KIRKUK UNIVERSITY JOURNAL FOR AGRICULTURAL SCIENCES

Improving Steviol Glycosides Production in *Stevia Rebaudian* Bertoni by Plant Tissue Culture Technology (Review)

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- Received date 05/07/2023 and accepted 24/07/2023.

Abstract

Plant tissue culture techniques have made tremendous progress in the production of various active compounds of interest, including Steviol Glycosides in Stevia. *Stevia rebaudiana* is a perennial herbaceous plant known for its sweet-tasting leaves that contain active substances used as natural low-calorie sweeteners called Steviol glycosides. Because of its high sweetness and low-calorie nature, it is an alternative to sugar. However, conventional cultivation of Stevia plants for the production of Steviol Glycosides faces challenges such as long growth cycles, changes in chemical composition, and environmental determinants. Therefore, plant tissue culture is an alternative for the production of large quantities of Steviol Glycosides by altering various factors such as nutrient media, growth regulators, and response. Photovoltaic and tissue culture methods have been successfully used for plant propagation as well as the production of high-quality glycosides. Through techniques such as callus culture, organogenesis, and somatic embryo culture, large quantities of cotyledons can be rapidly established from small plant parts in a relatively short time. With further advances in tissue culture techniques and an understanding of the physiology and genetics of stevia, the potential for large-scale production of glycosides using Tissue culture methods is a promising technique for the commercial use of this natural sweetener.

Keywords: *Tissue Culture, Stevia rebaudiana, Steviol Glycosides, natural sweetener, Active compouds.*

Citation: Mahdi, S., & Alsaedi, N. (2023). Improving Steviol Glycosides Production in Stevia Rebaudian Bertoni by Plant Tissue Culture Technology" (Review). *Kirkuk University Journal For Agricultural Sciences*,14 (3), 57-67, doi: 10.58928/ku23.14306

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Introduction

The Stevia plant, known by the scientific name *Stevia rebaudiana*, is a perennial herbaceous plant belonging to the family (Asteraceae). Stevia grows upright with a bushy appearance, plants can reach a height of 30 to 60 centimeters. Leaf shape: The leaves of Stevia are typically small, elongated, and lanceolate to oblong in shape and the leaves are arranged in an alternate pattern along the stem. The flowers are typically small and white, They form in clusters or inflorescences, often in loose panicles or cymes. The individual flowers have a pale purple throat corolla, adding a touch of color to the predominantly white blooms.

The origin of this plant goes back to certain regions in South America, such as Brazil, Paraguay, and Argentina. It was discovered by Moises Santiago Bertoni in 1877 and used by the Guarani Indians in South America. Its leaves are traditionally used for their sweet taste and effectiveness in treating diabetes.

The Stevia plant is distinguished by its small green and pointed leaves that contain naturally sweet active compounds known as (Steviol glycosides) and symbolized by them (GSs). These compounds are responsible for the sweet taste of the Stevia plant, as it has a high sweetness strength that reaches 200-400 times more than table sugar [1]. Steviol glycosides are not represented due to their complex structure in the human body, so they do not contain calories. In addition, its leaves contain several other effective compounds such as terpenoids (Amerin, Limonene, Kaurenoic Acid, Carvacrol, Phenols (Catechin, Apigenin, Chlorogenic Acid, Quercetin, Ferulic Acid, Cinnamic Acid, Leteolin-7-O-Rutinoside, Carbohydrates (L-Arabinose, D-Mannose, D-Xylose, L-Rhamnose), Fats (Palmitic Acid, Stearic Acid, Linoleic Acid), Protein (Serine), Vitamins (Ascorbic Acid, Niacin, Riboflavin, Thiamine) And Minerals (Calcium, Magnesium, Sodium Manganese, Selenium, Cobalt, Phosphorus, Zinc, And Potassium) [2].

Stevia is mainly used as a natural substitute for sugar in foods and beverages, especially in cases of desire to reduce calorie intake and control blood sugar levels. 3] It is believed to have the ability to improve the health of the immune and cardiovascular systems. Studies have shown that Stevia leaf extracts act as antimicrobial [4], antioxidant [5], antiviral [6], and anti-inflammatory [7].

