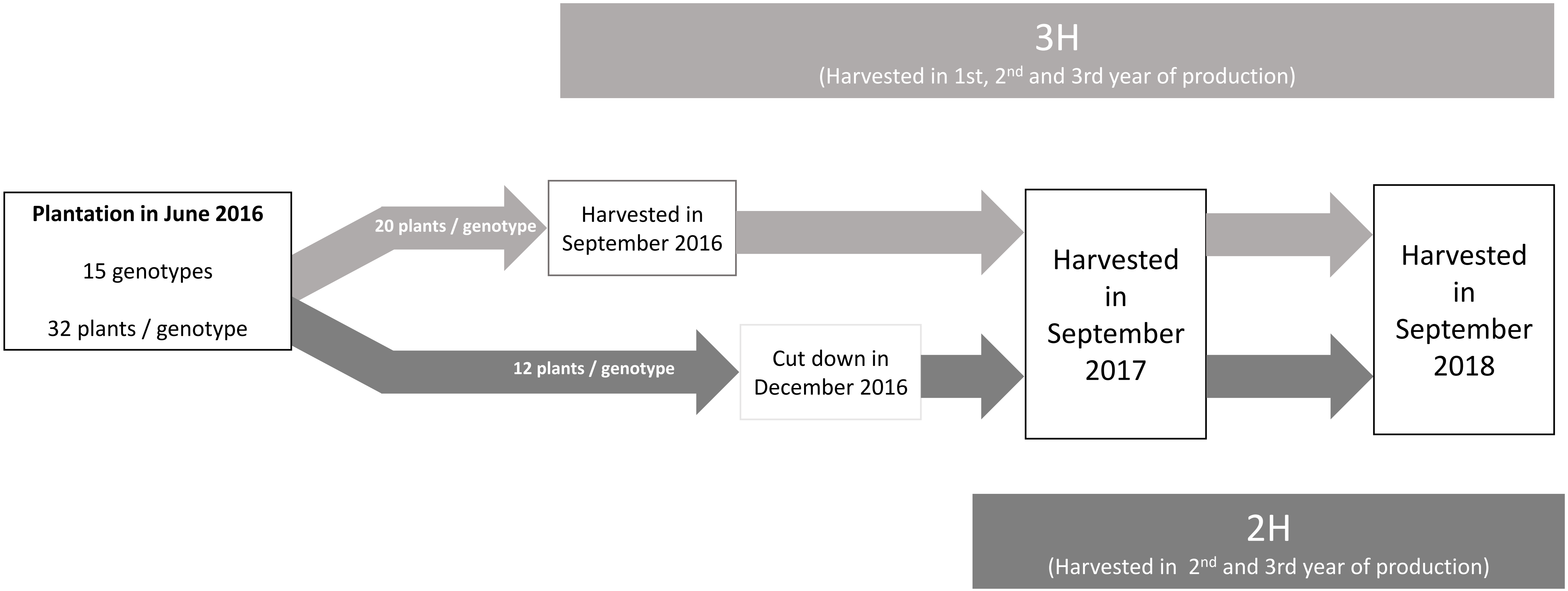
Warnes, G., Bolker, B., Bonebakker, L., Gentelman, R., Liaw, H.A., Lumley, T., Maechler, M., Moeller, S., Schwartz, M., Venables, B., 2016. Package “gplots.”

Wickham, H., Chang, W., Henry, L., Lin Pederson, T., Takahashi, K., Wilke, C., Woo, K., Yutani, H., Dunnington, D., 2016. Ggplot2: elegant graphics for data analysis. Springer-Velag New York.

Wilson, D.R., Sinton, S.M., Wright, C.E., 1999. Influence of time of spear harvest on root system resources during the annual growth cycle of asparagus. Acta Hortic. 313–320. https://doi.org/10.17660/ActaHortic.1999.479.43

Woelwer-Rieck, U., Lankes, C., Wawrzun, A., Wüst, M., 2010. Improved HPLC method for the evaluation of the major steviol glycosides in leaves of Stevia rebaudiana. Eur Food Res Technol 231, 581–588. https://doi.org/10.1007/s00217-010-1309-4

Zachokostas, P., 2016. Effects of Agricultural Techniques on the Quantitative and Qualitative Characteristics of Stevia Rebaudiana Bertoni, under Mediterranean Conditions (Karditsa, Thessaly, Greece). APAR 3. https://doi.org/10.15406/apar.2016.03.00105



