IMPROVEMENT OF STEVIA (Stevia rebaudiana BERTONI) AND STEVIOL GLYCOSIDE THROUGH TRADITIONAL BREEDING AND BIOTECHNOLOGICAL APPROACHES

Article *in* SABRAO journal of breeding and genetics · March 2021

CITATIONS
2

READS
452

4 authors:

Sudad Al-Taweel
College of Agriculture Engineering Sciences - University of Baghdad- IRAQ
48 PUBLICATIONS **42** CITATIONS
SEE PROFILE

Clara Azzam
Agricultural Research Center, Egypt
68 PUBLICATIONS **139** CITATIONS
SEE PROFILE

Khaled A M Khaled
Faculty of Agriculture Beni-Suef University
26 PUBLICATIONS **68** CITATIONS
SEE PROFILE

Rania A Aziz
Agricultural Research Center, Egypt
9 PUBLICATIONS **16** CITATIONS
SEE PROFILE

REVIEW

SABRAO Journal
of Breeding and Genetics
53 (1) 88-111, 2021

IMPROVEMENT OF STEVIA (*Stevia rebaudiana* BERTONI) AND STEVIOL GLYCOSIDE THROUGH TRADITIONAL BREEDING AND BIOTECHNOLOGICAL APPROACHES

S.K. AL-TAWEEL[1]*, C.R. AZZAM[2], K.A. KHALED[3], R.M. ABDEL-AZIZ[4]

[1]Medical and Aromatic Plants Research Unit, College of Agriculture Engineering Sciences, University of Baghdad, Al-Jadiriya, Baghdad, Iraq
[2]Cell Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt
[3]Genetics Department, Faculty of Agriculture, Beni-Suef University, Egypt
[4]Sugar Crops Research Institute, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt.
*Corresponding author email: sudad.altaweel@coagri.uobaghdad.edu.iq
Email addresses of coauthors: clara.azzam@arc.sci.eg, khaled.adly@agr.bsu.edu.eg, snowrosa114@gmail.com

SUMMARY

Stevia rebaudiana Bertoni, widely known as sweet leaf, is a perennial herbal species of the Asteraceae family, which is widely used as a natural sweetener in many countries around the world due to the great demand for its steviol glycosides (SVgly) contents particularly rebaudioside A (Reb. A). This review article shows some important selected data accessible in previous scientific studies on crop improvement and SVgly production in stevia by traditional breeding methods and biotechnological breeding approaches. This review article reflects on improve the production of *Stevia rebaudiana* by using different breeding methods. *S. rebaudiana* plants are conventionally propagated through cuttings, but this traditional method cannot produce a large number of plants. The seeds of this species are smaller in size and the germination percentage is very low with a significant problem of low fertility and typically displays a low rate of germination. The seed yield and poor germination ability are some of the significant constraints caused by self-incompatibility. Therefore, modern techniques of propagation such as *in vitro* regeneration or tissue culture are needed to enhance the production for this important species. A real breeding key of stevia is developing SVgly and yield. It is attractive to develop and select a new variety with a higher content of stevioside and a higher Rebaudioside A (Reb. A). Recurrent selection is a particularly appropriate method for the enhancement of stevia quantitative traits in cross-pollinated species. Heterosis may also be a method for breeders to produce new improved varieties. The mutation method could play a significant role in the breeding improvement programs of stevia. Breeding of polyploidy could be valuable as they have larger leaves and potential for higher SVgly than the standard diploid. Modern techniques can also be explored, such as molecular markers, HPLC and mass spectrometry, to assist in quicker breeding for higher glycoside and yield traits.

Keywords: Stevia, breeding methods, mutation, polyploidy, propagation, molecular genetics

Key findings: This review article synthesizes available data and information from the scientific literature for crop improvement and increased SVgly production in stevia (*Stevia rebaudiana* Bertoni) by traditional breeding methods and biotechnological approaches. The main objective is to present the different breeding methods to improve the production of *Stevia rebaudiana* in order to identify new adaptable superior's genotypes.

Manuscript received: October 24, 2020; Decision on manuscript: December 18, 2020;
Accepted: January 27, 2021.

Communicating Editor: Dr. Sanun Jogloy

INTRODUCTION

Stevia rebaudiana (Bertoni), is a perennial herbal species of the Asteraceae (Composite) family (Ucar *et al*., 2016). Originating in South America at Paraguay country, it is widely used mostly as a natural sweetener in many countries around the world, including South and North America, South Europe, Korea, Thailand, China, India, and Bangladesh. Stevia becomes more prevalent in several developing and developed countries as a significant crop for nontoxic, nonnutritive, high-potency production of sweeteners. Stevia (*Stevia rebaudiana* Bert.) is a perennial and their leaves contain steviol glycosides (SVglys), which gives it a high sweetening property (Singh and Rao, 2005). The digestive system could not directly absorb that content; thus, it can be used by people with diabetes freely. Furthermore, it does not have many calories, so it is suitable for obese people who worry about weight loss. Several chemical compounds are callable to be manufactured out of stevia what makes it more attractive, especially in sweet taste manufacturing.

Stevia is a natural sweetener with low-calories, which has potential application as a sucrose substitute in the human diet and the stevia can be consumed in large quantities. Previous studies recommend the *in vitro* growing circumstances could meaningfully impact the crop biosynthesis pathways and biomass of stevia of SVglys. Stevia micro-propagation is the most effective technique to get these plants from an industrial point of view. Optimization of the media culture *in vitro* culture circumstances with the procedures of propagation, acclimatization and sterilization is a significant point in the tissue culture applied application.

The sweet extract was purified in 1931 to extract Stevioside. After that, the chemical structure was discovered in 1952 as a diterpene glycoside. Stevioside could be described as a glycoside, including an aglycone, the Steviol moiety, attached to three glucose molecules. Other SVgly were isolated in the 1970s, including Rebaudioside A (Reb. A), with a higher sweetening potency than stevioside (Barriocanal *et al*., 2008). stevioside has various medicinal properties like the fungal and inhibition of bacterial growth, it could be an anti-hypersensitive, anti-cancerous and anti-hyperglycemic agent. Its protective dental caries and has prevents properties, as claimed in many previous kinds of literature globally, that showed many aspects of crop enhancement, such as the development of new varieties, disease resistance, cultivation, seed germination, vegetative propagation, seeds production, improvement of glycosides quality and quantity.

Worldwide demand for both purified SVglys and stevia dried leaves is gradually increasing (Angelini *et al*., 2016) and a further increase is highly predictable in the future, as metabolic disorders for many diseases such as diabetes type-II and obesity are becoming more prevalent. Despite the stevia global market size characterized by rapid progress, the agricultural production of this crop is still problematic and insufficient to meet the

growing global demand. The stevia market estimates to reach $781.61 million in 2023. China is considered the stevia largest producer and manufacturer followed by the U.S. the largest stevia consumer, followed by North American countries. In terms of consumption, stevia yields remain low and unstable in many countries, where the crop is of recent domestication and performed by small- and medium-sized farmers, because of lack of appropriate adapted and accessible varieties, high input costs (in particular for planting and establishment), limited expertise in the cultivation, poor disease control and lack of irrigation. In order to improve the competitiveness of stevia production, it is essential to produce higher-performing and yielding crops, which are more tolerant to drought, extreme weather conditions and a range of biotic stresses. Therefore, genetic improvement with the development of varieties with higher leaf and SVglys yields and greater resistance to abiotic and biotic stresses. In comparison with the most and currently known cultivars, are important goals in stevia breeding (Tavarini *et al*., 2018).

Traditional breeding methods of stevia such as selection for the most anticipated traits engaged with molecular research, *i.e.,* polyploidization, selective markers joint determination with exact traits, or essential interference in the plant genome to constructing a transgenic diversity, all those approaches contribute to improve stevia in both genetic and commercial issues. It is essential to understand the inheritance and fundamental natures of a given trait to improve each stevia trait. Similarly, metabolic paths ways analysis, enzymes determination of those paths, genes regulation approaches accountable for the procedures could provide important information, which can assist in developing the anticipated traits (Yadav and Guleria, 2012). In the improvement of breeding efficiency, the genetic inheritance of glycosides is useful. Some of the responsible genes from the Steviol glycoside pathways have been identified (Brandle *et al*., 2002). However, many of them remain unknown. Most stevia accessions report as morphologically different from one another, indicating a right amount of phenotypic diversity available for breeding (Othman *et al*., 2015). Stevia plant's ability to biosynthesize SVgly is the most characteristic trait. Hence, this species breeding programs emphasized on compounds composition and percent modification. Because of the distinct bitter taste of Stevioside, breeders have studied decreasing its effect in the total SVglys. For instance, Reb. A, Reb. C and Reb. D compositions could be increased so that the sweet taste is reached. The overall peak content of SVgly is in the leaves, then traits may be modified to get high leaf-stem mass ratio and leaf yields economically. It is significant to breed traits of stevia characterized by high variability in populations by cross-pollination and high heritability, which consider susceptible to modification by selection (Brandle and Rosa, 1992).

Similarly, Reb. A and stevioside content are undesirably associated, while the contents of Reb. A and C and are positively correlated. A single dominant gene controls the presence or absence of Reb. A, while a higher number of loci regulate its amounts. A single additive gene determines the Reb. A and C ratio and these traits under segregation (Brandle, 1999).

Stevia is a self-incompatible plant and its flowers have sporophytic self-incompatibility (Raina *et al*., 2013). Deficient germination percent and low seeds yield potential are some of the significant problems caused by self-incompatibility. Stevia seeds low germination, viability and fertilization are the main factors that affect their quality, quantity and growth of SVgly and the ratio of the total stevioside and Reb. A, that limit its agriculture from the seeds. Mostly, plants with required characteristics are spread by vegetative propagation *via* stem cutting and by micropropagation using tissue culture practices. However, at the same time, these methods need a

long time, large scale, handling and tools, which restrict the wide-scale production of planting substance. It is recommended in the upcoming research to focus on fertile or viable seed production with improved germination and the developing new seed varieties that have sufficient stress adaptability in the different climatic conditions, improved leaf: stem ratio for successful cropping and high SVglys production, with the high Reb. A percentage compared with other glycosides. In the case of the self-pollinated plant with high cross-pollination chances and variation in a given trait, so the selection is a helpful method to improve the economic traits in new varieties. Throughout three decades of stevia breeding programs, the amount of Steviol glycoside has been increased from 2-10% to 20% of the dry matter in stevia leaves (Brandle and Rosa, 1992). In stevia breeding programs, selection should make before the flowering stage on mature plants.