With the increasing rates of diabetes and obesity worldwide and the risks associated with artificial sweeteners, people are turning to natural sweeteners. As a result, sweeteners and medicines produced from the Stevia plants are now available in the market and have an increasing demand. It is worth noting that the Stevia plant is considered safe to use and has no side effects. Known negativity, however, a doctor should be consulted before using it regularly, especially for people with special health problems or taking certain medications.

Traditional agricultural methods are not sufficient to meet the growing demand for Stevia growers. For example, seed cultivation is an ineffective method, as it produces a heterogeneous group with different concentrations of SGs in plants, which affects the sweet taste of the leaves, as well as weak seed germination due to lack of Self-compatibility also hinders the large-scale production of Stevia. Therefore, to overcome the problems of conventional cultivation, other propagation methods have been used, most notably plant tissue culture [8]. This review presents a comprehensive study of the tissue culture of the Stevia and the methods used to enhance the production of the active substance SGs.

Botanical description and cultivation

Stevia is a new global genus from Asteraceae family native to northeastern Paraguay. The genus consists of herbaceous plants and shrubs that grow at elevations of 500 to 300 meters above sea level and is distributed from the southern United States to Argentina and the Brazilian highlands through Mexico, the Central American states, and the Andes Mountains. In South America [9] among the 230-261 species of the genus Stevia, the sweet diterpene glycosides ent-kaurene are found in *S. phlebophylla* and *S. rebaudiana* but the highest level of sweetness is found in S. rebaudiana, conferring industrial and economic importance on them [10], Stevia is cultivated in Japan, India, China, Thailand, Taiwan, Korea, Brazil, Malaysia, Hawaii, California, Canada, and other parts of Asia and Europe [11].

Stevia is from plants of the plant R. Al-Qusayr, as flowers usually appear from January to March and from September to December in the northern and southern hemispheres, respectively [13]. It is grown in the tropics and subtropics at an average temperature of 24 degrees Celsius [14] It grows well in sandy soils with a pH of 6.5-7.5 and gives a leaf yield for eight years and the leaves are harvested six times a year and each plant gives a yield of approximately 15-35 g of leaves [15].

Steviol Glycosides properties

Steviol glycosides are natural sweeteners extracted from plants. These compounds are widely used as sugar substitutes due to their intense sweetness and low-calorie content. Here are some of the main properties of Steviol Glycosides:

1. Very sweet: Steviol glycosides are about 200 to 350 times sweeter than sucrose (table sugar) on a weight basis and have economic importance so they are used in the food and beverage industry.
2. Low-Calorie Content: Steviol glycosides contribute virtually no calories to the diet as they are not metabolized by the body as they pass through the digestive system without being absorbed.
3. Thermally Stable: Steviol glycosides are relatively stable under heat and can be used in cooking and baking without being affected.
4. pH Stability: Stable across a wide pH range making it suitable for use in various food and beverage products including acidic and alkaline compounds.
5. Non-fermentable: It is not fermented by oral bacteria, which means that it does not contribute to or stimulate tooth decay. This property makes it a desirable substitute for sugar in mouthwash products.
6. Limited aftertaste: Sweeteners containing Stevia can sometimes exhibit a mild aftertaste and are often described as licorice-like. However, advances in Steviol Glycoside purification technologies have helped reduce their aftertaste, making them more palatable to consumers.
7. Stability in Beverages: It has good stability in a wide range of beverages including sodas, juices, and teas, and does not easily dissolve or precipitate out of solution over time.
8. Glycemic index: Steviol glycosides have little effect on blood sugar levels. It does not raise blood glucose or insulin levels, making it suitable for individuals with diabetes, the obese, or those on a low-glycemic diet.

