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An interdisciplinary approach towards sustainable and higher steviol glycoside production from in vitro cultures of *Stevia rebaudiana*

Vartika Srivastava, Rakhi Chaturvedi *

Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India

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ABSTRACT

Stevia rebaudiana is one of the vastly acclaimed commercial plant in the world and belongs to Asteraceae family. The exclusive advantage of *Stevia* over artificial sweeteners is impeccable and targets its potentiality to the presence of diterpene glycosides. Moreover, the flaunting sweetness of steviol glycosides with associated medicinal benefits, turns the plant to be one of the most economic assets, globally. As compared to vegetative propagation through stem-cuttings, plant tissue culture is the most suitable approach in obtaining true-to-type plants of superior quality. During last few decades, significant in vitro propagation methods have been developed and still the research is ongoing. The present review discusses the tissue culture perspectives of *S. rebaudiana,* primarily focusing on the mineral nutrition, growth regulators and other accessory factors, motioning the optimum growth and development of the plant. Another crucial aspect is the generation of sweeter varieties in order to reduce the bitter-off taste, which is noticed after the consumption of the leaves. The in vitro cultures pose an efficient alternative system for production of steviol glycosides, with higher rebaudioside(s) content. Moreover, the review also covers the recent approaches pertaining to scale-up studies and genome editing perspectives.

1. Introduction

The rich biodiversity of mother nature has been an exquisite gift to mankind and other life forms on earth. Utilization of flora for agriculture, feed or ethnomedicine, has been dated since centuries. Thereafter, the journey of local and instinctive application of plants for battling and subsiding diseases is on a progressive way. From mild cold to chronic diseases, people have begun shifting their medication from synthetic drugs to natural therapies. Owing to this, industries and pharmaceutical companies have turned up their focus to plant-based drugs. Unfortunately, these growing needs of human civilization have severely affected the natural flora, causing a major reduction in their population leading them at risk of extinction.

Moreover, continuous climatic and environmental changes have led to severe modifications in plant growth, development and their metabolic profile. Entrepreneurs, research scientists and their technological advancements have initialized the largescale propagation of medicinal plants with their sustainable use and germplasm conservation. Plant tissue culture technology is most feasible and effective way in achieving extensive plant propagation in short duration and space with desirable traits, along with down streaming processing of novel secondary metabolites.

In the chronicles of events of plant tissue culture, it was Henri-Louis Duhumel du Monceau in 1756 who discovered the wound healing capacity of plants (Monceau 1756), followed by Haberlandt who initiated the tissue culture of isolated leaf tissues of *Lamium purpureum* and *Eichhornia crassipes* (Haberlandt, 1902; Bhojwani and Razdan, 1996; Thorpe, 2007). Thereafter, a lineage-walk from discovery of callus culture, suspension culture to development of largescale plantations via shoot-tip culture, marks a remarkable phase. The application of in vitro cell culture technology went well beyond micropropagation, and embraced all the in vitro approaches that were relevant or possible for the particular species, and the problems being addressed. Over recent years, underlying molecular mechanisms behind dedifferentiation and redifferentiation have gradually unfolded. It has been speculated that auxin induces cascade of transcription factors where activation of *LBD* transcription factor (TF) by *ARF7* and *ARF19* TFs was carried followed by activation of *E2Fa* TF by *LBD* TF to hark back to S-phase of cell cycle (Berckmans et al., 2011). In a study on *Solanum lycopersicum* revealed major changes during the reprogramming of cell in case of

* Corresponding author.
E-mail address: rakhi_chaturvedi@iitg.ac.in (R. Chaturvedi).

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dedifferentiation and redifferentiation such as upregulation of carbohydrate, protein and fatty acid biosynthetic pathways as well as pentose phosphate pathway (PPP) and TCA cycle Kumari et al. (2017). On the contrary, metabolites with respect to cell wall lignification was downregulated signifying that the callus is soft, friable and less-rigid in nature as compared to organized cultures. In this context, secondary metabolite profile also varies during the course of de/re-differentiation. Few molecules are upregulated while yield of few molecules get subsided. Organized cultures pose added advantage in obtaining similar quantity of metabolites as their donor mother plants until they are not affected by somaclonal variations. On the other way out, unorganized callus show variability in chemical profiling of metabolites, nevertheless, biochemical alterations for scale-up of compounds is possible only by this methodology (Kefi, 2018). Elicitation studies could also be performed as yield enhancement strategies of target secondary metabolites.

In the narrative of the aforementioned facts, present review details around the interplay of specific factors, such as explant type, macro- and micronutrients, cytokinin-auxin cross-talks, and accessory elements on the growth profile of micropropagated shoots of *Stevia rebaudiana.* The review also spotlights bioreactor studies on micropropagation of *Stevia* for obtaining higher leaf biomass. Concerning the yield enhancement strategies of steviol glycoside, two biotechnology approaches are discussed pertaining to genome editing analyses and scale-up strategies. It has been observed that apart from genes and transcription factors, expression of miRNAs is also gaining significance. Since steviol glycoside content is intertwined with the abovementioned conditions, the review discusses their effectual role on steviol glycosides production and upregulation of associated genes with a major focus on the effect of biotic and abiotic elicitors on its yield. These important parameters have not been highlighted altogether in earlier published reports.

1.1. *Stevia rebaudiana*

S. rebaudiana is 250–300 times sweeter than sucrose and is prioritized substitute for artificial sweeteners. Native to Guarani region of Paraguay, it was discovered by Moisés Santiago Bertoni in 1905. However, the intake of the plant began since early 1960 s and is well propagated in Japan, California, China, Thailand, Taiwan, Korea, Brazil, UK, Israel, Philippines, Canada, Hawaii, Ukraine and India (Brandle et al., 1998; Sivaram and Mukundan, 2003). Studies have reported that excessive usage of sweeteners doesn't increase the risk of diabetes rather improper food habits with excessive sugar intake disturbs the lipid profile of the body (Basciano et al., 2005). Dietary monitoring by intake of natural sweeteners would thereby reduce the threats of the diseases. The leaves of the plant, as well as purified steviol glycosides, pursue therapeutic aspects including anti-diabetic, hepato-protective, anti-carcinogenic, anti-hypertensive, antioxidant, anti-tuberculosis, immunomodulatory, anti-inflammatory, anti-cariogenic, neuro-protective, vasodilator, as well as non-toxic to the reproductive system. There are detailed reports discussing the aforesaid functional properties of the plant (Brahmachari et al., 2011; Ferrazzano et al., 2016; Gupta et al., 2016; Lemus-Mondaca et al., 2012; Talevi, 2021). Recently, wound healing effects have also been evaluated using aqueous extracts of *S. rebaudiana*. It led to a decrease in wound surface area, macrophages, and lymphocytes, while, the extract improved the number of blood vessels and fibrocytes (Abbasi et al., 2021).(Fig. 1).

2. Steviol glycosides

Steviol glycosides (SG) are the group of diterpene glycosides with glucose moieties attached to the basic aglycone non-glucosidic unit (Fig. 2). These moieties are attached at C-19 (R_1) and C-13 (R_2) positions at the aglycone component, which contains β-glucose, α-rhamnose and xylose as sugar substitutes. Based on this, β-glucose-based glycosides are steviolmonoside, steviolbioside, rubusoside, rebaudioside B, rebaudioside D, rebaudioside E, rebaudioside G, rebaudioside I, rebaudioside J, rebaudioside L, rebaudioside M, rebaudioside O. Stevioside is the first steviol glycoside discovered and extracted from the leaves of the plant and is 250-times sweeter than sucrose (Brandle et al., 1998; Crammer and Ikan, 1986) and non-glycemic in nature (Gantait et al., 2015; Suzuki et al., 1977). Further, other steviol glycosides were also discovered consisting of rebaudiosides' A-X of which rebaudioside A is the sweetest among all the steviol glycosides and is 300-times sweeter than sucrose. Rebaudioside M doesn't possess any bitterness or licorice after taste as compared to rebaudioside A, however, it is present in a low amount in the plant. While, α-rhamnose based derivatives are dulcoside A, rebaudioside C (dulcoside B), rebaudioside H, rebaudioside K, rebaudioside N. On the other hand, rebaudioside F represents the xylose-based derivative. These derivatives aid in providing regulatory effects to the aglycone unit. Table 1 enlists few steviol glycosides with varied R-groups and sweet potency in comparison to sucrose. Along with the sweetness, there is slight bitterness after taste, which is contributed by the presence of essential oils, tannins, flavonoids and sesquiterpene lactones (Goyal et al., 2010; Soejarto et al., 1983).