Moreover, it is still expensive and time consuming to determine the leaves' content of glycoside using HPLC(Sun, 2001). Biotechnological tools and genetic markers provide opportunities to improve the anticipated stevia traits.

In vitro tissue culture is usually utilized for the vast spread and gaining haploid genotypes in alternative culture. The classification of molecular genetic markers is intended to locate the loci in the stevia genotype that affects the biosynthesis of SVglys. It is similarly significant to comprehend more intensely the biochemical synthesis pathways of Steviol glycoside synthesis, which permits regulating gene expression. Encouraged mutations might be utilized for the trait's enhancement with little variability within the stevia population.

There is limited research in stevia trait discovery, also limited research on methods for active breeding, determining qualities of interest and estimating the heritability for critical traits such as glycoside content. A breeding method for stevia could adopt from other cross-pollinated crops chrysanthemum, which also are members of the Asteraceae family. To date, there are few stevia species, which have been considered a higher yield of SVglys. A crop is a variety in the breeding of a plant defined as a special use or ornamental feature. The attained homogeneity and stability, with high SVglys and superior traits, is the breeder's goal by the ways of breeding methods, for example, selection, cross breeding, polyploidization, or mutation induction.

BREEDING OBJECTIVES

A key breeding objective of stevia is developing the plant for improved SVgly and yield. It is attractive to design and select a new variety with a higher Reb. A content and a higher Reb. A to stevioside ratio to make stevia more suitable to the customers as a healthy natural sweetener with minimizing bitterness (Yadav *et al*., 2011). However, the recent articles and patents show that Reb. C, Reb. D and Reb. M are the most desirable. Reb. D was found to be less bitter than Reb. A (Allen *et al*., 2013). Additionally, Reb. M is also reported as a sweeter and less bitter compound than Reb. A (Prakash *et al*., 2014). The general objectives of stevia breeding programs and biotechnological breeding approaches:

(1) To increase the leaves, yield, and stability.
(2) To ensure high-quality value in terms of SVglys content and composition,
(3) To produce types with higher resistance and resilience against disturbance and stress and targeted to specific growing conditions and farming needs.
(4) To improve seeds fertility and germination.

The primary desired traits, to reach through traditional and biotechnological breeding approaches in stevia, can be summarized as mentioned by Tavarini *et al*. (2018) with some additional points as follows:

1- High leaf yield per unit area.
2- High leaf to stem ratio.
3- Rapid growth rate and regrowth capacity.
4- Enhance photosynthetic activity.
5- High SVglys content.
6- High content of specific demanded SVglys (*e.g.,* Reb. A and M).
7- The high ratio of Reb. A to SVglys. for the best sweetness quality.
8- High adaptability to a wide range of agro-climatic conditions.
9- Resistance to environmental stresses, pests and diseases.
10- Photoperiod in-sensitivity, which is related to flowering induction.
11- Self-compatibility for viable seeds production.
12- Developing new adaptable and high compatible lines in stevia crop.

CYTOLOGY OF STEVIA PLANT

The genus stevia has varied in their number of chromosomes, *Stevia rebaudiana*'s chromosomal number reported as 2n = 22 (Monteiro, 1980 and 1982 and Frederico *et al*., 1996). Chromosomal number was investigated in some different stevia`s strains (Figure 1). The cytotype tetraploid and triploid, which contain 2n = 44 and 2n = 33, also occur, are responsible for male sterility due to the abnormalities of chromosome through the formation of gamete. Grashoff (1972) performed a cytological study for some species of stevia and concluded that all the shrubby species contained n = 12 chromosome numbers in their gamete when herbaceous species in a lax paniculate cluster with flower heads recorded 2n = 22. Other authors observed values of 2n = 24, 33, 34, 44, 48, 66, 70 (Oliveira *et al*., 2004). While stevia deems a multi-basic, with x = 11, 12 and/or 17, or only x = 11 was recorded (Frederico *et al*., 1996). This variation mirrors a numeral structural abnormality (polyploidy & aneuploidy), at most inversions pericentric (Frederico *et al*., 1996). All studied stevia species were diploid except the Colombian *S. elatior* (X = 11 and 2n = 66), a pre-dominance of X = 11 between South American species observed, while x = 12 observed in Colombian (*S. Lucida)*, Argentinean (*S. jujuyensis)* as well as Brazilian (*S. organensis*), these may result from species with x = 11 through ascending aneuploidy (Oliveira *et al*., 2004).

GERMINATION AND SELF-INCOMPATIBILITY

Stevia is a short-day plant; the flower is a minimal size with white color. The plant could produce flowering just after forming at least four true leaves. In general, the plant needs around one month to pass through the several developmental flower phases relying on the variety x environment (Figures 2 and 3). Seeds have a significant problem of low fertility and typically display a low rate of germination. Hence, the seed yield and poor germination ability are some of the significant constraints caused by self-incompatibility (Lester, 1999 and Raina *et al*., 2013). Stevia potentially pollinated by insects (Yadav *et al*., 2011). The level of selfing is 0 to 0.5%, while the out-crossing ranges from 0.7 to 68.7% (Maiti and Purohit, 2008). The safe aspect of the flowers and the greater stigmatization of the style facilitate cross pollination (Das *et al*., 2015). Due to Stevia's self-incompatibility, seeds harvested from a single plant may represent a half-sib family. Infertile seeds are regularly bright or pale. Fertile seeds are usually dark-colored (Figure 3), whereas seeds quality was a challenge because stevia seed has a short shelf life (Brandle *et al*., 2002). A complex mechanism of seed multiplication characterizes of Stevia. This species is hermaphroditic, highly cross-pollinated, photo-periodically sensitive and produces tiny flowers in small capitulate with five white tubular flowers (Yadav *et al*., 2011). *Stevia rebaudiana* Bertoni has sporophytic self-incompatibility that may lead to reduce seed yield and quality, which are obstacles to large-scale crop establishment (Martini *et al*., 2016 and Raina *et al*., 2013). Stevia has two types of achenes, black and tan colored (Figure

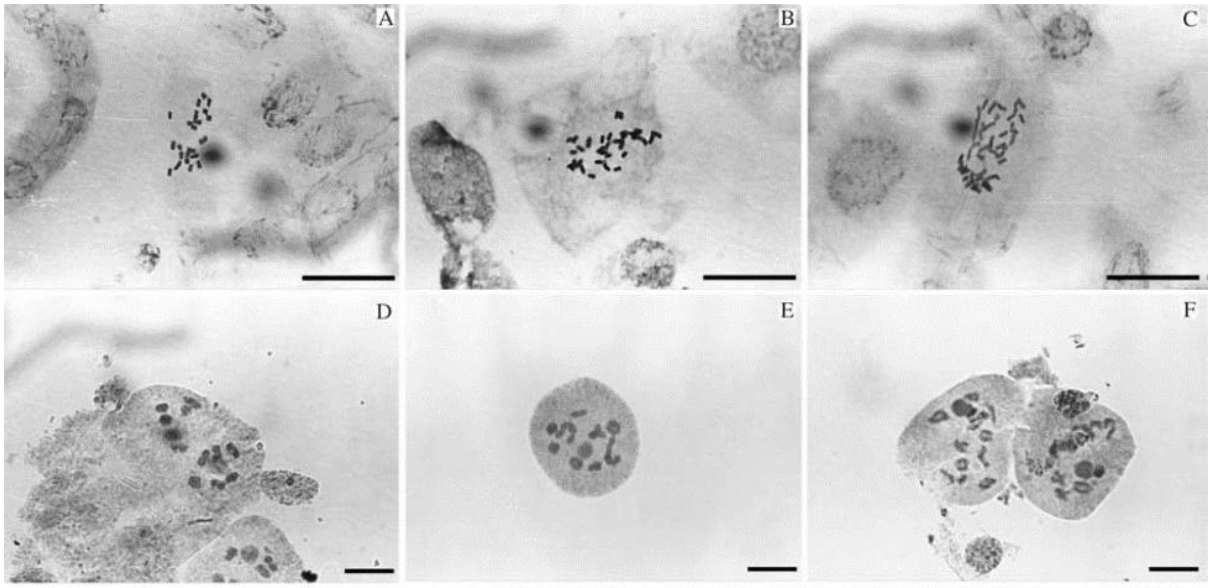

Figure 1. Mitosis (A-C), Meiosis (D-F) stained with reference to method described by Alexander (J-L) or by Acetocarmine for stevia. A, D = 2n = 22, B, E = 2n = 33 and C, F = 2n = 44. (Source Oliveira et al., 2004).

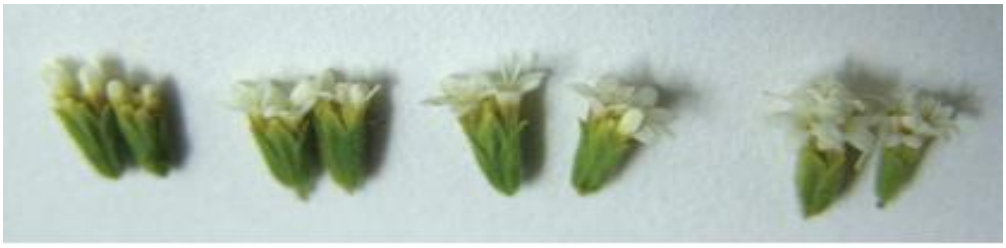

Figure 2. Different stages of flower opening (Yadav et al., 2011).

Figure 3. Stevia`s flowers and seeds.

Figure 4. Stevia`s seeds (a) Infertile seeds (b) Fertile seeds (Yadav et al., 2011).