Steviol Glycosides applications

Diabetes treatment: Steviol glycosides have been studied for their ability to help treat diabetes. Research indicates that they may have a glucose-lowering effect by enhancing insulin secretion and improving insulin sensitivity. They can be used as a sugar substitute in a diabetic diet to reduce calories and help control blood sugar levels [54]. Lowering blood pressure: Some studies have shown that Steviol Glycosides may affect lowering blood pressure, as they can act as vasodilators, which helps to relax and widen blood vessels, which may contribute to reducing high blood pressure [55]. Anti-inflammatory: Steviol Glycosides have shown anti-inflammatory properties in several studies. They have been found to inhibit inflammation and inhibit its pathways. These effects indicate their potential for use in the management of inflammatory conditions and diseases [56]. Antioxidant: Steviol glycosides have antioxidant properties, which can help eliminate harmful free radicals in the body. Antioxidants play a vital role in protecting cells from oxidative stress and reducing the risk of chronic diseases associated with oxidative damage [9]. Anti-cancer: Some studies have discovered the potential anti-cancer effects of Steviol Glycosides for their ability to inhibit the growth and proliferation of cancer cells and stimulate apoptosis [57,9]. Antibacterial and antimicrobial: Steviol glycosides have shown antibacterial and antimicrobial properties against certain strains of bacteria and fungi [57]. Steviol glycosides have a wide range of applications in the food, beverage, and nutritional supplement industry as well as in other fields such as pharmaceuticals, personal care supplies, and animal feed [57].

Steviol Glycosides

The following are several types of Stevia glycosides that are commonly found in the leaves of the Stevia. The main Steviol Glycosides include:

1. Stevioside: Stevioside is one of the most common Steviol glycosides. It consists of a Steviol molecule attached to glucose. It has a sweet taste and is often used as a standard for the sweetness of stevia.
2. Rebaudioside A (Reb A): Rebaudioside A is a Stevia glycoside found in Stevia leaves that has a very sweet taste and contributes to the sweetness of many sweeteners.
3. Rebaudioside B (Reb B): It is found in lesser amounts than Rebaudioside A
4. Rebaudioside C (Reb C): Found in Stevia leaves, it tastes bitter in low concentrations.
5. Rebaudioside D (Reb D): Reb D is a less abundant Steviol glycoside characterized by its high sweetness. It is often used with other glycosides to enhance sweetness and improve the taste of stevia-based sweeteners.
6. Rebaudioside M (Reb M): Reb M is another relatively less abundant steviol glycoside that is notable for its intense sweetness. It has a sugar-like taste and is often used with Reb A or Reb D to create a higher-intensity sweetener.
7. Dulcoside A: Dulcoside A is a Steviol glycoside that contributes to the overall sweetness of the leaves. It has a sweet taste although it is not sweet like some of the other glycosides mentioned above.

The technique of propagating Stevia by tissue culture and enhancing its content of Steviol glycosides

Stevia can be grown using the tissue culture technique or vegetative propagation. Tissue culture is an effective way to quickly and effectively multiply the number of plants by producing new plants from small cells or plant parts. Various types of plant tissue culture technologies have been developed such as in vitro propagation, genetic engineering, callus cultivation, and adventitious root cultivation for breeding, crossbreeding, propagation, and production of secondary metabolites of Stevia [16]. The most important technologies are summarized as follows:

1. Micropropagation (in vitro):

This method includes the use of small plant parts such as leaves, root tissues, and axillary buds to create new plants and grow them in artificial nutrient media (MS, DKW, B5) whose composition has been improved by adding nutrients and changing the concentrations of various growth regulators such as auxins (IAA, NAA, 2, 4D) and Cytokinins such as (TDZ, BAP, Kin) and gibberellins (GA3) and (IBA). These studies have shown good results in increasing the number of plants and the yield of effective compounds [18,19,20,21,22,23,24].

2. Callus use: Callus culture is a technique used in plant tissue culture to induce the formation of an undifferentiated mass of cells known as a callus. Callus can start from different plant tissues, including leaves, stems, or even embryonic tissues. Callus cultivation has been used extensively in several studies for the production of Steviol Glycosides and is a potential alternative to conventional cultivation methods for the large-scale production of these compounds. The use of callus dates back to the beginning of the twentieth century [31,32]. Several studies indicated that callus cultures of Stevia can be established from many different parts of the plant, including leaves, seed cotyledons, shoots of vegetative branches, somatic embryos, and flowers [21,33,34]. Here are some areas where callus transplantation has been explored in research:

Production of Steviol Glycosides:

Callus cultures: can be stimulated to produce Steviol Glycosides including key sweeteners such as stevioside and rebaudioside A. Researchers have optimized callus culture conditions, including nutrient media, and plant growth regulators to enhance the production of these sweet compounds [38,39].

Gene transfer: Callus cultures are an important target for gene transfer in Stevia. The researchers used callus cultures to introduce genes responsible for Steviol glycoside biosynthesis, such as the UDP-glucosyltransferase (UGT) gene, to enhance the production of specific Steviol Glycosides [40].