3H

(Harvested in 1st, 2nd and 3rd year of production)

Plantation in June 2016

15 genotypes

32 plants / genotype

20 plants / genotype

Harvested in September 2016

12 plants / genotype

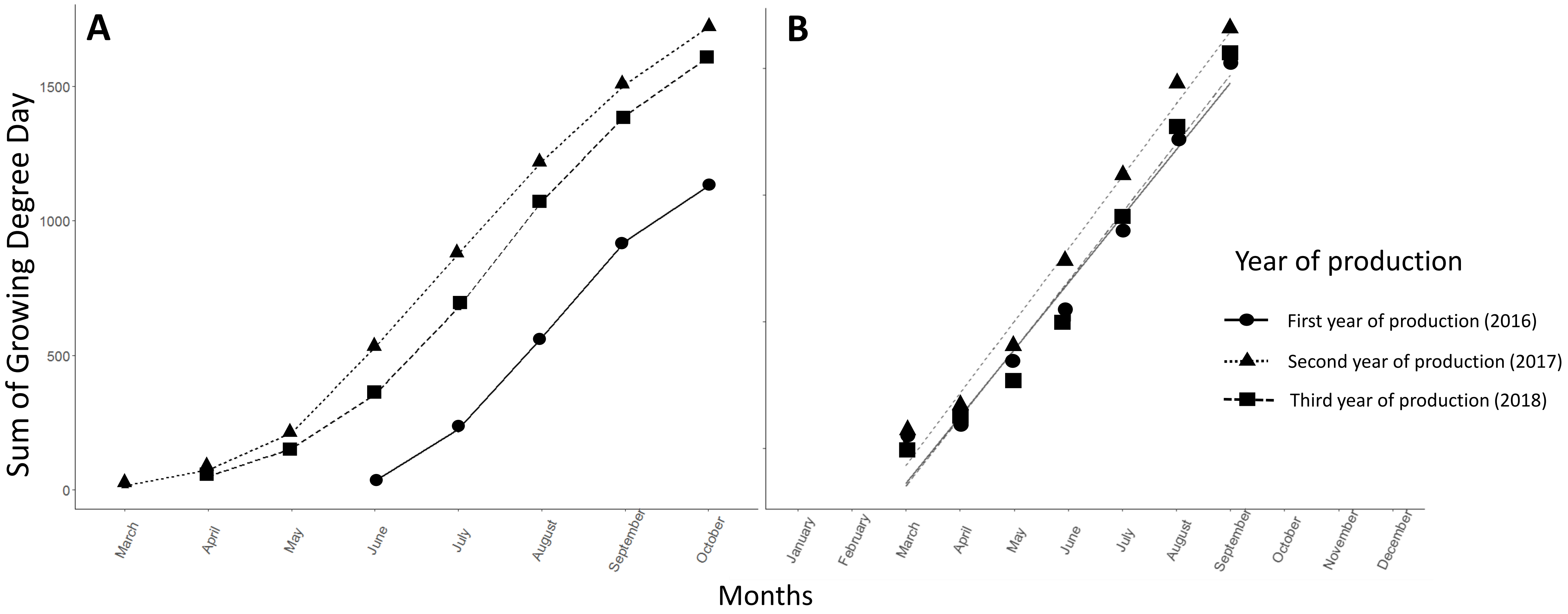
Cut down in December 2016

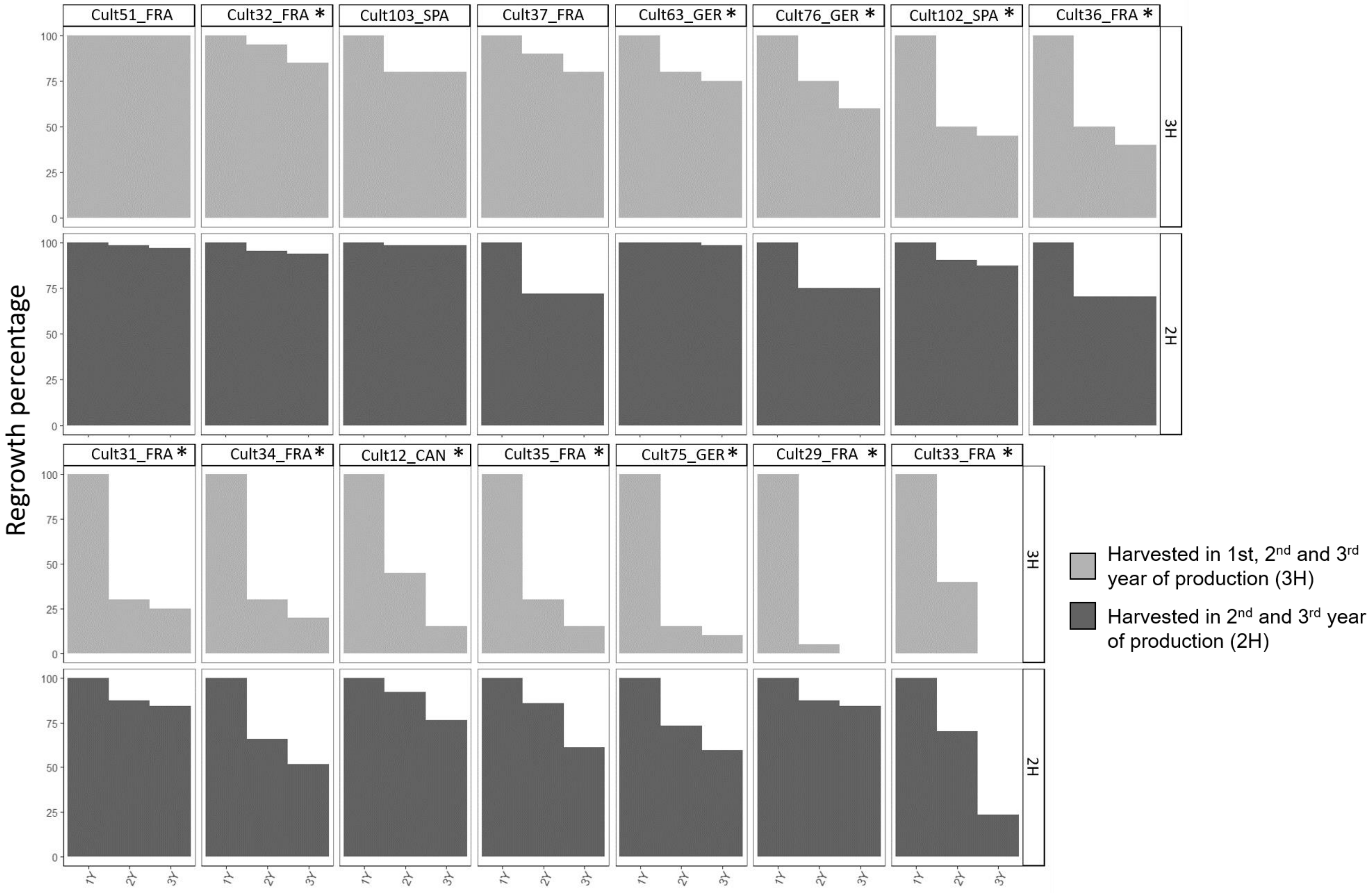
Harvested in September 2017