4). Martini *et al*. (2016) reported that the black type produced by cross-pollination and characterized by high germination, while the tan achenes, which do not have the seed embryo, are originated from self-pollination. Even if self-incompatibility is a genetic mechanism, which prevents and thus encourages allogamy, it can be problematic for breeding techniques to be employed for crop improvement. The possibility to select self-compatible lines enables more efficient breeding techniques, as well as, to select cultivars to be easily reproduced by seed. Stevia seeds have small endosperms and are wind dispersed. A contributor to the poor seed germination maybe that seed is harvested before maturity, but also the timing of flowering and pollination plays a role as well. Seeds are either black or tan. Stevia black seeds had a 59-86% germination rate and the tan one had 0% germination in a study by Raina *et al*. (2013). Khalil *et al*. (2014) reported that the fresh seeds have a normal germination percent (25.51– 40%) but lost its viability after a few days of storage. In order to improve the germination percentage, the seeds have irradiated with 2.5, 5.0, 7.5 and 10 Gy gamma doses. However, these treatments did not show any significant increase in seed germination. The germination percent of stevia seeds is varying significantly, it takes 4-6 days to start the germination and to reach two-thirds of 62-90% the final germination of at 28^{o}C light generally increases germination (Takahashi *et al*., 1996). Studies on seeds production and fertility improvement show that selection for high germination is possible. A mixed population of compatible genotypes should raise to obtain a fertile seed (Raina *et al*., 2013). The germination percentage and germination speed can be increasing by some pollination treatments and seeds pre-treatments such as seeds priming with plant growth regulators such as GA3 and Methyl Jasmonate (Mohamadian *et al*., 2018). Goettemoeller and Ching (1999) reported that all pollination treatments increased the black seeds germination over the control, such as cross-pollination by hand (92.0%), or by bees (78.3%), cross-pollination by wind (68.3%) and control (36.3%). However, it suggests that active flower manipulation is required for an active pollination process. A contributor to the poor seed germination maybe could be due to that seed is harvested before maturity, but also pollination and the time of flowering play a role as well. Light-emitting diodes (LED) reported influencing the germination rate of stevia seeds when controlling the light spectrum (Simlat *et al*., 2016). Blue LED wavelength (430-485nm) is shown to confer enhanced seed germination in comparison to red light (620-660nm). Blue LED light was also found to improve the growth of leaves and roots of young stevia plants. However, a red LED wavelength (620-660nm) increased stem length and roots but had the least effect on the synthesis of chlorophyll and carotenoids. Blue and red light are found to have an opposite effect on the activity of antioxidant enzymes. The highest fresh weight among the experiment found where a combination of red and white LED lighting used. Gorzi *et al*. (2020) indicated that seed priming

with S.A., Fe and Zn at a suitable concentration could promote the poor germination performance of stevia.

COLLECTION AND INTRODUCTION OF GENOTYPE METHOD

The introduction of a new genotype will be considered an important activity in breeding programs in developing countries. It can be seen as a source of eminent genes in this breeding program and growing genetic diversity. Methods of introduction may be seen as profitable crops. The researchers in the Collage of Agricultural Engineering Sciences - Baghdad University collected local and hybrid seeds and plant material from Egypt and Ever Stevia Co. (Canada) to introduce them to Iraqi country for future studies in stevia breeding and improvement programs.

SELECTION METHOD

In the case of the self-pollinated plant with high variation in an assumed trait, as shown in stevia, the selection is a helpful process to improve a new variety. After more than three decades of stevia breeding, cost-effective breeding traits of stevia were categorized based on reproducibility and heritability. Because of high heritability, they are liable to modify by-selection (Brandle and Rosa, 1992). For selection and breeding purposes in stevia, information about the interrelationships among characters are of crucial importance for the following reasons: (1) to have a right selection choice for characters of desirable genotypes under the planned breeding program for higher yield, yield components and qualitative traits, (2) to have information about magnitude and nature of variation present inaccessible materials and (3) to know the association among characters. Selection in a cross-pollinated species population such as recurrent selection will consider as a suitable improvement method to increase yield and total glycoside content (Yadav *et al*., 2011). The high level of natural variability owing to constant out-crossing if sufficient genetic variability was present in available germplasm to allow selection of lines with chemical and agronomic properties suitable for local production conditions development of composites and synthetic cultivars consider as the most effective and practical breeding method for stevia breeding (Yadav *et al*., 2011).

In stevia, high estimates of heritability, genotypic and phenotypic variability recorded for several important characters. Several studies have carried out in stevia in order to find character potentially, which be useful in assisting selection programs and demonstrate the requirement of yield and various growth parameters, in addition to SVglys content (Brand and Rosa, 1992). Recurrent selection is particularly appropriate to improve quantitative characteristics in cross-pollinated species with excessive variation within the population. This method includes the selection of healthier traits in contrast with the overall population and extra crossbreeding of the recombinants. Consequently, subpopulation procedures where alleles defining anticipated traits happen at a greater frequency than in the preliminary population. Selection and estimation of glycosides must make before flowering has exceeded 10% as SVgly have reached to the maximum value at this stage (Yadav *et al*., 2011). The consequence of sampling too early would not capture the full potential of the SVglys in the plant, considering that the high-performance liquid chromatography (HPLC) is a costly test to waste (Brandle and Rosa, 1992). HPLC can be used to determine glycoside percent to select superior plants with desirable compounds. Yadav *et al*. (2011) showed that intercrossing and selection amongst many required genotypes are the best method for refining quality traits in stevia. Numerous plant kinds with more enormous quantities of specific glycoside have previously patented, for instance, RSIT 94-1306, RSIT 94-75, RSIT 95-166-1 through the selection and intercrossing. The genetic divergence among stevia genotypes plays a vital role in the

selection of parents having more extensive variability for different traits (Yadav *et al*., 2011).

The research of genotypic and phenotypic diversity therefore plays a key role in improving stevia breeding program. In stevia, considerable genetic and phenotypic variability has been observed, with particular regard to plant size, flowering period, leaf yield and SVglys content and composition. The data showed by Abdelsalam *et al*. (2016) achieved high significant variations between nineteen genotypes concerning the morphological traits, which mean there is a wide genetic variation between these genotypes due to the open pollination for several years. These genotypes could be used in future breeding programs.

Brandle and Rosa (1992) claimed that these features provide a leaf yield and are efficiently important in stevia breeding with considerable diversity in large colonies and considerable heritage (h^2 = 62.1), leaf-to-stem ratio (h^2 = 78.8) and content of SVglys (h^2 = 76.6). Because of high heritability, they are liable to modify by selection. Heritability of agronomic traits and glycoside yield measured in four elite *S. rebaudiana* Bertoni populations from elite selections from the cutting plants grown in 2015. The larger sample size is always preferred when estimating the heritability and genetic gain and components. Low heritability estimation for some traits may be due to the limited population size for accurate estimation of those traits. To calculate the heritability for half-sib families, we resorted to the method by Isik *et al*. (2017) for narrow-sense heritability rather than the common genotype divided by phenotype.

Heritability for glycosides estimates is high when measured in percent of total SVgly and mg/g of dry weight, including the total SVglys, Reb. A, Reb. C, Reb. D and stevioside showed the highest gain per cycle for major glycosides while minor glycosides such as Reb. D showed lower gains per cycle. It was unusual that Reb. C showed higher gain per cycle than Reb A., considering that it is found to be in lower accumulation than Reb. A and stevioside (Parris *et al*., 2016). Gain from selection (20%) was estimated for agronomic and glycoside characteristics. Gain for subjective and objective measurements 50 days after planting were moderate to high for plant size, stem height and plant width, but low for leaf size. Late-season ratings at 100 days after planting showed lower heritability and gain than when estimated in June. Gain in yield (dry weight) was at 1.19 Mg/ha per breeding cycle. However, gain per breeding cycle for percent survival and lodging resistance were low. Gains were moderate to high for the glycoside compounds Reb. A, Reb. C, Reb. D, stevioside and total SVgly at 20% selection.

These expected gains for minor glycosides are large considering how minimal these compounds are present in a normal population. Typically, Reb. D is found at 1-2% of a glycoside profile and these gains show that you could increase them as much as 1.49 percent per breeding cycle, which would almost double the amount the first breeding cycle. With minor glycosides, it would be interesting to see how much can be improved. High levels of Reb. D and Reb. M are preferred by sweetener processors such as Cargill, however due to the complexity of the biopathway in stevia you would not get near 100% in a plant. Therefore, due to the limited concentration found in plants they have resorted to production via yeast fermentation (Cargill, 2015).

Current breeding work with stevia has resulted in increased glycoside concentration by 20%. There has also been progress in the ratio of Reb. A to stevioside, which was initially 0.36:1 and now is 0.96:1 in some cultivars (Brandle, 2001). It appears evident that the glycoside focus based on recent cultivar releases include improvements of Reb. A, Reb. D and Reb. M. Reb. A has been well utilized as a good choice for sweeteners such as Truvia®, with a focus on lowering stevioside. However, other sweeteners seem to utilize stevioside as a primary

sweetener. Reb. D and Reb. M are more of a recent focus in new cultivars of stevia based on recent releases although there is limited research on these compounds. Yield is also an important factor that is considered with all cultivar releases.

Overall, agronomic traits in stevia have variable heritability and gain from selection, but glycoside traits have moderate to high heritability and gain from selection. As a result, much gain in these desirable traits could result per breeding cycle. Yield in dry weight had moderate heritability, showing gain from selection of 1.19 Mg/ha/breeding cycle which suggests that initial gain when selecting for yield could be high. This refers to the gain based on 20% selection criteria, where only the top 20% of the population is selected per cycle. If a lower selection criterion been used such as 40%, lower gains overall would be expected with a higher standard deviation. Gains estimated give an idea on the initial expected gain, but will likely decrease per cycle as the traits improve. Yield of fresh weight was not determined due to negative estimates. A larger population size for detecting heritability should be used to further explore these traits, which might potentially eliminate negative estimates. However, this heritability and expected gains estimated, give plant breeders an idea of how to make progress and design field experiments to breed stevia.