Rhizogenesis: Root propagation refers to the process of root formation in plants. It can occur naturally through seed germination or be artificially induced through various techniques in plant tissue culture including the use of plant growth regulators, physical treatments, or manipulation of environmental conditions. Root formation plays a role Important in plant growth and important for plant reproduction, and nutrient uptake. In plant tissue culture, the root formation is often used to produce new plants from tissue cultures, somatic embryos, or callus cultures and is also important in gene transfer studies where root formation is necessary to create transgenic plants.

Some of the techniques commonly used to stimulate root formation in plant tissue culture include:

Treatment with Auxins: The most common way to induce root formation is to treat plant tissues with auxins. IBA and NAA are frequently used for rooting in tissue culture. These auxins can be used in different concentrations and different methods such as immersion, basic medium, or pre-soaking treatment [42].

Photoresponse: Some studies have shown that exposing cultured plant tissues to specific light spectrums can improve tissue growth and yield of secondary metabolites in the Stevia plant. Lighting methods have been improved and the use of specific and advanced light sources such as (light-emitting diode) to enhance growth and productivity, and its most prominent response can be summarized as follows:

- Some studies indicated that the use of low light intensity in plant tissue culture of Stevia plants can improve growth rates, and low intensity may contribute to stimulating cell divisions and developing tissues well [25,26,27].The effect of high intensity:

Some studies found that high intensity of the light may negatively affect the growth and development of plant tissues of the Stevia plant, as the stress resulting from high intensity of light may cause tissue damage and inhibit cell growth and multiplication [26].

- Optical spectra: The optical spectra may have a specific composition that affects the cultivated tissues of some herbal plants such as strawberries and stevia, as certain light spectra such as red and blue spectra can be used to enhance growth, multiply and stimulate the emergence of adventitious roots on plant tissues, which contributes to reducing the duration of the acclimatization phase of plants, as well as its effect on photosynthesis compounds and secondary metabolism compounds [28, 29, 30].

Wounds: Injury to plant tissues can promote root development. Cutting or damaging the base of the parent plant produces adventitious roots [43].

Dark environment: Maintaining plant tissue cultures in dark or low-light conditions can stimulate root initiation. Darkness is thought to promote the accumulation of auxins and other substances that stimulate root formation [44].

Interference of plant hormones: The interaction of various plant growth regulators, such as auxin and Cytokinin, can influence root formation, maintaining the proper balance between Auxin and cytokinin is critical for successful root formation [45].

Stimulating the emergence of somatic embryogenesis

It is a process in which the somatic plant cell undergoes a series of developmental changes to form an embryo, that is, it is a form of plant renewal that takes place in the laboratory and in appropriate conditions that guarantee the process of stimulating the development of somatic embryos and re-differentiating the plant cells to have the ability to develop into a complete plant. Redifferentiation takes place by manipulating growth conditions and using growth regulators such as auxins and cytokinins. The process of somatic embryonic development proved useful in propagating the Stevia plant outside the living body and increasing its content of secondary metabolites such as the high content of Steviol Glycosides or disease resistance. It provides a means of rapidly multiplying Stevia plants and allows a large number of plants to be produced in a relatively short period. As well as the use of somatic embryonic development for gene transfer and production of transgenic Stevia plants with high specifications and improvement of production [9,48].

Manipulation

Nutrient manipulation of *Stevia rebaudiana* culture in vitro can have a significant impact on the growth, development, and quality of young plants. By modifying the nutrient compositions of the culture medium, it is possible to improve growth and enhance certain traits of stevia. The following are some of the effects of nutrient change in tissue culture:

Macro elements: Adjusting the concentrations of nutrients, such as nitrogen (N), phosphorus (P), and potassium (K), can affect the growth and biomass production of Stevia. High nitrogen concentrations, for example, often increase the growth of cotyledons, while high concentrations of phosphorus can affect the growth and development of roots on cotyledons [49].

Micronutrients: Adding nutrients such as iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) to the culture medium can affect various physiological processes in stevia. These microelements play important roles in enzyme activities, chlorophyll synthesis, and other metabolic functions affecting the overall growth of plants [50].