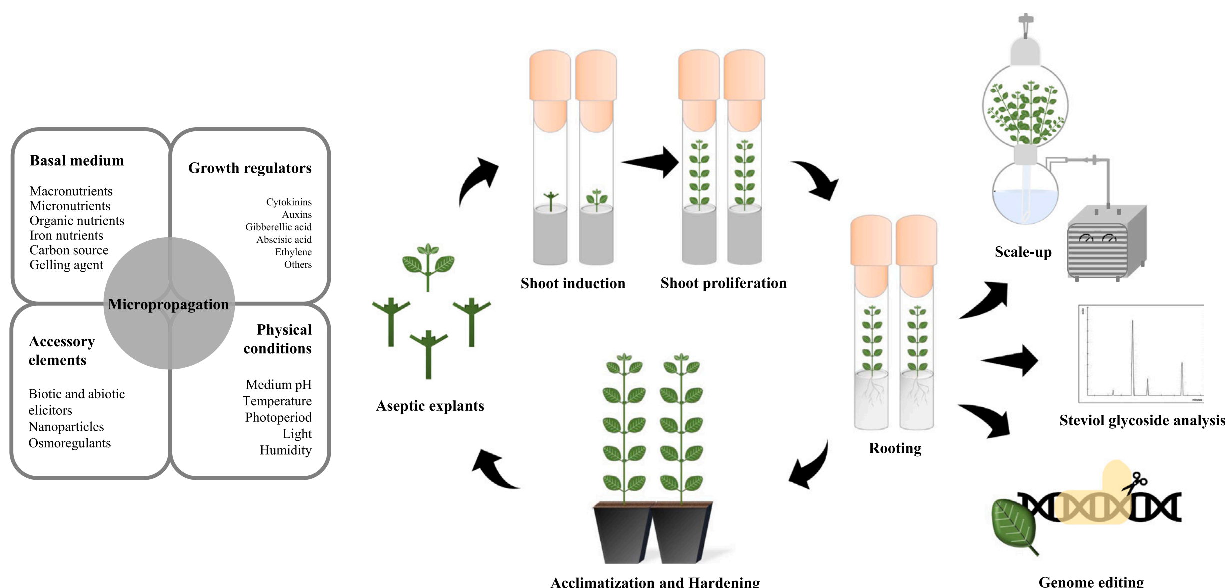

Fig. 1. Graphical overview of research outline and prospects of *Stevia rebaudiana*.

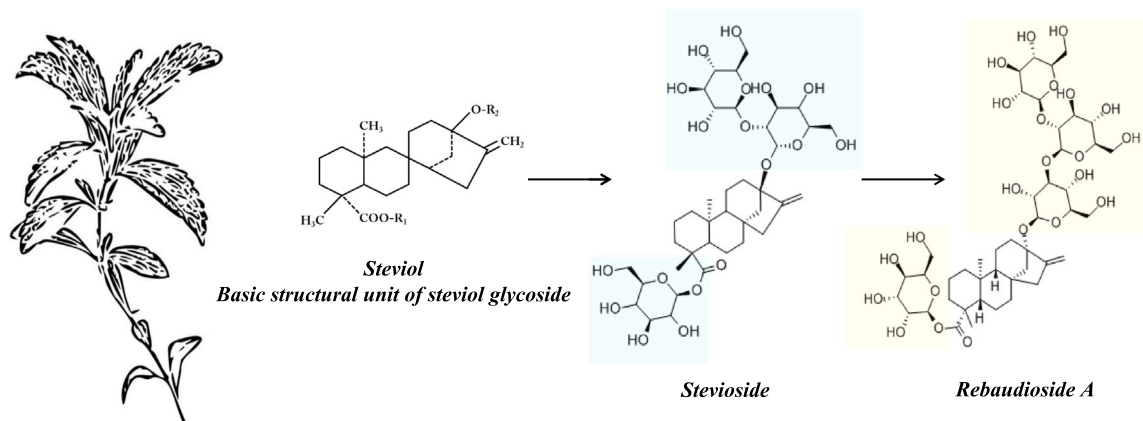

Fig. 2. Main steviol glycosides originating from steviol, the basic aglycone unit.

Tablee 1
Steviol glycosides with varied R-group moieties, molecular formula and sweet potency.

Steviol glycosides	R-Groups		Formula	Molecular Weight (g/mol)	Sweet potency*
	R1	R2			
Rebaudioside A	β-glc-	(β-glc)2-β-glc-	$C_{44}H_{70}O_{23}$	967.01	300
Rebaudioside B	H	(β-glc)2-β-glc-	$C_{38}H_{60}O_{18}$	804.88	150
Rebaudioside C	β-glc-	(β-glc, α-rha-)-β-glc-	$C_{44}H_{70}O_{22}$	951.01	30
Rebaudioside D	β-glc-β-glc-	(β-glc)2-β-glc-	$C_{50}H_{80}O_{28}$	1129.15	221
Rebaudioside E	β-glc-β-glc-	β-glc-β-glc-	$C_{44}H_{70}O_{23}$	967.01	174
Rebaudioside F	β-glc-	(β-glc, β-xyl)-β-glc-	$C_{43}H_{68}O_{22}$	936.99	200
Rebaudioside M	(β-glc)2-β-glc-	(β-glc)2-β-glc-	$C_{56}H_{90}O_{33}$	1291.30	250
Stevioside	β-glc-	β-glc-β-glc-	$C_{38}H_{60}O_{18}$	804.88	250
Steviolbioside	H	β-glc-β-glc-	$C_{32}H_{50}O_{13}$	642.73	90
Rubusoside	β-glc-	β-glc-	$C_{32}H_{50}O_{13}$	642.73	114
Dulcoside A	β-glc-	α-rha-β-glc-	$C_{38}H_{60}O_{17}$	788.87	30

*In comparison with sucrose; glc is glucose; xyl is xylose; rha is rhamnose

Being the key ingredients in food and pharmaceutical industries, the stability and safety prospects of the steviol glycosides is also crucial, as they are degraded or decomposed into other form of steviol glycosides. These glycosides are not metabolized in the alimentary tract, rather they break into steviol and glucose by the large intestinal flora. Glucose is metabolized by bacterial flora in large intestine and steviol is converted to glucuronide in liver and are excreted out (Geuns et al., 2007; Renwick and Tarka, 2008; Samuel et al., 2018; Sharma et al., 2009). Free steviol remnants get excreted out via faeces. Consumption of stevioside (administered orally) produce more steviol glucuronide than rebaudioside A. In a study by Nakayama et al. (1986), [^{3}H] stevioside was administered to Wistar rats, at a dose of 125 mg/kg. In the end, steviol was the major metabolite found in the faeces, while stevioside and steviobioside was not found. Glucose was excreted in expired air as carbon dioxide and water. Later, Cardoso et al. (1996) found the presence of steviol in the liver, kidney and intestine after intravenous administration with only $< 1.8\%$ found in the heart, stomach, muscle and testes. In another report, the authors tested the toxicological behaviour and metabolism pattern of rebaudioside A in humans as compared to stevioside. They found the maximum radioactivity with respect to steviol and minimum of steviol glucuronide, stevioside and rebaudioside A. Moreover, steviol along with stevioside and rebaudioside A was excreted out within 48 h via faecal matter (Roberts and Renwick, 2008). Rebaudioside A is successfully approved by US FDA and European Food Safety Authority (EFSA) to be used as dietary supplement or food additive. Joint FAO/WHO Expert Committee on Food Additives (JECFA 2005) confirmed non-genotoxic effects of stevioside and rebaudioside A on human intake. The dietary daily intake was proposed to be less than 6 mg/kg body weight for children and diabetic people. One of the *Stevia*-product, 'Rebiana', is commercialized in the market, which comprised of maximum content of rebaudioside A.

3. Biosynthesis of steviol glycosides

Biosynthesis of steviol glycosides share common precursor to gibberellic acid pathway. They are synthesized via methylerythritol 4-phosphate (MEP) pathway localized in the plastids of leaves (Fig. 3). From the common pathway, kaurenoic acid 13-hydroxylase (KAH) produce steviol by hydroxylation of (-)-kaurenoic acid, in endoplasmic reticulum. Subsequent transfer of glucose units is performed by plant UDP-glucosyltransferases (UGT) from UDP-glucose to steviolmonoside. There are three types of UGTs involved in the pathway, viz, *UGT85C2*, *UGT74G1*, *UGT76G1*, located in the cytosol. Further, steviol glycosides are translocated and accumulated in the vacuole. The probable transport from endoplasmic reticulum to vacuole remains unknown but hypothesized via vesicular trafficking, utilizing an electrochemical gradient or ATP for translocation of metabolites via vacuolar membrane (Brandle and Telmer, 2007). In a recent study, the authors proposed that steviol glycoside synthesis doesn't depend upon light and active photosynthesis, since, the expression profile for key diterpene glycosides were upregulated (*UGT85C2, UGT74G1* and *UGT76G1*) in dark conditions as well. The authors endorsed the similar transportation and localization of steviol glycosides in plant cells (Libik-Konieczny et al., 2020).