Harvested in September 2018

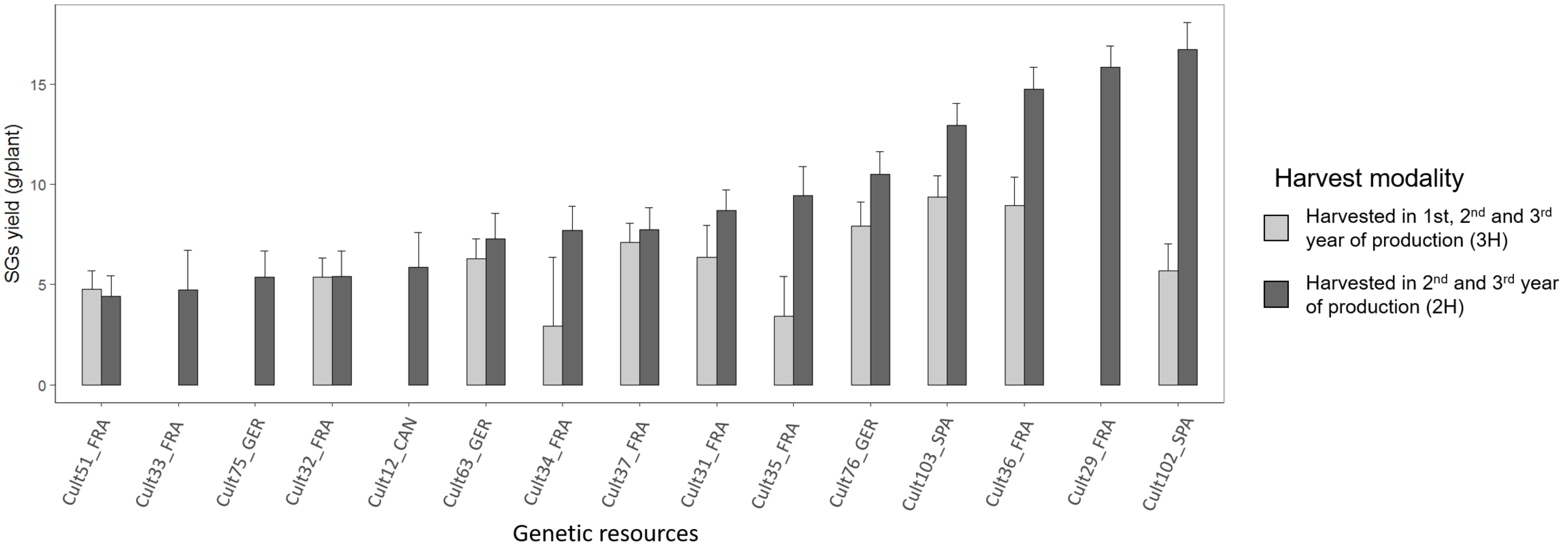
2H

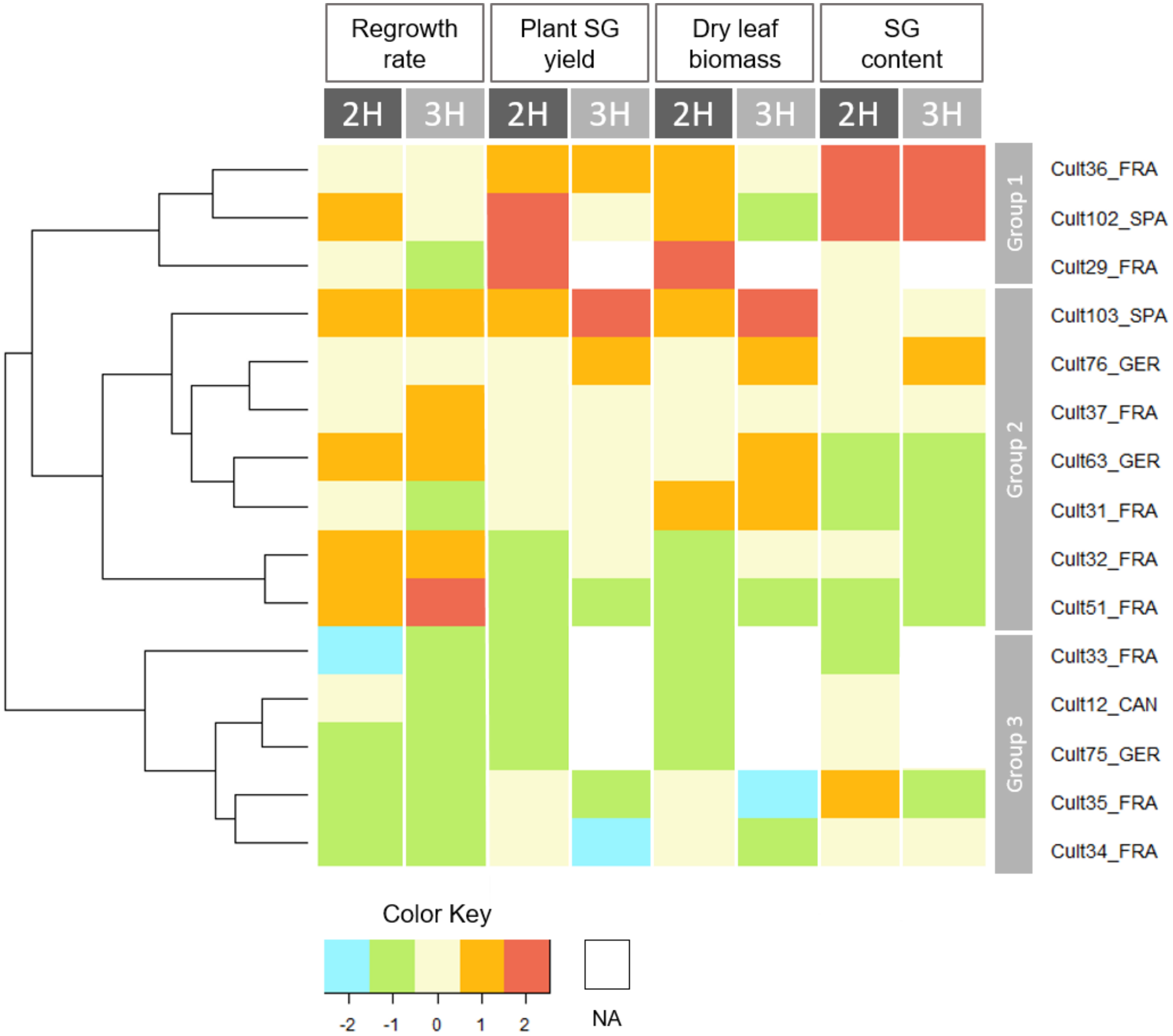
(Harvested in 2nd and 3rd year of production)