Organic Supplements: Organic additives such as plant extracts, vitamins, amino acids, or complex organic compounds can be incorporated into the culture medium to improve the growth and development of the seedlings. These supplements can enhance nutrient uptake, stimulate root growth, or act as growth stimulants and increase the concentration of the active substance, ultimately leading to better plant quality and higher survival rates during the acclimatization phase [51].

Carbon source: The carbon source in the culture medium, usually in the form of sucrose, provides energy for the growth of cotyledons. Sucrose concentrations can influence branch propagation, root development, and biomass production in general. High concentrations of sucrose often promote the multiplication of cotyledons, while low concentrations stimulate root growth [52].

Nitrogen source: Choosing a nitrogen source in the culture media can affect the growth and quality of Stevia, such as ammonium (NH4) or nitrate (NO3), and in turn affect root growth, increase biomass, and the formation of secondary metabolites, including Steviol Glycosides [53].

Conclusions

Tissue culture technology has been successfully used to multiply the number of Stevia and improve cultivars used in commercial cultivation. Several scientific studies have developed and improved tissue culture methods for stevia, and the most important results obtained are:

1. Developing Tissue Culture Protocols: Improved Stevia tissue culture protocols have been developed including using the leaves of the plant as a source of tissue for reproduction. This research has shown an increase in the growth rate, cellular proliferation, and the effectiveness of vegetative propagation.

2. Improving productivity: The yield of Stevia was improved using tissue culture, and the characteristics of plants producing active compounds were improved with higher concentrations of stevioside and an increase in the content of sweet substances.

3. Regeneration of old plants: Tissue culture can be used to regenerate old or diseased plants. The use of small plant parts, such as leaves and seed tissues, to produce new, healthy plants has been studied.

4. Using modern technologies: Modern technologies such as genetic modification and gene transfer are applied to the Stevia plant to improve desirable genetic characteristics such as increasing the content of sweet substances or reducing the content of unwanted compounds.

It should be noted that research in the tissue culture of the Stevia plant is still ongoing and developing, and recent studies may show more developments and new results in this field.

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تقنية زراعة الأنسجة النباتية لتحسين إنتاج Steviol Glycosides في نبات الستيفيا Bertoni *Stevia rebaudian* (مقالة)

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• تاريخ استلام المقالة 2023/05/26 وتاريخ القبول 2023/07/19

المستخلص

أحدثت تقنية زراعة الأنسجة النباتية تطورا هائلا في إنتاج المركبات الفعالة المختلفة ذات الأهمية بما في ذلك الجليكوسيدات في الستيفيا. *Stevia rebaudiana* هو نبات عشبي معمر معروف بأوراقه ذات المذاق الحلو والتي تحتوي على مواد فعالة تستخدم كمحليات طبيعية منخفضة السعرات الحرارية تسمى Steviol glycosides بسبب حلاوتها العالية وطبيعتها منخفضة السعرات الحرارية كبدائل للسكر. ومع ذلك فإن الزراعة التقليدية لنبات الستيفيا لإنتاج جليكوسيدات الستيفيول سيد تواجه تحديات مثل دورات النمو الطويلة، التغيرات في التركيب الكيميائي والمحددات البيئية.لذا تعد زراعة الأنسجة النباتية بديلاً لإنتاج كميات كبيرة من جليكوسيدات الستيفيول عن طريق تغيير العوامل المختلفة مثل الاوساط الغذائية ، منظمات النمو ، الاستجابة الضوئية وتم استخدام طرق زراعة الأنسجة بنجاح لإكثار النبات فضلاعن إنتاج جليكوسيدات عالية الجودة. من خلال تقنيات مثل زراعة الكالس، تكوين الأعضاء وزراعة الاجنة الجسمية يمكن إنشاء كميات كبيرة من النبيتات بسرعة من اجزاء نباتية صغيرة وبوقت قصير نسبيا ومع مزيد من التقدم في تقنيات زراعة الأنسجة وفهم علم وظائف الأعضاء وعلم الوراثة في الستيفيا فإن إمكانية إنتاج جليكوسيدات على نطاق واسع باستخدام طرق زراعة الأنسجة واعدة مما يفتح طرقًا جديدة للاستخدام التجاري لهذا المُحلي الطبيعي.

الكلمات المفتاحية :ستيفيا، ستيفيول جلوكوسيات، محلي طبيعي