4. Cultivation of *Stevia* and factors governing steviol glycoside content

The nutritional aspects of *Stevia* have initiated its propagation as a crop in Japan in 1968. *Stevia* cultivation and its demand as a dietary supplement and natural sweetener got amplified over the years. Main reservoir of steviol glycosides are leaves and factors, such as harvest period, cultivar, climate, soil characteristics and proper storage conditions, govern production of rebaudioside A, stevioside, and ratio of rebaudioside A to stevioside content in the plant (Zeng et al., 2013). *Stevia* grows properly in slightly acidic to neutral soil, which could be sandy, well-drained and organically rich in potassium and phosphorus rather than nitrogen (Vozhehova et al., 2021). Potassium (K) deficiency

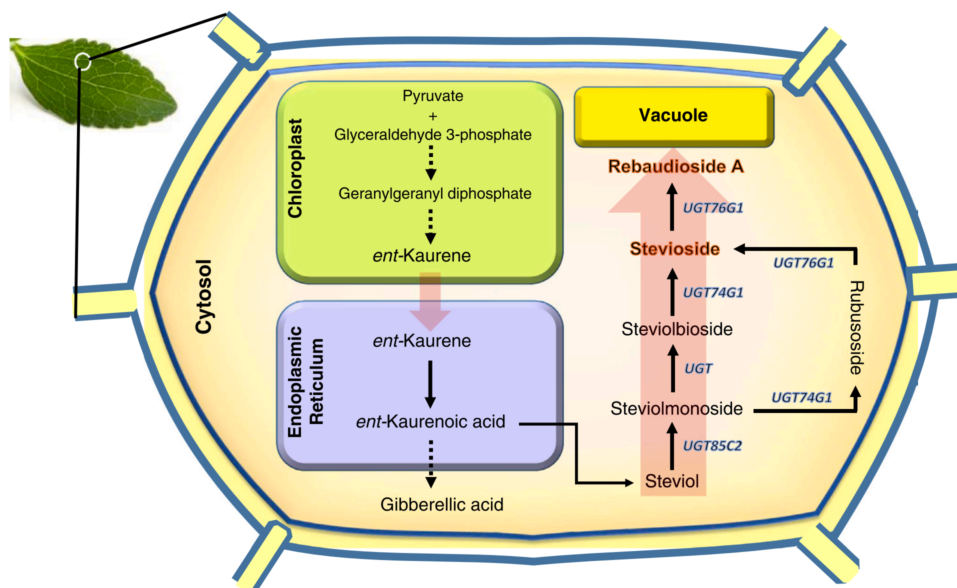

Fig. 3. Biosynthetic pathway of steviol glycosides.

was found responsible for the downregulation of gene expression profiles of key steviol glycosides, thereby, significantly reducing steviol glycosides content; however, it didn't affect the leaf biomass productivity (Sun et al., 2021). *Stevia* can tolerate mild frost but is extremely sensitive to water logging, excessive moisture and reduced aeration. Aerial part of the plant dries during winter climate, although axillary bud-break from the buried rhizome is observed, as favored by the mobilization of stored metabolites during warm temperatures of spring. This was correlated with the agronomic studies performed by Clemente et al. (2021), wherein, *Stevia* can be regarded as a semi-perennial crop and is grown in temperate areas of middle Italy. Moreover, crop rotation favors better plant growth, which reduces upon monoculture cultivation (Angelini et al., 2018; Ramesh et al., 2006). The plant grows well at an average temperature of 25 °C under long-day conditions, thus favoring the vegetative growth of the plant. Vegetative phase is important for optimum growth of the plant and high yield of steviol glycosides. In this context, Ghaheri et al. (2018) postulated variation in steviol glycoside content on the basis of their location, during the vegetative phase of *Stevia*. High rebaudioside A content was found in the upper leaves, unlike stevioside, which was found maximum in lower leaves. Moreover, in a report by de Andrade et al. (2021), among 12/12 h, 15/9 h and 16/8 h light dark photoperiods, the plant exhibited maximum rebaudioside A content, higher superoxide dismutase (SOD) and antioxidant effects, under 16/8 h photoperiod cultivation. The most prominent phase of harvesting leaves (mostly upper leaves) is the onset of flowering for obtaining highest yield of metabolites (Clemente et al., 2021; Pal et al., 2015). Authors have also stated that single harvest phase gives better yield of leaf biomass and compounds than multiple harvests, during a year (Moraes et al., 2013; Serfaty et al., 2013). Contrastingly, (Midmore and Rank, 2002) reported that multiple harvests provided better yield of biomass as compared to single harvest, provided replanting of the crop is performed every year. In India, production of dry stem and leaf biomass was on average 2.5 t/ha in the fourth year of planting, better than the initial three years (Megeji et al., 2005).

Moreover, farmers encounter weed growth as one of the problems of *Stevia* cultivation, thereby, decreasing the total biomass yield and surges the production cost. In India, summer and rainy seasons experiences 36.5% reduction in agricultural crop yield, which reduces to 22.7% during cold climates, as induced by various weed species (Shney and Babu 2008). Among few literatures, a recent study reported the weed control strategies, wherein, maximum weed control was obtained with herbicide sprays (Pendimethalin, glufosinate ammonium and prolan), while highest crop yield/leaf biomass was achieved using eucalyptus leaves as organic mulch (Taak et al., 2021).

Processing of the leaf biomass is also significant for determination of the highest yield of glycosidic content. According to the reports, drying of the leaves should be performed at ambient temperatures of 35–40 °C for 24–48 h under proper air circulation. Drying of leaves although enhances the phenolic content of the plant but it is rather easier for storage and commercialization of the leaves. Studies have been performed where steviol glycosides were extracted from fresh leaves, providing less hindrance from phenolics, but suffers stability issues as well (Lemus-Mondaca et al., 2016).

5. The need of plant tissue culture approach for propagation of *Stevia*

Market value of *Stevia* is already on a hike and demands proper method of propagation. Presently, intake of leaves of the plant increased to 5000 metric tonnes per year. Conventionally, it could be propagated via stem-cuttings or seed. Fresh seeds sown at an ambient temperature of 25 °C produces better germination rates. Long storage conditions, low temperatures and small seed size exaggerates loss in seed viability, negatively affecting its propagation (Ramesh et al., 2006; Randi and Felippe, 1981; Shock, 1982). Largescale cultivation of the plant via stem-cuttings is rather better alternative, possessing high survival rate. Yet, direct planting of the cuttings led to their low survival. Therefore, few researchers tested auxin pre-treatment to the stem-cuttings (IAA/-NAA at different concentrations), which resulted in increase in sprouting, average shoot length, number of leaves and number of roots (Ingle and Venugopal, 2009; Khalil et al., 2014b; Smitha and Umesha, 2012). In addition to this, Carneiro et al. (1997) examined the effect of different potting mixtures to attain maximum survival and growth of cuttings in green house and found maximum fresh and dry weight of the shoots in case of mixture of sand clay, soil, laying hen manure (10% v/v) and lime. Stevioside content and growth was found to be better in plants propagated via stem-cuttings (Yadav et al., 2011). Inspite of vegetative propagation, being the prevalent propagation method for *Stevia*, it is a cumbersome approach. It is highly labour intensive and requires larger land space. Moreover, seasonal changes offer great variability in plant biomass production and steviol glycoside content. Instead, plant tissue culture technology was found to be most suitable methodology, curbing the aforementioned limitations of conventional propagation methods. For obtaining high multiplication rates of in vitro plants, optimization of

key factors is important, which includes, explant type, basal macro and micro salt concentrations, growth regulators, additives and culture conditions. Few, particularly for *S. rebaudiana* have been discussed in detail. However, the present review also suggests media optimization strategies through statistical approach, which elaborates on the individual factors and their interactive studies.

5.1. Choosing the explant

Selection of appropriate explant is primarily important for bulk production of elite cell lines of target plant species. All plant cells have the ability of totipotency, giving rise to an entire plant, however, only axillary/apical buds are empowered for expressing entrenched parental genes, regenerating elite clones. Axillary bud proliferation via nodal segment culture is most widespread method of plant propagation. It involves development of shoot from pre-existing meristem and possess resistance to genetic changes even post-field transfer. Certain genes such as *LATERAL SUPPRESSOR (LAS)* and *REGULATOR of AXILLARY MERISTEMS (RAX)* genes, are responsible for initiation of meristematic potential of axillary meristem, followed by the process of axillary bud formation. Auxin(s) and cytokinin(s) have their influential role in axillary bud activation involving upregulation along with export of auxin from the bud, cell cycle reactivation, thereby, triggering the bud break (Müller and Leyser, 2011). Hence, obtaining disease-free clones, with higher rate of multiplication and regeneration potential via this methodology is relatively a rapid process and highly preferred on commercial scale.