A variety of plant breeding procedure has been used for better leaf yield and Reb. A concentration in the leaves, which appears that sufficient genetic variability exists to make significant genetic gains in leaf yield of Reb. A content and the ratio of Reb. A to stevioside. Seed is best stored at 0°C, but even under low temperature conditions, germination declines by 50% over three years (Brandle and Rosa, 1992). As a result, many gains in these desirable traits could result in the breeding cycle, Brandle (1999) studied the genetic control of the proportions of two of those glycosides, Reb. A and Reb. C suggesting that these two compounds, equally glycosylated, differing only like one sugar unit, are synthesized by the same enzyme. The results were used to propose a model for glycosylation of steviol glycosides. Many stevia varieties were developed by repetitive breeding and selection, which contained 2.56 times more Reb. A than SVglys. Nowadays, several varieties can have Reb. A content up to 80% of the total SVglys, with very little bitterness and aftertaste. In 2011 a patent was filled for a new and distinct cultivar called 'AKH L1', noted for its late harvesting ability, light green leaves, with more branching on the main stem (US PP23728 P3, 2013.) 'AKH L1' is also noted for high Reb. A with a high yield of dry leaves and disease resistance. The yields of the dried leaves were 4,500 kg/ha. This cultivar was a result of selection from a controlled breeding program in San Pedro, Paraguay. 'AKH L1' was bred by a controlled pollination of the female parent of 'Eirete' crossed with the male pollen parent of 'AKH/EM1', an unpatented cultivar. This new cultivar 'AKH L1' was a selection within a population of seedlings from this cross. The selection criteria were for high rebaudioside A with content more than 50% of the total steviol glycoside with high yield of leaves and resistance to leaf spot diseases (US PP23728 P3, 2013). Constructing genetic maps will permit the molecular selection procedures in stevia breeding programs depended on the genetic markers and may make the way easier for new research on the stevia genome organization and metabolism. The post-stage analysis should align such markers for economically meaningful characteristics and form them into plant material.

SYNTHETIC BREEDING METHOD

A synthetic cultivar refers to the cultivar produced by intercrossing clones or sib-lines obtained from a breeding population during several cycles of recurrent selection. The synthetic population is continually reproduced from specific parents, then it is left to open-pollinated to produce over a generation, it will

change its genetic make-up as a population. A synthetic cultivar propagated for only a limited number of generations and then must be reconstituted from the parental stock. In stevia, the development of synthetic varieties seems to be suitable methods for the development of new variety, which have high SVglys content. In order to achieve the synthetic parents, crosses were made between a large numbers of progeny plants in the field and then made a selection among these plants. Seeds were collected (12 families and 60 plants for each family) and planted in the field. Twenty plants from each family were selected according to their SVglys content by HPLC analysis according to their SVglys content and then four of these plants were further selected based on their SVglys content and other suitable traits such as high Reb. A. These four selected plants enclosed to cress pollinated by bees or hands at the flowering stage. At the end of the season, the seeds were mixed for new progeny (Wölwer-Rieck, 2019). Composites and synthetics can be used to capture part of the heterosis available. Brandle *et al*. (2002) has developed a synthetic variety A.C. Blackbird with a total SVglys from 14-18% and a high Reb. A ratio. The composites and synthetics are used to detect part of the available heterosis due to a high percentage of natural crossing and the absence of an appropriate control mechanism for pollination. Synthetic cultivar such as "AC Black Bird" and "PTA-444" are the most practical and efficient method of breeding (Yadav *et al*., 2011).

HETEROSIS BREEDING METHOD

Plant hybrids allow new genes to be present within the plant gene pool, leading to the genetic enrichment. In homogenous population's crop, heterosis breeding gets a positive gain. Heterosis may also be used to produce new varieties with better function use by breeders, *e.g.,* improvement of plant growth such as plant branching, Chlorophyll content, leaves branches ratio, SVglys production, Reb. A/ SVglys. In relation to increase in quality and quantity of yields, abiotic or biotic stress tolerance in the food industry, Criolla and Morita II are the most studied and known stevia hybrid varieties, the first appears to be the unique stevia diversity native to Paraguay, whereas the other variety has been nominated for its high Reb. A content. Other known and grown varieties in Paraguay include Eirete, an improved hybrid for harsh crops, Morita III, grown in Morita II and classified as a low water drought burden. Katupyris is a chosen variety in the Province of Paraguay for the cultivation of arid soils. Also, several new stevia varieties with improved desirable traits have been released, such as Sugar High A3 (Figure 5), Huny, Shur A3, Shou-2 with a high SVglys content and Reb. A (Ever Stevia Co. Canada).

According to the genetic development, a crossbreeding method using the hybrid vigor of F1 generation could be the advantage of comprehensive different stevia parents, increase the quality of stevia and substantially economic flow rate and bring new growth engines to the seed industry simultaneously. The CN105052503A (2014) patent mentioned the method for stevia hybrid seeding, it comprises following steps: (1) set up the parent's strain: in the field, cottage propagation carried out to the individual plant of stevia, form parent's strain that genotype is consistent with reproductive characteristic, (2) hybrid seeding: by two different parent's strains by alternate row field planting in the same field, carry out mutual natural hybridization, obtain stevia crossbreed (https://patents.google.com/patent/CN105052503A/en).

MUTATION BREEDING METHOD

Mutation breeding is a method that changes plant genes pools to accelerate the enhancement of plants considerably with anticipated traits in comparison with conventional approaches for breeding. The use of both chemical and physical mutagens will allow a much faster way of obtaining genetic diversity within a plant

Figure 5. Sugar High A3 (Hybrid variety) of Ever Stevia Co. Canada., the left photo: Vegetative stage, the right photo: Flowering stage.

population. Those approaches can be used when a given trait indicates minimal variability within the population. Numerous mutagenic agents, for example, physical ones as X-rays, gamma-rays, thermal neutrons, fast neutrons, or chemicals like sodium azide (SA), ethyl methane sulfonate (EMS), diethyl sulphate (DES), methyl nitrosourea (MNU) could be utilized to give beneficial mutations. Cobalt-60 gamma ray irradiation of stevia breeding lines was used. Gamma radiation does not affect the seeds of stevia germination but induces a higher suppresses root development (Ali *et al*., 2014). The interesting traits could only improve through mutation breeding when the population indicates fewer variations for interesting traits such as leaves yields or SVglys content. Khan *et al*. (2016) exposed stevia leaf explant to chemical and physical mutants of EMS and Gamma Radiation. The Gamma plants induced the double-fold Reb. A with lower stevioside content of 3.2 ± 0.22 percent dry wt. Among all treatment methods 0.95 KR gamma ray radiation and 0.4 percent v/v EMS were found to be most effective treatment for selection of variants *via* direct shoot bud induction in comparison to control plants. By contrast, plants exposed to EMS reported more than 1.5 and 2.0 increase in stevioside and Reb. A. The enhanced steviol glycoside profile was supported by the RT-PCR analysis of UGT74G1 and UGT76G1 that corresponds to stevioside and Reb. A biosynthesis, respectively. While plants exposed to EMS showed 5–6-fold increase in the UGT74G1 (RQ = 5.51 ± 0.5) and UGT76G1 (RQ = 6.61 ± 0.5) gene expression, the plants exposed to gamma radiation showed 5-fold increase in UGT76G1 (RQ = 5.29 ± 0.2) gene expression. Al-Maracy *et al*. (2016) studied the antimutagenic response of stevia to a mutagenic compound (E.M.S.). Results proved that stevia leaves ethanol extract has no mutagenic activity and it has an antimutagenic activity.

ARTIFICIAL POLYPLOIDY BREEDING METHOD

Polyploidization was effectively utilized for enhancing the yields of various crops. There are different methods to induction of polyploidy in plants such as seed treatments (Hanzelka and Kobza, 2001), Taweel *et al*., 2019), flower buds (Wu *et al*., 2013), apical meristem (Toruan-Mathius *et al*., 1995 and Manzoor *et al*., 2019). The ploidy level may be considered to be a major factor affecting the leaf shape. The leaf appearance of chimeras was very variable, including deformed or

asymmetric leaves. This may be due to the fact that, in chimeric plants, some portion of the leaf may be tetraploid and other parts may be diploid (Thao *et al*., 2003). A study conducted on colchicine-induced stevia polyploidy resulted in significant differences in traits such as leaf thickness, fresh and dry leaf weight and leaf area (Hedge *et al*., 2015). Also, delayed flowering would be a valuable characteristic in stevia, allowing for later and potentially more significant yields. Leaf area and yield were higher with the polyploidy than in the diploid control. Breeding of polyploidy could be valuable as they have larger leaves and potential for higher glycoside content than the standard diploid (Shuichi *et al*., 2001). Breeding of triploids conducted by crossing a tetraploid with a diploid (both with high Reb. A content), resulting in triploid progeny that had higher Reb. A content than either parent. Eight triploid cultivars were obtained and categorized by leaf shape based on needle-like or broad needle-like leaves. There was a correlation with Reb. A and stevioside content based on the leaf shape of triploids (Hata *et al*., 2001). Cultivars with broad needle-like foliage resulted in high stevioside content, whereas needle-like foliage had a high content of Reb. A across various plants. Therefore, phenotypic screening for glycosides like stevioside and Reb. A is possible (Hata *et al*., 2001). Triploids are additionally valuable to a plant breeder due to sterility, making it difficult for others to use their lines inbreeding (Allard, 1960). Therefore, polyploidy could be an additional tool used in developing elite lines with higher yield and or higher glycoside content.