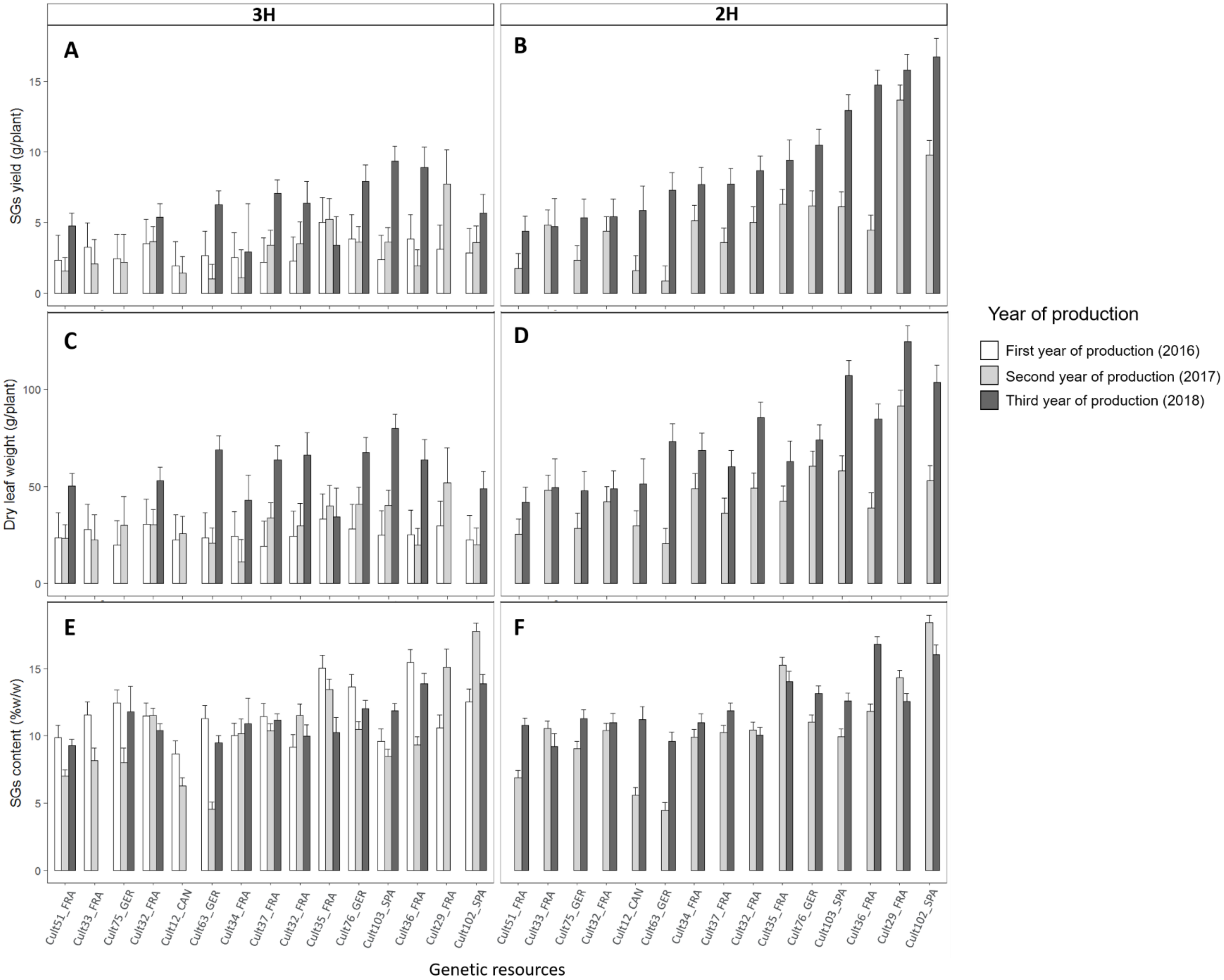

A
B
Sum of Growing Degree Day
0
500
1000
1500
Months
March
April
May
June
July
August
September
October
January
February
November
December
Year of production
First year of production (2016)
Second year of production (2017)
Third year of production (2018)