In case of *S. rebaudiana*, tissue culture was pioneered by Handro et al. (1977), who initiated callus development using leaf and stem explants and also initiated micropropagation using nodal segments on Murashige and Skoog (1962) (MS) basal medium. Thereafter, shoot organogenesis was established using leaf explants intervened by callus formation (Yang and Chang, 1979). Among the existing literatures on *Stevia,* maximum rate of multiplication was obtained using nodal segment generating 123 shoots per explant in 65 days (Thiyagarajan and Venkatachalam, 2012). Maximum usage of this explant holds mention in other literatures as well (Hwang, 2006; Debnath, 2008; Preethi et al., 2011; Khan et al., 2014; Khan et al., 2016; Lata et al., 2013a; b; Nower, 2014; Singh et al., 2014; Tufail et al., 2019). Moreover, availability of nodal explants is in myriad form, ideal for initiation of expansive aseptic cultures with similar genetic integrity.

Apart from this, shoot-tip explants were commenced for higher production of in vitro plants giving rise to maximum of 73.6 shoots per explant in 42 days. Selection of this explant leads to rapid genesis of virus-free clones, as opted by numerous findings (Beniwal et al., 2018; Hassanen and Khalil, 2013; Tamura et al., 1984; Ramírez-Mosqueda et al., 2016). Nevertheless, a few researchers also focused upon substantial production of in vitro plants via indirect organogenesis. In a study, leaf explants produced granular yellowish-green callus in 84% of the cultures on 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BAP) supplemented MS medium, regenerating to maximum of 106 shoots per explant in 28 days (Khalil et al., 2014b). Although, the process resulted in higher production of in vitro plants, the study was devoid of any genetic integrity investigations.

5.2. Impact of basal salts on growth of Stevia

Growth regulators, as name suggests, have wide influence on proper development of the plant, however, basal mineral salts affect overall growth of the plant by modulating group of interactions between the explant and the medium components (Williams, 1993, 1995). Moreover, mineral salts govern the sensitivity of cells towards growth regulators as well. In a study, Jansen et al. (1990) evaluated the counter-acting behaviour of calcium against inhibitory effects of 2,4-D during somatic embryogenesis. In *Oryza sativa*, authors found that the regeneration capacity of immature embryo-derived callus varied with reference to changes in 2,4-D levels fortified with N_6 and MS basal media. Somatic embryos decreased in former while the phenomenon remain unchanged in the latter case (Koetje et al., 1989).

Influence of basal medium components on organogenesis of *S. rebaudiana* has been mentioned in several literatures. In a study, effect of copper on shoot proliferation rate of *Stevia* was examined, wherein, $CuSO_4$ was supplied to the induction and proliferation MS media, giving rise to 4-fold rise in shoot proliferation rate. The authors observed 37.4 and 9.2 average number of shoot buds per explant with and without supplementation of additional $CuSO_4$ (Jain et al., 2009). Similarly, Kalpana et al. (2010) inferred formation of 16.4 (medium + 0.5 μM $CuSO_4$) and 27.2 (medium + 1 μM $CuSO_4$) mean shoot buds, using nodal and leaf explants. Apart from this, influential role of adenine sulphate was illustrated by Khan et al. (2014), wherein, 62 average number of shoots was obtained with average length of 20 cm, representing large-scale cultivation of the plant. Adenine sulphate is purine nucleotide and degradation product of cytokinins. It possesses cytokinin-like effects and diminish the degradation of cytokinins in the medium (Van Staden et al., 2008). It is a rapid source of nitrogen than inorganic nitrogen, therefore it is incorporated as cytokinins coadjuvant for stimulating somatic embryogenesis, adventitious shoot formation and caulogenesis (Singh and Patel, 2014).

On the other hand, in a recent report, effect of nitrogen and phosphorus levels was studied on shoot growth and rebaudioside A and stevioside contents. The authors summarised increased shoot length, number of internodes, number of roots and root length, while decreased levels in number of leaves, fresh weight, rebaudioside A and stevioside yields, upon additional nitrogen fortification to the medium. Alike, phosphate supplementation has similar effects in case of shoot length, number of roots and root length. However, enhanced rebaudioside A, stevioside and steviol yield was observed upon 3.5, 0.5 and 2 mM of phosphate supply (Magangana et al., 2018). On the contrary, Tavarini et al. (2015) postulated enriched rebaudioside A content and better rebaudioside A/stevioside ratio upon application of nitrogen (150 kg/ha) to in vivo plants with improved photosynthetic CO_2 assimilation, stomatal conductance, RUBISCO enzyme activity, photosynthetic nitrogen-use efficiency (PNUE), and PSII efficiency.

In addition to the individual components, basal medium change had differential effects on in vitro developed shoots. Predominantly MS medium has been used by varied researchers, while quite a few literatures used WPM basal medium as well, which is known to have low salt strength (Bayraktar et al., 2016; Moharramnejad et al., 2019). A significant statistical media optimization study was performed which reported higher in vitro shoot length and leaf number with 3 × minor salts and reduced nitrogen levels, as compared to MS basal medium in 4-weeks of growth cycle (Poothong et al., 2018). In our laboratory, we unveiled Driver and Kuniyaki Walnut (1984) (DKW)) medium to be more promising than MS basal medium, in obtaining multiple, multinodal and elongated shoots. Continuous subcultures in glass culture vessels on MS basal medium resulted in rosette-like multiple shoots with low multiplication rates. Signs of hyperhydricity could also be observed, which was absent in case of DKW based medium. This inference is in corroboration in case of recent reports on *Cannabis sativa* (Holmes et al., 2021; Page et al., 2021). DKW medium attributes to high calcium (Ca^{2+}) and sulfate (SO_4^{2-}) levels with no potassium iodide (KI), could be decisive factors for higher shoot proliferation rate. However, DKW medium was used in modified form, wherein, vitamin and iron constituents of DKW basal medium were replaced with those of MS basal constituents.

5.3. Effect of cytokinins on bud proliferation and shoot growth

Bud-break phenomenon occurs upon activation of cytokinins, and polarization of auxin off the bud, along with up-regulation of axillary bud-associated genes. Upon post-decapitation, (removal of apical dominance), their cytokinins levels gets exaggerated at the nodal regions and subsequently at the buds (Müller and Leyser, 2011).

Dormancy halts and the cells eventually begin their cell cycle by their smooth transition to S-phase. As an example, in *Pea*, before removal of apical bud, the axillary buds were arrested in G_1 phase of cell cycle and undergo multiple rounds of cell arrest. After 6–9 hrs of decapitation, the mRNA expression of cell cycle genes (PCNA, cyclinB, cyclinD, and *cdc2*) enhanced in all parts of axillary buds (Shimizu and Mori, 1998).

Keeping this in mind, in majority of the micropropagation methodologies adopted for *Stevia* cultivation, the authors supplied cytokinins for efficient bud break and shoot proliferation. Among the cytokinins, BAP was mostly chosen for the study, alone or in combination with 6-furfurylaminopurine (kinetin). Both gave satisfactory shoot proliferation rate, however, the former provided better response. Ferreira and Handro (1988) obtained 50 shoots per explant in 60 days, via indirect organogenesis using leaf-disc explants, whereas, Hassanen and Khalil (2013) reported 43.9 shoots/explant, using shoot-tip explants in 42 days. In this respect, Mangena (2020) speculated that shoot proliferation is a resultant of cumulative effect of biosynthetic metabolites, aided via benzyladenosine 5-mono, di-, tri-phosphates, which are converted conjugates of BAP. Conversely, higher BAP concentration is also a noticeable issue, in induction of hyper-hydric shoots with flappy leaves. Furthermore, other cytokinins such as kinetin and thidiazuron (TDZ) display reasonable substitutes for shoot proliferation of *Stevia*. In an earlier study, multiple shoot formation (40 shoots per shoot-tip explant) was reported in 50 days on MS medium supplemented with 10 mg/L kinetin (Tamura et al., 1984). Similarly, Das et al. (2011) assessed multiple type of explants for obtaining multiple shoots, of which, shoot-tip explant gave 11.1 no. of healthy shoots on kinetin (2 mg/L) supplemented medium. On the contrary, TDZ which represents urea-type cytokinin is required in minimal quantities and enhances the accumulation of endogenous cytokinins. Although, it could also be responsible for upliftment of production cost of in vitro plants. Lata et al. (2013b) induced shoot organogenesis in 96% of *Stevia* explants leading to 60.3 shoots per explant in 4-weeks, using 1 μM TDZ. The regenerants were eventually checked for their clonal fidelity using ISSR molecular markers.