Stevia triploids were obtained via colchicine seeds treatment or by crossing between diploid and the tetraploid plants. The chromosomes were analyzed during mitosis and diakinesis in *Stevia rebaudiana*. All of the strains had 2n = 22, except for two, which had 2n = 33 and 2n = 44. Pairing at diakinesis was n = 11_{III} and n = 11_{IV}, that corresponded to 2n = 33 and 2n = 44, respectively. Oliveira *et al*. (2004) registered that colchicine solution was applied to the seedling growing points to induce the Tetraploidy to eight times resulted in mutation up 31.25%. Triploids in stevia are associated with a wider material and larger leaves of a Rebaudioside (Shuichi *et al*., 2001). The number of chromosomes is a controversial subject in this species for example, Oliveira *et al*. (2004) have reported 2n = 22 in this species with zero percent of pollen viability in 2n=22 cytotype, which prevents direct-sowing and requires plants to be grown as transplants. Stevia tetraploids have more significant and thick leaves, which could hypothetically result in a rise of biomass yields. The color of the leaf was much more in-depth and improved the test, which showed that the percentage of SVglys content in leaves of 15.7%, but it was 10.8% for diploid. Both polyploids also had non-functional pollen (Oliveira *et al*., 2004). Flow cytometry technology tends to provide the greatest benefit in plant breeding and is primarily ideal for very quick and basic markers such as colchicine polyploidization for ploidy manipulation. Mahdi (2012) reported that the stevia Tetraploidy plants were induced by 1.5%, 2% of colchicine, respectively, according to flow cytometry records statistics of *Stevia rebaudiana* with increasing in leaves stevioside content from 5.57% in control to 14.98% in 2% colchicine treatment.

Seed Propagation

Crop propagation is no traditional propagation technique because of the issue of low germination power, low seed production and genetic insulation. Using seeds to create a new variety of crops of stevia is much successful in the selective breeding programs, especially in tropical climates, where there is no climatic restriction on the length of the growing season to produce viable seeds in stevia flowers, which is required to be fertilized by pollen from another plant. Artificial pollination in any way such shaking plants for producing sib lines to increase seed

Figure 6. Stevia`s leaves and stem cutting.

production by using field cages or high density of bees (three to four hives per hectare) recommended for good seed production (Oddone, 1999). The immature seeds may also contribute to poor germination. In the determination of the successful pollination time, receptiveness for stigma, pollen-grain germination, ovule survival and finally setting in stevia, the temperature, light and the obscure mechanism perhaps play a key role. It can also be inferred that many aspects control the environment of stevia's seeds. The protandrite, lower pollen viability, lack of pollen germination nutrients and hard-style tissue inhibit pollen germination and pollen tube development beyond the stigma surface and thereby suppress seed environment. Pollination, seeds fertility for high fertile seeds production studies suggest that high germination percent are possible breeding programs that depend on selected compatible lines with good interaction in the flowering time (Gantait *et al*., 2018). All flowering, seeds maturation time, pollination methods with fertilization occurrence and environmental factors play an essential role in stevia seeds production.

VEGETATIVE PROPAGATION

Improvements in propagation techniques will be beneficial to stevia industry and plant breeders. Seed germination is generally low, however, one report showed that a stevia selection did not produce viable seed at all and therefore vegetative propagation was required to reproduce this line (Shock, 1982). Production of stevia through the seeds was not beneficial because seeds were not getting a high germination rate due to their infertility. Javad *et al*. (2015) used different concentrations of auxins such as naphthalene acetic acid (NAA.) and indole butyric acid (IBA) to improve the rooting of all kind of stevia stem cutting such as soft, semi hard (Figure 6) and hard woody ones. Results were showed that the highest number of roots per cutting observed to be 34.9 from 3% NAA solution, with maximum root number, root length, shoot length and the number of nodes per cutting. Cuttings of *Stevia rebaudiana* responded well in NAA treatment as compared to the IBA treatment. IBA concentration 2.10, 2.30 and 2.46 mM had a more substantial influence on stem cutting rooting than the other concentrations and the young apical stems should use for the replication of stevia stem cuttings (Abdullateef and Osman, 2011) and the micro- cutting were promoted the rooting of stevia cutting under mist-chamber propagation box and treatment with IBA (Osman *et al*., 2013). In order to enhance survival, stem cuttings were also dipped in different plant growth regulators (PGRs) solution. Only

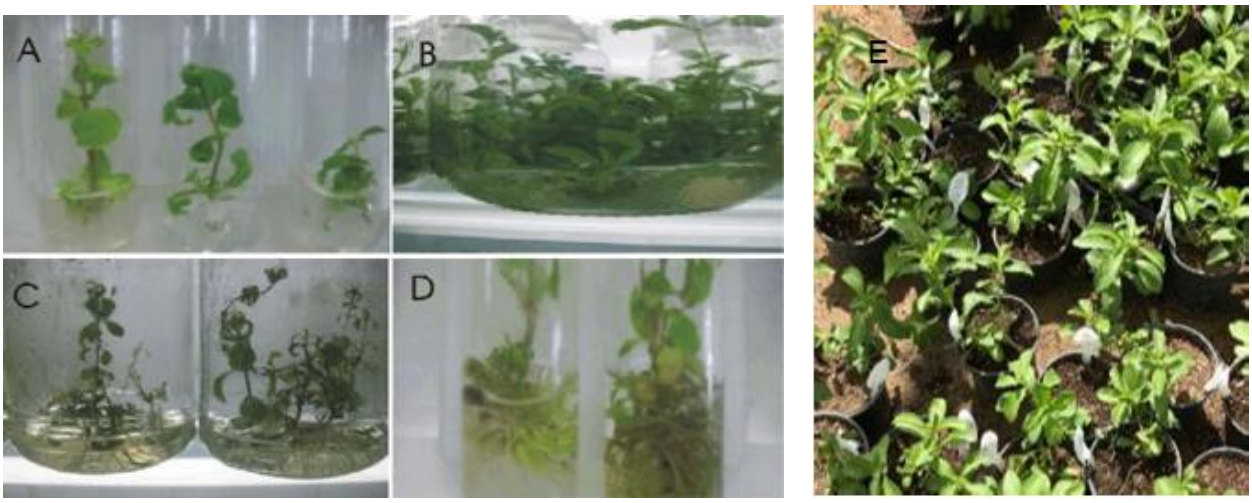

Figure 7. Micro-propagation of stevia (*Stevia rebaudiana*): (A) Starting stage, (B) Multiplication of shoots, (C) Rooting stage1 (D) Rooting stage2 (E) Plantlets of Micropropagation (Abdel-Aziz and Al-Taweel, 2019).

treated cutting with IBA at 1000 ppm showed a higher survival (33%) than control (11.1%) (Khalil *et al*., 2014). Javad *et al*. (2015) concluded that NAA and IBA were a better choice for enhanced rooting and vegetative propagation of *Stevia rebaudiana* through stem cuttings. However, vegetative propagation is labor-intensive.

IN VITRO PROPAGATION

In vitro propagation protocol (tissue culture) described for efficient propagation of *Stevia rebaudiana*, a medicinally and commercially sweet producing plant. Micro-propagation is considered as an effective method for stevia breeding (Figure 7). Tissue culture is the most efficient fastest and method of obtaining a large number of plants from the industry point of view. The plants are homogenous in composition and SVgly contents and plants free of diseases (Sivaram and Mukundan, 2003). Tissue culture techniques are successful methods of stevia propagation, where various plant parts, including leaves, shoot primordia, auxiliary shoots and intermodal explants (Yadav *et al*., 2011). Cell-suspension cultures developed for the mass propagation of stevia for field production (Ferreira and Handro, 1988). Recent advancement such as an improved 500 l bioreactor was developed to mass-propagate shoots of stevia, producing 64,600 g of shoots from 460 g of propagules (Akita *et al*., 1994). Once the desired cultivar developed, this mass-propagation technology is beneficial to accelerate the breeding and commercial production. This method would help decrease the cost of clonal propagation and make it cost-effective for growers to plant elite cultivars rather than growing from seed. Researchers could also use this technology to produce extensive experiments of clonal material that was previously difficult due to limitations in labor, the beneficiary of the method further improved by the fact that stevia characterized its self-incompatibility. Stevia pollinates by wind and insects. So, the percentage of pollinated flowers is low in this plant. The seeds have a low germination percentage (Liona- Tsakalidi *et al*., 2012). The generative propagation results in varied genotypes and phenotypic traits, what does not allow for obtaining a homogenous population in

Figure 8. Leaf size of (the left plant) Polyploidy and (the right plant) Diploid plant. (Yadav, et al., 2011).

terms of such essential traits as the content of SVgly and chemical composition, searching for solutions within traditional vegetative reproduction/ propagation methods requires many efforts and their efficiency is limited with the availability of the genetic material. The tissue culture technique became one of the essential tools in plant breeding. Callus is essential in rapid mass replication, variability generation, cell suspension culture, cell line culture persistence and secondary metabolite production. Masri *et al*. (2019) reported that the treatment (MS+1.0 mg-1 2, 4-D +0.75 mg-1 N.A.A.) gave the highest values of fresh callus weight in stevia and half strength MS medium with one mg-1IBA found to be the optimum medium for root formation, so it gave an excellent root formation (88.67%), highest roots number/shoot (6.24) and highest root length (2.90 cm).

Tissue culture technique became one of the most critical tools in plant breeding. Plant tissue culture technique is one such biotechnological approach, which will improve both qualitatively and quantitatively the productivity of the stevia plant as well as free disease plants. *In vitro* culture regeneration of plants can be accomplished through embryogenesis or organogenesis. In stevia, regeneration has been accomplished by organogenesis from diverse explants, for example, leaves, axillary shoots, stem tips, suspension cultures and anthers (Flachsl and *et al*., 1996). Abdel-Aziz and Al-Taweel (2019) reported that the stevia root system and post acclimatization of rooted plantlets could be improved by pre-acclimatization treatments using growth retardants, Alar (B9: succinic acid 2-2-dimethylhydrazine) and Cycocel (CCC: chloroethyl trimethyl ammonium chloride) in the culture medium (Figure 8). So, growth retardants increased the survival percentage in acclimatization in greenhouse conditions and, this protocol could be useful in producing a true-to-type plant and use *Stevia rebaudiana* medicinally and commercially. It has contributed many biotechnologies, mostly for the propagation of problematic species (species having problems in their natural propagation), rapid propagation of selected and superior genotype and also genetic mutants through somaclonal variation as well as the synthesis of secondary metabolites, which is an

integral part of food fragrance and pharmaceutical industries. On the other hand, callus masses can sometimes yield the highest number of secondary metabolites (Das *et al*., 2010). It enables the massive vegetative replication and spread of plants that cannot be replicated by micro-propagation or somatic embryogenesis under typical conditions.