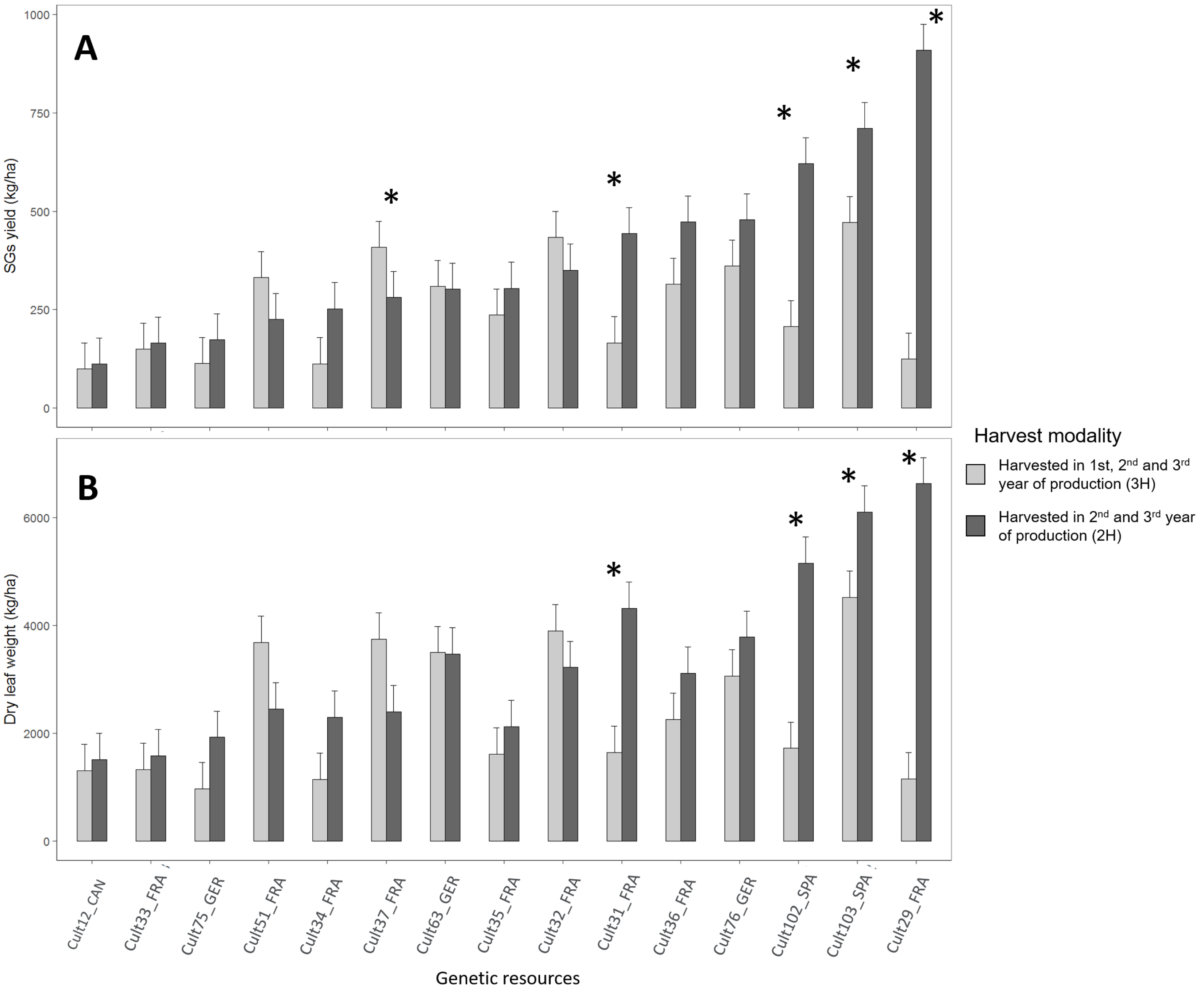


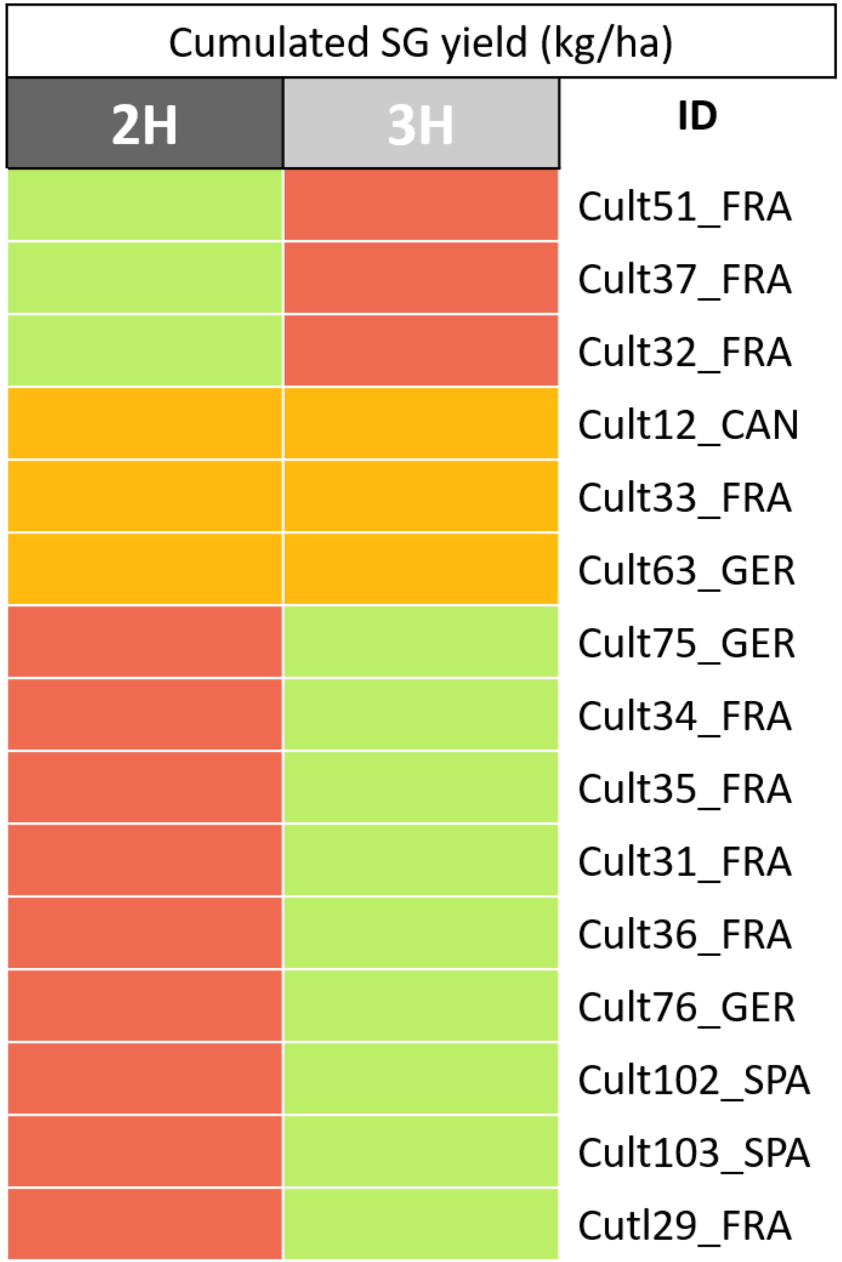
Regrowth percentage
Cult51_FRA
Cult32_FRA *
Cult103_SPA
Cult37_FRA
Cult63_GER *
Cult76_GER *
Cult102_SPA *
Cult36_FRA *
Cult31_FRA *
Cult34_FRA *
Cult12_CAN *
Cult35_FRA *
Cult75_GER *
Cult29_FRA *
Cult33_FRA *
3H
2H
0
25
50
75
100
1Y
2Y
3Y
Harvested in 1st, 2nd and 3rd year of production (3H)
Harvested in 2nd and 3rd year of production (2H)