5.4. Cumulative effect of auxins and cytokinins on shoot proliferation

Apart from the ally relations of cytokinins, their cross-talk with auxins is one of the crucial features of tissue culture. In a study, synergistic use of BAP with IAA, facilitated enhanced axillary bud proliferation due to upward movement of nutrients (Black and Osborne, 1965). In case of *S. rebaudiana*, several authors speculated auxin-cytokinin(s) blend for better shoot proliferation (Banerjee and Sarkar, 2010; Hwang, 2006; Janarthanam et al., 2009; Röck-Okuyucu et al., 2016; Sivaram and Mukundan, 2003; Yang et al., 1981). In another study, Thiyagarajan and Venkatachalam (2012) reported the highest shoot proliferation rate, yielding 123 shoots/explant (with average height of 6.3 cm) in 65 days, wherein, the nodal explants were inoculated on MS medium supplemented with 4.4 μM BAP and 2.8 μM indole-3-acetic acid (IAA). The authors formulated BAP alone and in combination with kinetin, of which, the latter gave 94.5% of multiple shoot induction (16.7 shoots/explants). In another experimental setup, BAP along with 0.5 mg/L of IAA, indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) was tested and IAA came out to be superior in imparting no basal callusing, when used along with BAP. Another paradoxical feature of auxin was found to be fortification of lateral bud growth and elongation of stunted shoots, focusing on the internodal regions (Sachs and Thimann, 1967). Our laboratory also ventured the usage of BAP and IBA and observed 54-fold multiplication rate (calculated as number of shoots × number of nodes per nodal explant containing two opposite axillary buds, at the end of the multiplication cycle) in 42 days using single nodal explant. An average of 10 shoots of ~7.1 cm shoot length was obtained after inoculation of single nodal segment in the optimized medium composition (Fig. 4). Likewise, other reports mentioned the use of IAA, NAA and IBA as preferred auxins for shoot proliferation. Of these, Taleie et al. (2012) reported 8.5 cm average shoot length upon inoculation of nodal segment explant on MS + 8.9 μM BAP + 5.7 μM IAA, while Karim et al. (2008) demonstrated 6.6 cm heightened shoots on MS + 4.4 μM BAP + 5.4 μM NAA. Another known fact about auxin are, it causes significant elongation of shoots, where, the internodes are properly developed. It has universal inhibitory effects on lateral bud outgrowth or under-developed/short shoots (Libbert, 1954; Snow, 1937; Thimann, 1937). Therefore, it is clearly stated that development of micropropagation protocol for largescale cultivation of plants depends upon several factors such as explant type, concentrations and kinds of growth regulators, their combinations and the culture conditions. Nevertheless, in the findings of suitable media composition, role of basal

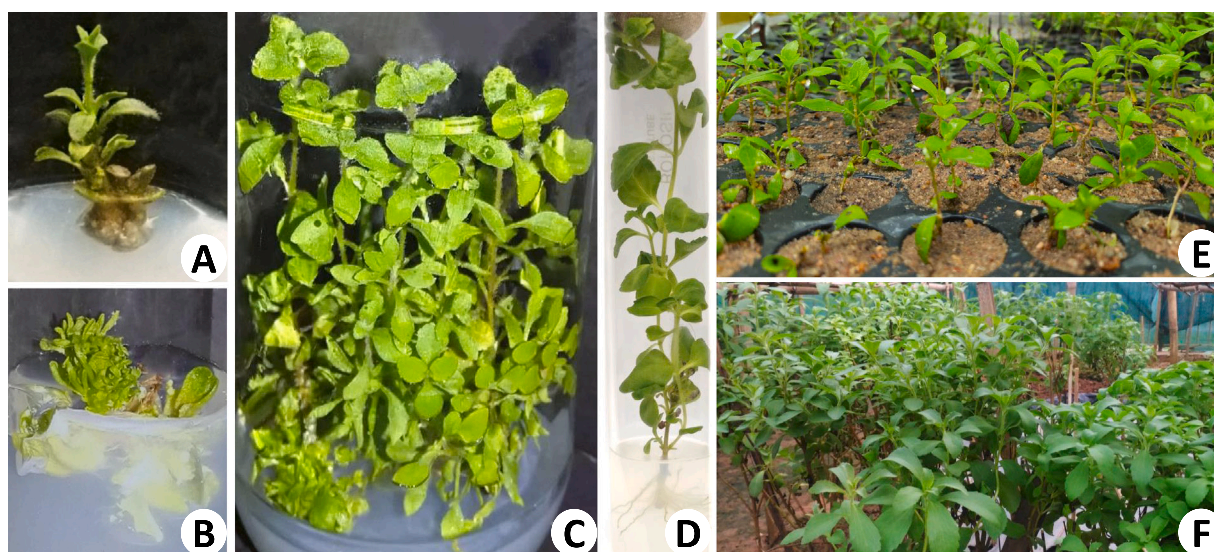

Fig. 4. Schematic representation of micropropagation through nodal segment culture of *S. rebaudiana*, (A) Two-week-old shoot proliferating from nodal segment inoculated on DKW_1 + 5 μM BAP (Bar=0.25 cm), (B) A nodal segment cultured on MS + 10 μM BAP showing hyperhydricity and flappy leaf structures (Bar=0.5 cm), (C) Multinodal elongated multiple shoots obtained on DKW_1 + 5 μM BAP + 0.05 μM IBA in 6-weeks (Bar=1 cm), (D) Root induction in regenerated shoot cultured on MS + 0.5 μM IBA (Bar=0.7 cm), (E) In vitro plants kept for acclimatization in sand:soil (3:2) in greenhouse (Bar=2.8 cm), (F) Hardened plants growing in field conditions (Bar=2.5 cm).

medium or salts play a vital role.

5.5. Clonal fidelity assessment

Micropropagated regenerants developed using nodal and shoot-tip explants bear similarity with the parent plant and pose negligible chances of somaclonal variations, as compared to the ones generated via indirect organogenesis. However, in the course of generation of true-to-type clones, onset of somaclonal variations could be observed in plant species, such as banana or plantains. The phenomenon ascribes to numerous aspects, such as explant source, stress, effect of hazardous sterilants, media components including growth regulators, carbon source and even environmental factors such as temperature, light or humidity variations. Since auxin and cytokinin balance triggers the bud break and shoot proliferation, it could indirectly force the cell to proceed to the next phase, bypassing any repair mechanism, prompting variations at chromosomal level. Another probable reason are the epigenetic disparities with respect to histone methylation or demethylation, DNA methylation, small RNAs and transposable elements (Bednarek and Orłowska, 2020; Morrison et al., 1988).

Hence, the integrity investigation of in vitro plants is an essential agreement and could be uncovered via DNA based molecular marker or cytological marker system. According to PCR based markers, Random amplification of polymorphic DNA (RAPD) analysis is a rapid technique involving amplification of repetitive DNA sequences of plant genome while Inter Simple Sequence Repeats (ISSR) deals with amplification of relatively small inter-sequence repeats between microsatellite nucleotide fragments. The latter provides better resolution and reproducibility in genome mapping experiments. Nonetheless, both of the methods don't require prior knowledge of genome statistics of plant or any organism (Bornet and Branchard, 2001; Gostimsky et al., 2005). The amplified products represent the banding profiles and reveals the monomorphism or polymorphism levels among the regenerants and the host plant (Fig. 5). Besides, cluster analysis has also been reviewed as another way for categorizing the in vitro clones on the basis of their affiliation and represented in the form of dendogram (Melchinger et al., 1992). Moreover, in a study, set of 107 expressed sequence tags based simple sequence repeat markers (EST-SSR) were developed consisting of tri-repeat (most abundant being $(ATG/CAT)_n$), tetra-repeat and penta-repeat. Among the unigene-derived microsatellites, 85% SUGMS

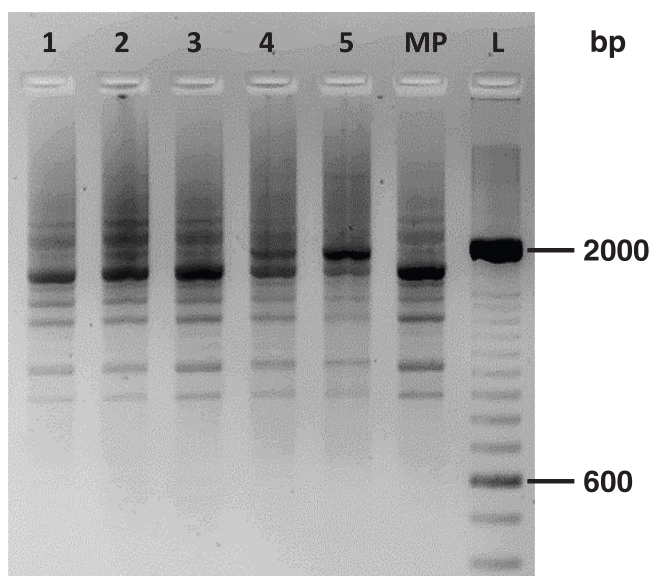

Fig. 5. Clonal fidelity analysis of in vitro regenerants using ISSR molecular marker (UBC-808). Lane 1–5: In vitro raised clones; Lane MP: Mother plant; Lane L: 100 bp ladder.

markers were identified, pertaining to steviol glycoside synthesis, vegetative to flowering phase transition phase and response to osmotic, heavy metal, biotic and abiotic stress (Bhandawat et al., 2015).