Even if crossbreeding is possible, hybrid plants with physical or morphological barriers to pollination are equally developed by in-vitro crops and somatic hybrids. The development of haploid and dual-haploid species, e.g. homozygous species, is made possible by other and microspore cultures. *In vitro* practices are used to cause mutations in plant cells and genetic change and eventually to obtain plants that are free of viruses. El-Motaleb *et al*. (2013) observed that shoot tip explants in stevia were more efficient than nodal explants in shoot proliferation estimated as the percentage of shooting, length of shoots hoots, so the shoots that were produced by the addition of 1.0 mg/L B.A. were skinny and contained many lateral shoots, while that produced by the addition of 2.0 mg/L kin were healthier, with dark green leaves and full stem. All shootlets were rooted onto MS medium supplemented with different concentrations of IBA, 0.5 mg/L IBA was the best concentration for 100% rooting and gave the highest number of roots, taller root length and fastest rooting response compared to other concentrations. Masri *et al*. (2019) study an efficient method of callus induction and plant regeneration of a new sweetening crop plant (stevia) using different explants with different growth media. They found that all studied media-induced callus for all explants, but MSc4 (MS+1.0 mg-1 2,4-D +0.75 mg-1 NAA) gave the highest values of fresh callus weight.

Only, calli obtained from the Msc4 callus induction medium gave the best response to regenerate a sufficient number of shoots. Half strength MS medium with one mg-1IBA was found to be the optimum medium for root formation. It gave an excellent root formation (88.67%), the highest roots number/shoot (6.24) and the highest root length (2.90cm) as well as anther cultures, *i.e., in vitro* cultures shaped from immature anthers, are utilized in gaining haploid plants. It may be used to generate dual haploids, *i.e.* fully homozygotically plants. Specimens of homozygotic populations might be used for hybridization in terms of a certain function. Stevia has successfully obtained regeneration of plants from anthers (Flachsland *et al*., 1996). Biotechnological methods are expected to bring improvement in stevia (Hassanen and Khalil, 2013).

GENETICS and MOLECULAR BREEDING

Stevia was developed by molecular markers and markers identification, which related to particular traits to create new support of plant breeding methods. It allows anticipated characteristics of the readily recognized molecular marker to be determined earlier in plant development. This removes mature plants and greatly decreases the time taken for the determination of the characteristics of a specific specimen (Hassanen and Khalil, 2013).

Molecular markers used to identify genotypes (variants, somaclons, cultivar, clones and hybrids) rapidly in plants with the high-solving ability and low-cost labor (Reddy *et al*., 2002, Perry, 2004 and Khaled *et al*., 2015). Marker assisted selection (MAS) such as randomly amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), amplified fragment length (AFLP), start codon targeted marker (SCoT) and simple sequence repeat (ISSR), are used immensely to identify the artificial and natural outputs crossing of plants (AL-Taweel *et al*., 2019, Azzam *et al*., 2019, Khaled *et al*., 2018, Aziz and Khaled, 2017, Khaled *et al*., 2015, Shasany *et al*., 2005, Ruas *et al*., 2003, Rajora and Rahman, 2003 and Wolfe *et al*., 1998).

The polymorphisms among individual plants are scored by the absence or presence of a specific

fragment. RAPD markers are used for constructing a map depending on the genomic polymorphism of stevia (Aziz and Khaled, 2017). Among the different DNA-based markers accessible, ISSR appears that it gives quick, reproducible, primary and economical methods in breeding, taxonomy on the molecular level and analysis of genetically diverse.

A linkage map of stevia has been constructed with 183 random amplified polymorphic DNA (RAPD) markers with 21 linkage groups covering 1389 cM (Yao *et al*., 1999). However, as much as 1250 cM has yet to map. The authors found 35.5% polymorphism and 62.5% 1:1 segregation for the markers. Currently, simple sequence repeat (SSR) and single-nucleotide polymorphism (SNP) markers have replaced RAPDs, so this RAPD map has minimal use at this point because new advanced technologies are more efficient and provide more information. RAPD markers are also challenging to replicate. However, the previous linkage map suggests a high level of polymorphism in the genome of stevia despite its limited coverage of the genome. The polymorphism level that detected was varied among stevia samples, while it was 44.70%, as reported by Hassanen and Khalil (2013), when they compare the RAPD genetic profiles between mother plants and regenerated plantlets. On the other hand, Chester *et al*. (2013) mentioned that the polymorphism level was about (67.24–92.40%). In the harmony of these findings, Thiyagarajan and Venkatachalam (2015) used RAPD-PCR for investigation of the phytochemical variation and polymorphism of *Stevia rebaudiana* genomic-DNA, where Moktaduzzaman and Rahman (2009) used RAPD for adjusting auxins as well as cytokines concentration in *S. rebaudiana* regeneration and some clonal variations analysis.

The marker-assisted selection has been deployed in most major crops and would help aid in more efficient breeding progress in stevia (Staub *et al*., 1996). Genetic maps for stevia were created with the use of the RAPD (Randomly Amplified Polymorphic DNA) technique in 1999 (Sun, 2001). New advanced technologies such as next-generation sequencing and transcriptome can be more efficient in characterizing the genomics of stevia. Chester *et al*. (2013) studied the genetic and metabolic variability in *S. rebaudiana* among different regions of India using random amplified polymorphic DNA (RAPD) markers. They found that ten out of 20 primers screened which most informative, amplification products of the genotypes produced a total of 87 scorable bands (67 polymorphic), whereas the genetic similarity (GS) coefficient is 0.01-0.08 and polymorphism values is 67.24-92.40% showed significant variability. RAPD has become an essential technique for population genetic studies since the amplified products provide a random representation of both non-coding and coding regions across the whole genome (Abdelsalam *et al*., 2016). Among many molecular used for working on stevia, RAPD markers were the first type used for constructing stevia genomic linkage map (Yao *et al*., 1999). RAPD analysis (Williams *et al*., 1990) is one of the PCR reactions (polymerase chain reaction) that usually dominant. Chen *et al*. (2013) found 143 UDP-glucosyltransferase unigenes by using RNA-Seq, including those likely involved in Steviol glycoside biosynthesis, which indicates that new technology such as RNA-Seq can be useful in species that have not explored. Haraz (2016) has studied the genetic variability and relationships of different genotypes of stevia (*Stevia rebaudiana*) based on RAPD analysis. Initial screening of a large number of RAPD primers with nineteen genotypes of stevia (*Stevia rebaudiana*) resulted in eight RAPD primers that produced informative and polymorphic products resolvable by agarose gel electrophoresis. Abdelsalam *et al*. (2016) used eight oligonucleotide primers in random amplified polymorphic DNA.s (RAPD) analysis of Stevia genotypes. SCoT techniques (cDNA-SCoT) are used for gene differential expression in plants (Wu *et al*., 2013). The cDNA-SCoT method has advantages, in comparison with

others, for its efficiency and it is cheaper, simpler and faster (Luo *et al*., 2014). A cDNA-SCoT marker used successfully for *Phoenix dactylifera*, *Saccharum officinarum*, Mangiferaindica, sugarcane, barley, olive tree, *Dendrobium officinales* and stevia (Munns and Tester, 2008; Chen *et al*., 2013; Bosily *et al*., 2018; AL-Janabi and Al-Rawi, 2018 and AL-Taweel *et al*., 2019). At the same time, it is used to assess genetic diversity (Al-Qurainy *et al*., 2015). Al-Taweel *et al*. (2019) reported that the cDNA- SCoT markers technology might work well and identify and develop the salt stress tolerance for new lines and varieties in the stevia crop.

CONCLUSION

Stevia has the potential to become an essential crop in the future as a natural sweetener due to valuable sweetening and medicinal compounds such as Reb. A, C, D and M. and antioxidant, antibacterial, antifungal and may be antiviral compounds according to many studies around the world. It allows anticipated characteristics of the readily recognized molecular marker to be determined earlier in plant development. This removes mature plants and greatly decreases the time taken for the determination of the characteristics of a specific specimen. Self-incompatibility knows in stevia, but poor seed germination continues to be an issue with commercial production, where additional research is needed to explore the flowering physiology. Elite clonal varieties exist but come with a higher cost as a plant than the cost of seed. However, if clonal lines offer higher quality and yield than a seed cultivar would than clonal lines may be cost-effective, mutation breeding can also be explored for cultivar development in stevia, as it has been successful with other crops. Polyploidy has reported having benefits in stevia breeding, but research is limited as to the effect on glycoside production. Polyploidy can potentially further improve cultivars that are currently available. Genome editing through new techniques in biotechnology such as ISSR, RAPD, cDNA-SCoT could prove useful in developing varieties and eliminating bitter compounds. Current estimates of genetic variance components and heritability have indicated great potential in breeding for glycosides and yield. Additional heritability estimate studies should conduct using large populations, as it can be a beneficial tool for stevia breeding and can help increase breeding efficiency. Also, further identifying trait correlations can help breeder's ineffective selection.

Modern techniques can also be explored, such as molecular markers, HPLC and mass spectrometry, to assist in quicker breeding for higher glycoside and yield traits. Glycoside evaluation by HPLC is practical but expensive, which limits the ability of large populations to be analyzed. Phenotypic screening methods would be valuable to help identify the best plants in a breeding population without the need to test them all *via* HPLC. Hence, varieties that have a higher leaf-to-stem ratio are desirable because they yield higher quantities of SVglys per unit of harvested plant biomass. A higher relative growth rate and a better capacity to regrow after each harvest, with higher chlorophyll content and photosynthetic rate, to get a new leafy shoot rapidly, thus allowing multiple harvests per year. The accumulation of SVglys occurs in active photosynthetic tissues (mainly in leaves). Therefore, quality is related to the improvement of SVglys content and of specific SVglys (namely Reb. A, D and M) as a measure of taste in addition to a higher content of antioxidant compounds too that should be considered in breeding strategies. The stevia plant is still one of the plants that recently have domesticated around the world, especially in the Middle East, including Iraq country. The researchers aim to improve stevia's growth and SVglys production by traditional breeding methods and biotechnological breeding approaches to deriving a new tolerant variety or varieties that have good growth traits and high SVglys and Reb. A. content (Figure 9).