SGs yield (g/plant)
0
5
10
15
Cult51_FRA
Cult33_FRA
Cult75_GER
Cult32_FRA
Cult12_CAN
Cult63_GER
Cult34_FRA
Cult37_FRA
Cult31_FRA
Cult35_FRA
Cult76_GER
Cult103_SPA
Cult36_FRA
Cult29_FRA
Cult102_SPA
Genetic resources
Harvest modality
Harvested in 1st, 2nd and 3rd year of production (3H)
Harvested in 2nd and 3rd year of production (2H)


Regrowth rate
Plant SG yield
Dry leaf biomass
SG content
2H
3H
2H
3H
2H
3H
2H
3H
Cult36_FRA
Cult102_SPA
Cult29_FRA
Cult103_SPA
Cult76_GER
Cult37_FRA
Cult63_GER
Cult31_FRA
Cult32_FRA
Cult51_FRA
Cult33_FRA
Cult12_CAN
Cult75_GER
Cult35_FRA
Cult34_FRA
Group 1
Group 2
Group 3
Color Key
-2
-1
0
1
2
NA


3H
2H
A
B
C
D
E
F
SGs yield (g/plant)
0
5
10
15
Dry leaf weight (g/plant)
0
50
100
SGs content (%w/w)
0
5
10
15
Year of production
First year of production (2016)
Second year of production (2017)
Third year of production (2018)
Cult51_FRA
Cult33_FRA
Cult75_GER
Cult32_FRA
Cult12_CAN
Cult63_GER
Cult34_FRA
Cult37_FRA
Cult32_FRA
Cult35_FRA
Cult76_GER
Cult103_SPA
Cult36_FRA
Cult29_FRA
Cult102_SPA
Genetic resources


A
B
SGs yield (kg/ha)
0
250
500
750
1000
Dry leaf weight (kg/ha)
0
2000
4000
6000
Cult12_CAN
Cult33_FRA
Cult75_GER
Cult51_FRA
Cult34_FRA
Cult37_FRA
Cult63_GER
Cult35_FRA
Cult32_FRA
Cult31_FRA
Cult36_FRA
Cult76_GER
Cult102_SPA
Cult103_SPA
Cult29_FRA
Genetic resources
Harvest modality
Harvested in 1st, 2nd and 3rd year of production (3H)
Harvested in 2nd and 3rd year of production (2H)
*



Cumulated SG yield (kg/ha)		
2H	**3H**	**ID**
🟩	🟥	Cult51_FRA
🟩	🟥	Cult37_FRA
🟩	🟥	Cult32_FRA
🟧	🟧	Cult12_CAN
🟧	🟧	Cult33_FRA
🟧	🟧	Cult63_GER
🟥	🟩	Cult75_GER
🟥	🟩	Cult34_FRA
🟥	🟩	Cult35_FRA
🟥	🟩	Cult31_FRA
🟥	🟩	Cult36_FRA
🟥	🟩	Cult76_GER
🟥	🟩	Cult102_SPA
🟥	🟩	Cult103_SPA
🟥	🟩	Cutl29_FRA

Tables:

Table 1: List of the 15 genetic resources of *Stevia rebaudiana* in field conditions in the south-western of France (Cosson et al., 2019)

Name	Providers	Country
Cult75_GER	EUSTAS gene bank	Germany
Cult76_GER	EUSTAS gene bank	Germany
Cult63_GER	EUSTAS gene bank	Germany
Cult33_FRA	Oviatis	France
Cult34_FRA	Oviatis	France
Cult102_SPA	Oviatis	France
Cult103_SPA	Oviatis	France
Cutl35_FRA	Oviatis	France
Cult29_FRA	Oviatis	France
Cult12_CAN	Oviatis	France
Cult36_FRA	Oviatis	France
Cult37_FRA	Oviatis	France
Cult31_FRA	Oviatis	France
Cult32_FRA	Oviatis	France
Cult51_FRA	Stevia store	Paraguay