Another DNA based methodology is flow cytometry, which allows to venture the total nuclear DNA content and ploidy determination of mother plant and regenerated in vitro plant samples. Above all, for better understanding of genetic relatedness within a population, the probable route could be usage of multivariate analysis, delimiting the probabilities of inaccuracy in outcomes.

Pertaining to *S. rebaudiana*, a few manifestations have examined and proved the genetic reliability of their in vitro plants with their parental lines summoning upon molecular markers, such as ISSR (Das et al., 2011; Lata et al., 2013a; Singh et al., 2014, 2017a) and RAPD (Khan et al., 2016; Modi et al., 2012). In another report, the similarity coefficients of banding profiles aided in generation of dendogram using UPGMA method (Khan et al., 2016). Similarly, Soliman et al. (2014) generated banding profiles of *S. rebaudiana* using ISSR primers, wherein, samples represented different subcultured clones and according to the phylogenetic tree analysis, increase in number of subculture (specifically beyond 5th subculture) gradually enhanced the somaclonal variations among the samples. In another communication, the authors employed ISSR, RAPD, Amplified fragment length polymorphism (AFLP) and isozyme analysis for exploring between growth retardant treated (alar and cycocel) and non-treated *Stevia* regenerants (Hassanen and Khalil, 2013). Although, the work used multivariate analysis, the article was shortage of multiple samples runs, which could increase the efficacy of the work.

In addition to these techniques, usage of flow cytometry has emerged as qualitative way in defining the genetic stability of tissue culture derived elite clones by their corresponding ploidy. With reference of suitable standard plant, we could also estimate the genome size of target plant species as $2\ C_a = (C_1/C_2) * 2\ C_b$, where, $2\ C_a$ is value of 2 C DNA content (pg) of experimental plant system, C_1 is average G_0/G_1 peak channel of experimental plant system, C_2 is average G_0/G_1 peak channel of known (standard) plant species and $2\ C_b$ is value of 2 C DNA content of known (standard) plant species (Amaral-Silva et al., 2016). In *S. rebaudiana*, genome size have been evaluated to be 2.72 pg/2 C using tomato as internal standard. The technique was performed for selection of tetraploids after colchicine treatment was provided to *Stevia* seeds (Yadav et al., 2013). Similar to the aforesaid report, our laboratory also proved its genome size to be 2.03 pg/2 C using *Vigna radiata* as known standard. Ploidy status as denoted by their respective G_1 values was devised of in vitro regenerants which was found alike to the mother plant (Fig. 6). In conclusion, the reported literature clearly stated the applications of multivariant molecular methods for determination of genetic integrity profiles.

5.6. Assessment of steviol glycoside content in in vitro plants

The in vitro plants are multiplied clones of the mother plant providing surplus plant material throughout the year irrespective of environmental fluctuations. Therefore, they represent a constant source of steviol glycosides too. There are few reports mentioned in Table 2, summarizing the possible effects of growth regulators and other multiple factors on steviol glycoside content in mother plant and in vitro cultures of *S. rebaudiana*. The industrial demands also favor the production of in vitro clones with higher rebaudioside A, D and M content and better rebaudioside A/stevioside ratio. This reduces the off-flavor lingering after taste due to several plant components such as, lipids, proteins, phenolics, pigments, spathulenol and other monoterpenes, sesquiterpenes and labdane diterpenes. In regards with the in vitro plants, Bondarev et al. (2001) revealed that in vitro plants had lower content of steviol glycosides than the mother plants and the in vitro cell cultures showed minor quantities of the target diterpene glycosides. On the contrary, there are a few reports which verified in vitro grown cultures of *S. rebaudiana* to be rich in rebaudioside A content (Rajasekaran et al.,

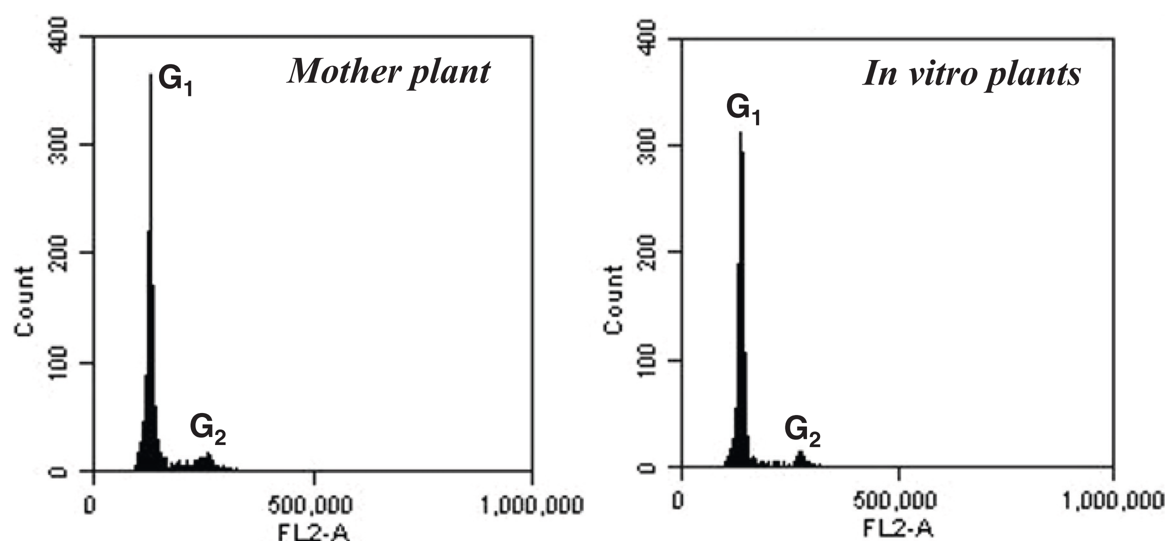

Fig. 6. Flow cytometry analysis of in vitro regenerants and the mother plants displaying similar G_1 channel position.

Table 2
Effect of major nutrients affecting the micropropagation of *S. rebaudiana* and the comparative assessment of steviol glycoside content in mother plant and in in vitro regenerants.

S. No	Plant part	Medium	Tissue culture response	Mother plant		In vitro plants		References
				R_A	Stevioside	R_A	Stevioside	
Effect of basal salts								
1.	Nodal explants	MS medium with variations in nitrogen (N) and phosphate (P) levels	Maximum shoot length of 6 cm on 0.5 N and 0.5 P media	0.0	0.0	270 mg/g FW on MS basal medium	740 mg/g on 0.5 P medium	Magangana et al. (2018)
2.	Shoot-tip	MS basal medium	NR	24.5 *	25.4 *	1.9 *	3.3 *	Ladygin et al. (2008) **(i)**
3.	Meristem	MS basal medium	NR	12.0 *	24.9 *	1.9 *	3.3 *	Bondarev et al. (2001) **(ii)**
Effect of cytokinin								
4.	Nodal explant	MS + 1 mg/L kinetin	2 no. of shoots in 3-weeks	10.4%	5.4%	11.7%	4.7%	Yücesan et al. (2016a) **(iii)**
5.	Nodal explant	MS + 1 mg/L kinetin	2 no. of shoots in 3-weeks	4.7%	6.9%	5%	6.7%	Yücesan et al. (2016b) **(iv)**
6.	Leaves	MS + 0.2 mg/L TDZ	53.2 no. of shoots per explant in 3-weeks	3.2%	5.1%	3.3%	5.2%	Lata et al. (2013a)
7.	Nodal explant	MS + 0.01 mg/L TDZ	11 no. of shoots per explant	0.0	7.0%	0.0	9.2%	Singh and Dwivedi (2014)
Effect of cytokinin + auxin								
8.	Leaves	MS + 0.5 mg/L kinetin + 2 mg/L IAA	23.4 no. of shoots per explant in 6-weeks	0.0	12.0 *	0.0	10.7 *	Hwang (2006)
9.	Nodal explant	WPM + 2.2 µM BAP + 5.4 µM NAA	8 no. of shoots in 4-weeks	0.0	0.0	12.2 * (obtained on PGR-free medium)	34.0 * (obtained on PGR-free medium)	Röck-Okuyucu et al. (2016)
10.	Nodal explants	MS + 8.8 µM BAP + 5.7 µM IAA	11.2 maximum number of shoots	4.9%		3.6%		Sivaram and Mukundan (2003)
11.	Leaf-derived callus	MS + 8.8 µM BAP + 9.8 µM IBA	–	0.0		5.8%		
12.	Shoot-tip	B5 + 4.4 BAP µM+ 0.8 µM NAA	28 no. of shoots per explant	0.04%	0.03%	0.1%	0.0	Giridhar et al. (2010) **(v)**
13.	Nodal segment originated primary callus	¼ MS + 0.5 mg/L kinetin + 1 mg/L IBA + 50 mg/L activated charcoal + 100 mg/L PVP + 1 mg/L GA_3	20.0 no. of shoots in 4-weeks	0.0	7.0%	0.0	9.2%	Singh et al. (2014)
Effect of accessory elements								
14.	Nodal explants	MS + 1 mg/L ZnO nanoparticles	4.6 cm maximum shoot length	0.0	0.0	3.6%	1.2%	Javed et al. (2017b)
15.	In vitro shoots	½ MS + 250 mg/L casein hydrolysate + 0.1% methanol	NR	0.0	0.0	10.5 mg /g DW	42.0 mg /g DW	Álvarez-Robles et al. (2016)