Figure 9. The new tolerant varieties of stevia in the fields of Collage of Agricultural Engineering Sciencs, Baghdad-Iraq.

Breeders could grow the amount of sweeteners in the leaves, followed by the Reb. A: stevioside ratio to Improve stevia and phytochemical characterization. The key goal of plant breeders dealing with the improvement and exploitation of this natural sweetener supply is *rebaudiana* with higher Reb. A level.

REFERENCES

Abdel-Aziz RA, Al-Taweel SK (2019). Effect of plant growth retardants on stevia (*Stevia rebaudiana* Bertoni) acclimatization produced *in vitro*. *Plant Arch.* 19(1): 1275-1284.

Abdelsalam NR, Haraz ASM, Haraz AEK, Saleh MSH, Elsheikh AE (2016). Genetic improvement through selection of different *Stevia rebaudiana* genotypes. *Alexandria Sci. Exch. J*. 37(1): 10-25.

Abdullateef RA, Osman M (2011). Effects of stem cutting types, position and hormonal factors on rooting in *Stevia Rebaudiana* Bertoni. *J. Agric. Sci.* 4(1): 49-57.

Akita M, Shigeoka T, Koizumi Y, Kawamura M (1994). Mass propagation of shoots of *Stevia rebaudiana* using large scale bioreactor. *Plant Cell Rep. Jpn.* issue 13: 180-183.

Ali A, Abo Shosha AA, Kassem MKM, El-Dabaawy EEM (2014). Biotechnological Studies on Gamma Irradiated stevia (*Stevia Rebaudiana*) Plant under abiotic stresses. *4th Int. Con. Rad. Res. Appl. Sci., Taba, Egypt*. 13-17 Oct. pp: 95-109.

AL-Janabi AS, Al-Rawi TKH (2018). Effect of irrigation terminating time on gene expression OeDGAT1, OeFAD2 and OeFAD6 and oil quality and quantity in some olive cultivars. *SABRAO J. Breed. Genet.* 50(3): 329-343.

Allard RW (1960). Principles of plant breeding. *John Wiley and Sons, Inc. (New York, Chichester, Weinheim, Brisbane, Singapore, Toronto)*, pp. 485.

Allen AL, Mc Geary JE, Hayes JE (2013). Rebaudioside A and rebaudioside D bitterness does not covary with Acesulfame K bitterness or polymorphisms in TAS2R9and TAS2R31. *Chemosensory perception* 6(3): 106-117.

Al-Maracy SH, Ali MK, Mandour AE, Fayed AH, Elmaghraby SS (2016). Mutagenic and antimutagenic response of stevia (*Stevia rebaudiana* Bertoni.) plant extract. *Zagazig J. Agric. Res.* 43(3):837-847.

Al-Qurainy F, Khan S, Nadeem M, Tarroum M (2015). SCoT marker for the assessment of genetic diversity in Saudi Arabian date palm cultivars. *Pak. J. Bot.* 47(2): 637-643.

Al-Taweel SK, Abdel-Aziz RM, Rabea KM, Khaled KAM (2019). Studying cDNA SCoT in response to salinity stress in *Stevia rebaudiana* Bertoni. *SABRAO J. Breed. Genet.* 51(3): 281-294.

Al-Taweel SK, Al Amrani HA, Al-Rawi TKH (2019). Induction and flow cytometry, GC-MS identification of tetraploids through colchicine treatments in datura stramonium L. Plant Archives 19(1) :241-250

Angelini LG, Martini A, Passera B, Tavarini S (2016). Sweeteners, pharmacology, biotechnology and applications. *Reference Series in Photochemistry, ed. J.-M. Merillon and K.G. Ramawat, Springer Reference.*

Azzam CR, Zaied KA, Abd El-Hadi AH, Nasr El-Din MM (2019). Genetic relationships among ten sunflower inbred lines based on ISSR and RAPD analyses. Egypt. J. Plant Breed. 23(4):547– 563.

Aziz R, Khaled K (2017). *Stevia rebaudiana* bertoni; rapid micro-propagation, stevioside accumulation and genetic fidelity using ISSR markers. *J. Agric. Chem. Biotechnol*. 8(12): 295-301

Barriocanal LA, Palacios M, Benitez G, Sussam B, Jimenez JT, Nora Jimenez, Vicenta R (2008). Apparent lack of pharmacological effect of steviol glycosides used a sweetener in humans. A pilot study of repeated exposures in some normotensive and hypotensive individuals and in Type 1 and Type 2 diabetics. *Regul. Toxicol. Pharmacol*. 51(1): 37-41.

Bosily MA, Noaman MM, El-Banna MN, Azzam CR, Nassar MA (2018). Breeding for barley resistance to leaf rust disease using marker-assisted selection. Proceeding of the 7th Field Crops Research Institute Conference. 18-19 Dec. 2018, Giza, Egypt: 397-437.

Brandle J (1999). Genetic control of rebaudioside A and C concentration in leaves of the sweet herb. *Stevia rebaudiana*. *Can. J. Plant Sci*. 79(1): 85-91.

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

Brandle JE, Richman A, Swanson AK, Chapman BP (2002). Leaf E.S.T.s from *Stevia rebaudiana*: a resource for gene discovery in diterpene synthesis. *Plant Mol. Biol.* 50: 613-622.

Brandle JE, Rosa N (1992). Heritability for yield, leaf: stem ratio and stevioside content estimated from a landrace cultivar of *Stevia rebaudiana*. *Can. J. Plant Sci.* 72(4): 1263-1266.

Cargill`s stories (2015). New zero-calorie sweetener hits the market. Cargill's EverSweet™ sweetener promises to be a boon for food makers and consumers*. https://www.cargill.com/ story/new-zero-calorie-sweetener-hits-the-market.*

Chen MH, Zhang BQ, Song XP (2013). cDNA SCoT analysis of differentially expressed genes in sugarcane induced by Leifsoniaxyli subsp*. Xyli. Acta Agron. Sin*. 39(9): 1119-1126.

Chester K, Amboli ETT, Parveenand R, Ahmad S (2013). Genetic and metabolic diversity in *Stevia rebaudiana* using RAPD and HPTLC analysis. *J. Pharm. Biol.* 51(6): 771-777.

Das A, Biswas M, Mandal N (2010). An economic analysis of stevia (*Stevia rebaudiana* Bert.) cultivation through stem cutting and tissue culture propagate in India. *Trends Agric. Econ.* 3(4): 216-222.

Das A, Saha BN, Maji A, Kumar S, Kumar M, Mandal N (2015). Reproductive phenology and factors affecting reproductive success in stevia (*Stevia rebaudiana* Bert.). *New Agric.* 26(2): 247–255.

El-Motaleb MA, El-Hady MAS, El-Kholy MA, Badr A (2013). *In vitro* propagation of *Stevia rebaudiana* Bertoni in Egypt. *J. Appl. Sci. Res.* 9(8): 4597–4605.

Ferreira CM, Handro W (1988). Production, maintenance and plant regeneration from cell suspension cultures of *Stevia rebaudiana* (Bert.) Bertoni, *Plant Cell Rep.* 7: 123-126.

Flachsland E, Mroginski L, Davina J (1996). Regeneration of plants from anthers of *Stevia rebaudiana* Bertoni (Composite) cultivated *in vitro*. *Biocell* 20(1):87-95.

Frederico AP, Ruas PM, Marin-Morales MA, Ruas CF, Nakajima JN (1996). Chromosome studies in some stevia Cav. (Compositae) species from Southern Brazil. *Braz. J. Genet.* 19(4): 605-609.

Gantait S, Das A, Banerjee J (2018). Geographical distribution, botanical description and self-Incompatibility mechanism of genus stevia. *Sugar Tech*. 20: 1-10.

Goettemoeller J and Ching A (1999). Seed germination in *Stevia rebaudiana. In J. Janick, ed. Perspectives on new crops and new uses. ASHS Press, Alexandria, VA. pp. 510–511*

Gorzi A, Heshmat O, Abdolamir B (2020). Effect of stevia (*Stevia rebaudiana*) seed priming treatments with salicylic

acid, iron and zinc on some germination traits and photosynthetic pigments under drought. *Iranian J. Seed Res.* 6(2): 125-135.

Grashoff JL (1972). A systematic study of the North and Central American species of stevia. University of Texas, USA.

Hanzelka P, Kobza F (2001). Genome induced mutation in *Callistephus chinensis* Ness. - evaluation of plant fertility and seed characteristics. *Hort. Sci*. 31(1): 22-26

Haraz ASM (2016). Genetic improvement through selection of *Stevia rebaudiana* under Egyptian conditions. *M.sc thesis. Faculty of agriculture (Saba basha), Alexandria University. Egypt.*

Hassanen SA, Khalil RM (2013). Biotechnological studies for improving of stevia (*Stevia rebaudiana* Bertoni) *in vitro* plantlets. *Middle East J. Scien. Res*. 14(1): 93-106.

Hata S, Yomo T, Fujita S (2001). Breeding of triploid plants of stevia with high rebaudioside A content. *Jpn. J. Trop. Agric.* 45 (4): 281-289.

Hedge SN, Rameshsing CN, Vasundhara M (2015). Impact of stevia (*Stevia rebaudiana* Bertoni) Polyploidization on leaf yield and attributes. *The Bioscan* 10(2): 609-611.

Isik F, Holland J, Maltecca C (2017). Genetic data analysis for plant and animal breeding. *Ebooks Springer, Chapter 4. 395pp.*

Javad S, Basharat S, Shaheen S, Aftab A (2015). Effect of auxins on propagation of honey crop: *stevia rebaudiana* (bertoni). *Pak. J. Sci.* 67(4): 340-345

Khaled KA, El-Arabi NI, Sabry NM, El-Sherbiny S (2018). Sugarcane genotypes assessment under drought condition using amplified fragment length polymorphism. Biotechnology. 17(3):120-7.