*mg/g DW; RA: Rebaudioside A, RB: Rebaudioside B, RC: Rebaudioside C, RD: Rebaudioside D, RF: Rebaudioside F; PGR: plant growth regulator; NR: not reported; PVP: Polyvinylpyrrolidone
i) Reported 0.9 mg/g DW R_C; **ii)** Reported 1.6 mg/g DW R_C and 0.9 mg/g DW R_B; **iii)** Reported 2.7% R_C; **iv)** Reported 1.4% R_C, 0.4% R_D, 0.2% R_F and 0.7% dulcoside A; **v)** Reported 0.02% RC, 0.09% steviolbioside and 0.02% dulcoside A

2007; Yücesan et al., 2016b). In this corroboration, our laboratory also appraised improved yield of key steviol glycosides in the in vitro regenerants (10.7% rebaudioside A and 5.2% stevioside) than the mother plant (5.8% rebaudioside A and 3.8% stevioside). Overall, ratio of rebaudioside A/stevioside improved to 2.08 and 1.51 in the in vitro and mother plants, respectively.

The effect of micronutrients is a crucial factor and affects both growth associated and non-growth associated metabolite production. As per our study, the macronutrients (Ca^{2+}, Mg^{2+}, SO_4^{2-}) and micronutrients (Zn^{2+}, Cu^{2+}) present in the DKW medium, could be decisive factors in enhanced steviol glycoside content in in vitro plants. Precisely, effect of individual minor or major salt components on productivity of steviol glycosides, is still a part of unexplored or ongoing studies. Most recently, in a report, copper (Cu), iron (Fe) and zinc (Zn) was supplemented to *Stevia* plants in pots. Of these, addition of 2 ppm of Zn significantly increased rebaudioside A/stevioside ratio, however, all three micronutrients improved steviol glycoside content in plants (Baroni-nezhad et al., 2021). Similar to this, 10 ppm of copper nanoparticles (CuO NPs) stimulated the production of rebaudioside A and stevioside by 2-fold and 3-fold, respectively, as compared to control (without CuO NPs treatment) (Javed et al., 2017a). In another manifestation, iron nanoparticles in lower doses of 45 µg/L enhanced the yield of rebaudioside A (4.9 mg/g DW) and stevioside (4.2 mg/g DW), respectively, along with improvement in plant growth and antioxidant effects (Khan et al., 2020). The fundamental role of nitrogen as available source in soil on *Stevia* growth and steviol glycosides production have been studied

Table 3
Influential role of elicitors on in vitro plant growth, steviol glycoside content and associated genes.

S. No.	Elicitor concentration and treatment duration	Control*	Effect on in vitro plant growth/induction of adventitious roots	Other remarkable features	Target gene upregulation	Fold increase in SG production as compared to control*	Reference
1.	200 mg/L chitosan for 4-weeks	In vitro shoots on MS + 2 mg/L BAP medium with no elicitors.	Maximum number of shoots (5.5 shoots per explant)	–	NR	Rebaudioside A (5.0-fold)	Rasouli et al. (2021)
	100 mg/L methyl jasmonate for 4-weeks		Significant increase in shoot length was observed			Stevioside (1.2-fold)	
2.	1.5 mg/L chitosan for 4-weeks	MS + 0.25 mg/L IAA medium	Maximum number of shoots (2.4 shoots per explant)	–	–	–	Thakur et al. (2021)
	Alginate for 4-weeks	MS + 0.25 mg/L IAA medium	–	Maximum *UGT74G1* expression observed in 1.5 mg/L PEG treatment	*UGT76G1* and *UGT74G1* in case of 1.5 mg/L alginate	Rebaudioside A (6.8-fold) in case of 0.5 mg/L alginate; Stevioside (5.1-fold) in 2 mg/L alginate	
3.	High far-red light for 1 h/day for 3 weeks in TIS-RITA®	½ MS with 1 mg/kg kinetin in TIS-RITA®	89.7% shoot biomass as compared to 76.9% in control	–	*ent-KO, ent-KS, ent-KAH13, UGT85C2, UGT74G1, UGT76G1*	Rebaudioside A (1.3-fold) and stevioside (1.6-fold)	Melviana et al. (2020)
4.	250 µM hydrogen peroxide for 3 days	½ MS liquid medium with 0.2% sucrose and 0.5 mg/L IBA.	–	Highest flavonoid content was noticed	NR	Rebaudioside A + stevioside (2.4-fold)	Alvarado-Orea et al. (2020)
5.	Mannitol for 28 days	MS basal medium	Optimum shoot proliferation was noticed on MS basal medium	–	*UGT76G1* (20 g/L mannitol) and *UGT74G1* (50 g/L mannitol)	Maximum Rebaudioside A and stevioside in 30 g/L and 20 g/L mannitol concentrations	Ghaheri et al. (2019)
6.	0.5 mg/L methyl jasmonate for 45 min	Normal roots	88.2% adventitious root induction frequency	Maximum (84.4%) DPPH free radical antioxidant effect	NR	Rebaudioside A (1.5-fold); Stevioside (1.8-fold)	Kazmi et al. (2019)
7.	50 µM salicylic acid for 3-weeks	WPM basal medium	Maximum number of shoots (3.7 shoots per explant) with 8.3 cm shoot length, 8.9 no. of nodes and 18.4 no. of leaves	50 µM methyl jasmonate also affected stevioside content	NR	Stevioside (4.7-fold)	Moharramnejad et al. (2019)
8.	10 mM H_2O_2 for 6 h	MS basal medium without H_2O_2	NR	–	NR	Rebaudioside A and stevioside (2.3-fold)	Javed et al. (2018)
9.	90 mg/L salicylic acid for 96 h	In vitro shoots on MS basal medium with no treatment	NR	–	*UGT74G1* and *KA13H*	Stevioside (1.2-fold)	Tahmasi et al. (2017)
	60 mg/L salicylic acid for 48 h				*UGT74G1* and *UGT76G1*	Rebaudioside A (1.7-fold)	
10.	1 g/L yeast extract for 4-weeks	In vitro shoots on WPM basal medium with no treatment	Maximum number of shoots (3.9 shoots per explant)		NR	Stevioside (8.7-fold)	Bayraktar et al. (2016)
	100 µM chitosan for 4-weeks		Maximum shoot length (14.7 cm) with maximum number of leaves per explant (23.4)	Maximum leaf biomass		Stevioside (4.5-fold)	
	0.5 g/L alginate for 4-weeks		Shoot length of 10.4 cm is achieved with 14.8 leaves per explant	–		Rebaudioside A (0.55-fold); Stevioside (9.2-fold)	
11.	0.1% methanol for 4-weeks	½ MS + 250 mg/L casein hydrolysate	NR	–	–	Rebaudioside A and stevioside (2-fold)	Álvarez-Robles et al. (2016)

NR: Not reported; TIS: Temporary immersion SystemTable 4 Scale up strategies of in vitro cultures of S. rebaudiana in bioreactor and its impact on steviol glycosides yield

lately. The authors elucidated decreased *Stevia* growth and biomass and increased steviol glycosides content (49.9% rebaudioside A, 84.8% rebaudioside C and 46.6% stevioside), respectively, upon nitrogen deficiency (Sun et al., 2021). Elseways, in another report, Ahmad et al. (2018) postulated that lower pH levels (5.1) of the submerged root cultures enhanced stevioside (79.5 mg/g DW) and rebaudioside A (13 mg/g DW) content, while higher dulcoside production (2.5 mg/g DW) was found higher at pH 5.8.