Khaled KA, El-Demardash IS, Amer, EAM (2015). Genetic Polymorphism among some sugarcane germplasm collections as revealed by RAPD and ISSR analyses. *Life Sci. J.* 12(3): 159-167.

Khalil SA, Zamir R, Ahmad N (2014). Selection of suitable propagation method for consistent plantlets production in *Stevia rebaudiana* (Bertoni). *Saudi J. Biol. Sci*. 21(6): 566–573.

Khan SA, Rahman LU, Verma R, Shanker K (2016). Physical and chemical mutagenesis in *Stevia rebaudiana*: variant generation with higher U.G.T. expression and glycosidic profile but with low photosynthetic capabilities *Acta Physiol. Plant.* 38(1): 63-72.

Lester S (1999). An introduction to phenomenological research. *Stan Lester Developments, Taunton. http://www.sld.demon.co.uk/resmethy.*

Liona-Tsakalidi A, Kaspiris G, Salahas G, Barouchas P (2012). Effect of salicylic acid (SA) and gibberellic acid (GA) pre-soaking on seed germination of stevia (S./evict predict (I) under salt stress. *J. Med. Plants Res*. 6(3): 416-423.

Luo C, He XH, Hu Y, Yu HX, Ou SJ, Fang ZB (2014). Oligo-dT anchored cDNA–SCoT: A novel differential display method for analyzing differential gene expression in response to several stress treatments in mango (*Mangifera indica* L.). *Gene* 548(2): 182-189.

Mahdi S (2012). Induction of genetic variability by Colchicine treatment in *Stevia rebaudiana* Bertoni, *M.Sc Thesis. University of Agricultural Sciences, Bengaluru, India.*

Maiti RK, Purohit SS (2008). Stevia: A miracle plant for human health. *Ebook Agrobios (India) Jodhpur, India.*

Manzoor A, Ahmad T, Bashir MA, Hafiz IA (2019). Studies on colchicine induced chromosome doubling for enhancement of quality traits in ornamental plants. *Plants (Basel).* 8(7): 194-199.

Martini A, Tavarini S, Macchia M, Benelli G, Canale A, Romano D, Angelini LG (2016). Influence of insect pollinators and harvesting time on the quality of *Stevia rebaudiana* (Bert.) Bertoni seeds. *Plant Biosystems* 151(2) 1-11.

Masri MI, Amein MMM, Abdel- Aziz RM, Saye DO (2019). Callogenesis and plant regeneration *via in vitro* culture of *Stevia rebaudiana* explants, *Egyptian J. Plant Breed*. 23(1):65– 76.

Mohamadian E, Kianmehr H, Somagh HA, Mahjor NAN, Safari F, Safarzaden A (2018). Effect of Methyl Jasmonate Pre-Treatment on germination indices and biochemical traits of stevia seedling (*Stevia rebaudiana*) under salt stress. *Iranian J. Seed. Res*. 5(1): 101-117.

Moktaduzzaman M, Rahman SMM (2009). Regeneration of *Stevia rebaudiana* and analysis of somaclonal variation by RAPD. *Biotechnol.* 8(4): 449-455.

Monteiro R (1980). Taxonomia ebiologia da reproducao da *Stevia rebaudiana* Bert.

Ph.D. thesis, Univ. Estadual de Campinas, Brazil. [English abstract.]

Monteiro R (1982). Estudos cromossoˆ micos em *Stevia rebaudiana* Se´ rie Multiaristatae no Brazil. *Rev. Bras. Bot*. 5: 5-15. [English abstract.]

Munns R, Tester M (2008). Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59:651-681.

Oddone B (1999). How to grow stevia. *Guarani Botanicals, Inc., Pawcatuck, Connecticut* 17(4): 1-30.

Oliveira V, Forni-Martins ER, Magalhaes PM, Alves MN (2004). Chromosomal and morphological studies of diploid and polyploid cytotypes of *Stevia rebaudiana* (Bertoni) Bertoni (Eupatorieae, Asteraceae). *Genet. Mol. Biol.* 27(2): 215-222.

Osman M, Samsudin NS, Faruq G, Nezhadahmadi A (2013). Factors affecting micro cuttings of stevia using a mist-chamber propagation box. *The Scien. World J.* 2013(4):940201:10 pages*. http://dx.doi*. *org/10 .1155 / 2013/940201*

Othman HM, Osman M, Zainuddin Z (2015). Morphological assessment of *Stevia rebaudiana* Bertoni a accession in IIUM's germplasm as initial material for stevia breeding. *Aust. J. Basic Appl. Sci*. 9(25): 1-9.

Parris CA, Shock CC, Qian M (2016). Dry Leaf and Steviol Glycoside Productivity of *Stevia rebaudiana* in the Western United States. *Hort. Sci.* 51(10): 1220-1227.

Perry DJ (2004). Identification of Canadian durum wheat varieties using a single PCR. *Theor. Appl. Genet.* 109 (1): 55-61.

Prakash I, Markosyan A, Bunders C (2014). Development of next generation stevia sweetener: Rebaudioside M. *Foods.* 3(1): 162-175.

Raina R, Bhandari SK, Chand R, Sharma Y (2013). Strategies to improve poor seed germination in *Stevia rebaudiana*, a low calorie sweetener. *J. Med. Plants Res.* 7(24): 1793–1799.

Rajora OP, Rahman MH (2003). Microsatellite DNA and RAPD fingerprinting, identification and genetic relationships of hybrid poplar (Populus x canadensis) cultivars. *Theor. Appl. Genet*. 106: 470-477.

Reddy MP, Sarla N, Siddiq EA (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica* 128: 9-17.

Ruas PM, Ruas CF, Rampim L, Carvalho VP (2003). Genetic relationship in Coffea species and parentage determination of interspecific hybrids using ISSR (Inter-Simple Sequence Repeat) markers. *Genet. Mol. Biol*. 26: 319-327.

Shasany AK, Darokar MP, Dhawan S, Gupta AK (2005). Use of RAPD and AFLP markers to identify inter- and intraspecific hybrids of Mentha. *J. Hered*. 96: 542-549.

Shock C (1982). Experimental cultivation of Rebaudi's Stevia in California. University of California, Oakland, 1-9.

Shuichi H, Tsuneo Y, Satoshi F (2001). Breeding of triploid plants of stevia (*Stevia rebaudiana* Bertoni) with high rebaudioside A content. *Jpn. J. Trop. Agriç*. 45(4): 281-289.

Simlat M, ŚlęzakMaria P, Moś M, Worcol M, Skrzypek E, Ptak A (2016). The effect of light quality on seed germination, seedling growth and selected biochemical properties of *Stevia rebaudiana* Bertoni. *Scien. Hort*. 211: 295-304.

Singh SD, Rao GP (2005). Stevia: the herbal sugar of 21' century. *Sugar Tech*. 7(1):17-24.

Sivaram I, Mukundan U (2003). *In vitro* culture studies on *Stevia rebaudiana*. *Vitro Cell Dev F3iol-Plant.* 39(5): 520-523.

Staub JE, Serquen FC, Gupta M (1996). Genetic markers, map construction and their application in plant breeding. *Hort. Sci*. 3: 729–41.

Sun J (2001). Method for breeding hybridized seed of *Stevia rebaudiana [on-line]. China (SIPO) Patent No. CN1237720-A.*

Takahashi L, Melges E, Carneiro JWP (1996). Germination performance of seeds of *Stevia rebaudiana* under different temperatures. *Rev. Bras. Sementes* 18: 6-9.

Tavarini S, Passera B, Angelini LG (2018). Crop and Steviol Glycoside improvement in stevia by breeding, in Steviol Glycosides: cultivation, processing, analysis and application in food. *Chapter* 1, pp.1-31.

Thao NTP, Ureshino K, Miyajima I, Ozaki Y, Okubo H (2003). Induction of tetraploids in ornamental Alocasia through colchicine and oryzalin treatments. *Plant Cell Tiss. Org. Cult*. 72: 1925.

Thiyagarajan M, Venkatachalam P (2015). Assessment of genetic and biochemical diversity of *Stevia rebaudiana* Bertoni by DNA fingerprinting and HPLC analysis. *Ann. Phytomed.* 1: 79-85.

Toruan-Mathius N, Pratiwi T, Hutabarat T (1995). Somaclonal variations in *Stevia rebaudiana* Bertoni irradiated with Co-60 gamma rays. *Menara Perkebunan*, 63(2): 33-42.

Ucar E, Ozvigit Y, Turgut K (2016). The effects of light and temperature on germination of stevia (*Stevia rebaudiana* Bertoni) Seeds. *Turk. J. Agric. Res*. 3(1): 37-40.

Williams J, Kubelik A, Livak A, Rafalski J, Tingey S (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18(22): 6531–6535.

Wolfe AD, Xiang QY, Kephart SR (1998). Assessing hybridization in natural populations of Penstemon (Scrophulariaceae) using hypervariable inter simple sequence repeat (ISSR) bands. *Mol. Ecol.* 7(9)**:** 1107-1125.

Wu JM, Li YR, Yang LT (2013). cDNA-SCoT: A novel rapid method for analysis of gene differential expression in sugarcane and other plants. *Aust. J. Crop Sci.* 7(5): 659-664.

Wölwer-Rieck U (2019). Steviol Glycosides: cultivation, processing, analysis and applications in food. *Ebook, The royal society of chemistry C.P.I. crop (U.K.). Ltd. Croydon, UK. pp. 209.*

Yadav AK, Singh S, Dhyani D, Ahuja PSA (2011). Review on the improvement of *Stevia rebaudiana*. *Can. J. Plant Sci.* 91: 1-27. doi:10.4141/CJPS10086

Yadav SK, Guleria P (2012). Steviol glycosides from stevia: biosynthesis pathway review and the application in foods and medicine. *Crit. Rev. Food Sci. Nutr*. 52: 988-998.

Yao Y, Ban M, Brandle J (1999). A genetic linkage map for *Stevia rebaudiana*. *Genome* 42: 657–661.