5.7. Role of growth regulators, elicitors and accessory compounds on in vitro cultures and steviol glycosides production

Elicitors are biotic and abiotic factors that instigate stress responses in plants, thereby, enhancing secondary metabolite production. These molecules bind to the cell-surface receptors on the plasma membrane, depolarize it and activates the antiport K^+/H^+ channel. The ion exchange persuades the efflux of Cl^-, which signals the activation of plant defence pathways. It also activates the G-protein coupled receptors and mitogen activated protein kinase (MAPK) cascades. These signal transduction pathways ultimately activate transcription factors and genes involved in synthesis of secondary metabolites (Narayani and Srivastava, 2017). Pertaining to *S. rebaudiana*, there are only few manifestations highlighting the stimulating potential of biotic, abiotic elicitors, chemicals and nanoparticles on shoot proliferation rate of the plant, steviol glycoside content and expression of associated genes (Table 3). In this regard, Bayraktar et al. (2016) evaluated shoot proliferation parameters with fortifications of alginate, casein hydrolysate, chitosan, pectin, salicylic acid, methyl jasmonate and yeast extract in different concentrations. Of these, only yeast extract (1 g/L) and alginate (2 g/L) produced higher number of shoots as compared to control. Nevertheless, each elicitor enhanced stevioside yield, while rebaudioside A was detected in alginate treatment only. Similar to this, Tahmasi et al. (2017) found intensified productivity of stevioside (38.3 mg/g DW) and rebaudioside A (2.9 mg/g DW) upon 90 and 60 g/L of salicylic acid treatment. Among the genes involved in steviol glycoside biosynthetic pathway, it upregulated *KA13H* and *UGT74G1* and downregulated *UGT76G1* gene expression. In another research work on green-house *Stevia* plants, foliar spray of 0.1 mM salicylic acid significantly increased the leaf number by 2.8-fold, upregulated *UGT76G1* and *UGT74G1*, thereby, raising rebaudioside A and stevioside content, respectively. While, 1500 mM chitosan spray downregulated *UGT76G1*, thus, reducing rebaudioside A yield (Vazquez-Hernandez et al., 2019). Similar to this, effect of chitosan together with NaCl was observed in reports of Gerami et al. (2020) affecting steviol glycoside production in green house grown *Stevia* plants. Highest rebaudioside A was noticed in treatment combination of 0.4 g/L chitosan and 150 mM NaCl salt. Whereas, high stevioside content was found in 0.4 g/L chitosan and 50 mM NaCl based treatment.

Besides, in another report, methyl jasmonate-elicited adventitious roots displayed highest 6.5 mg/g and 4.2 mg/g DW of rebaudioside and A stevioside, respectively. The report mentioned least steviol glycoside content in callus and higher content in elicitor-induced adventitious roots (Kazmi et al., 2019). Alike the previous observation, Mejía-Espejel et al. (2018) induced in vitro calli from leaf segments of *S. rebaudiana* and elicitated them with different concentrations of salicylic acid, methyl jasmonate, citric acid, ascorbic acid, BAP and 2,4-D. Of these, 10 mM salicylic acid promoted 34.6-fold increase in rebaudioside A content as compared to mother plant leaves, while stevioside got increased by 9.8-times, upon 100 mM of salicylic acid treatment. Another report on the effectual elicitor study was performed by Thakur et al. (2021), wherein, the authors inferred 2 mg/L and 0.5 mg/L alginate in aggravating stevioside by 5-fold and rebaudioside A by 7-fold, respectively. Whereas, 1.5 mg/L alginate remarkably increased the expression of *UGT76G1* and *UGT74G1* genes as well. Increase in fresh weight of biomass was however influenced due to addition of 1 mg/L yeast extract. Moving ahead in testing the effect of elicitors on gene expression of steviol glycosides has been reported by Lucho et al. (2018). In their study, in vitro shoots were grown in hydroponic system and were elicited at different exposure time. The relative gene expression was analyzed for plastid 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, condensing steps for production of kaurenoic acid and specific UGTs. In summarization of later two part pf biosynthetic pathways, paclobutrazol was observed to upregulate *KAH*, *UGT85C2* and *UGT74G1* genes at different exposure times. While methyl jasmonate elicited *UGT85C2* and *UGT76G1* genes only at 24 h of exposure. On the other hand, spermidine stimulated *KAH*, *UGT85C2*, *UGT74G1* and *UGT76G1* genes at different exposure time. Salicylic acid treatment had only mild influential role.

Apart from elicitors, urea as growth stimulus was used in a study generating 44.6 shoots in 40 days. The authors proposed urea as economic alternative to urea-type cytokinins, thidiazuron, forchlorfenuron and N-phenyl-N′-benzothiazol-6-yl urea (Khan et al., 2016). In addition to this, in a recent study, the authors have also speculated the role of light and dark conditions and different types of osmoticums on genes involved in steviol glycoside biosynthesis as well as yield of steviol glycosides. They found that *UGT85C2* and *UGT74G1* activity was upregulated in hairy roots cultured in 175 mM sucrose in dark conditions. Stevioside had better productivity in control conditions (85 mM sucrose and cultured in dark), while rebaudioside A was found in higher concentration in all experimental conditions (Libik-Konieczny et al., 2020). In this regard, Ahmad et al. (2021) established adventitious root cultures of *S. rebaudiana* and exposed the cultures with differential sucrose concentrations. They found highly branched adventitious root cultures with maximum biomass at 50 g/L sucrose and dulcoside A (12.24 mg/g DW) content at 40 g/L sucrose. Quite the reverse, maximum rebaudioside A (24.6 mg/g DW) and stevioside content (73.9 mg/g DW) was observed at low (10 g/L) sucrose. Ghaheri et al. (2019) reported that 30 g/L and 20 g/L mannitol improved rebaudioside A and stevioside content, respectively, however, plant growth was negatively affected at all the supplemented mannitol concentrations. In another report of Khan et al. (2021), NaCl salt stress of 50, 75 and 100 mM to in vitro shoot and callus cultures upregulated the expressions of *UGT85C2*, *UGT76G1* and *UGT74G1*, significantly increasing rebaudioside A expression by 67%, followed by changes in the stevioside expression as well. In a review about the effects of osmoregulants, 5% polyethylene glycol (PEG) increased rebaudioside A content (4.5%) as compared to stevioside (1.8%) upon elicitation given to in vitro suspension cultures of *S. rebaudiana*. In the same report, proline (7.5 mM) improved rebaudioside A content to 4.4%, while stevioside content showed negligible increase of 0.6% (Gupta et al., 2015). Similar to this study, gibberellic acid (2 mg/L) was found to have profound effect on adventitious root biomass and steviol glycoside production (Ahmad et al., 2020). According to biosynthetic pathway of steviol glycosides, the negative feedback suppresses the synthesis of GA_3, which directs *ent-kaurene* towards production of steviol glycosides. GA_3 has been reported to augment the expression of *ent-kaurene*, ent-*KS1*, *ent-KAH*, *UGT76G1*, *UGT74G1* and *UGT85C2,* respectively (Tavakoli et al., 2019). In a recent study by Saptari et al. (2022), daminozide (GA_3 inhibitor) reduced stem and internode length of in vitro shoots cultured in temporary immersion bioreactor. Meanwhile, leaf size and biomass were increased, while SG content was improved to 2-fold as compared to control medium without daminozide. The compound has inhibited GA_3 biosynthetic pathway and diverted towards steviol glycoside pathway. As mentioned above, light intensities have prominent effect of SG gene expressions and content. This was studied by Melviana et al. (2020) which reported effect of high far-red LED light in TIS RITA bioreactor system. The far-red light was exposed to in vitro shoots for 1 h and biomass productivity improved to 0.25 g/L/d as compared to culture without LED. The expression of *UGT76G1, UGT74G1, UGT85C2, ent-KO, ent-KS, ent-KAH13* genes enhanced.

6. Yield enhancement strategies for shoot organogenesis and steviol glycosides production

6.1. Scale-up strategies for shoot organogenesis of Stevia

Bioreactor seems to be an optimum choice for scale-up of cells and is widely used in case of cell suspension, hairy/adventitious roots and embryo cultures. Although, the initial set-up for largescale propagation was performed in *Begonia* through micropropagation in 300 ml shake-flask in 1981 (Takayama and Misawa, 1981). Hitherto, modifications in mode of culture, vessel type and capacity, impeller and sparger type has been developed. Availability of nutrients and oxygen in homogeneous way accelerates plant growth and metabolite production. Real-time monitoring of key parameters (pH, temperature, dissolved oxygen, aeration and agitation rate) provides better process control. Despite, in vitro cultures face hyperhydricity and shear problems and show slight morphological deformations, as well. With technological improvements, temporary immersion systems have been developed to overcome such issues, with add-on benefits of least contamination, semi-automation and being less complex. It also reduces the task of frequent subculturing. With standardization of immersion time and frequency, not only the optimum growth of in vitro regenerated shoots is achieved, but also the survival rate of the plantlets during soil acclimatization is high as compared to the in vitro plants grown in semi-solid or liquid submerged medium (Etienne and Berthouly, 2002; Yang and Yeh, 2008). In this section, we have discussed scale-up strategies of *S. rebaudiana* by different bioreactor methods (Table 4). The manifestations are majorly concerned with temporary immersion systems, recipient for automated temporary immersion system (RITA®) and the twin-flasks system (BIT®). The system offers ease propagation with no signs of phenolic exudation.

The first report upon *Stevia* propagation via bioreactor was published in 1994. Authors obtained 64.6 kg of shoots using 460 g of inoculum (140-fold increase) in 500 litres of bioreactor, in a two-step process. Successful acclimatization of 90% of the healthy grown shoots was achieved (Akita et al., 1994). In another report, Kalpana et al. (2009) utilized liquid culture system for *Stevia* propagation. Multiple shoot formation with 37 average number of shoots of 5.2 cm average shoot length was observed in 3-weeks. However, the in vitro shoots were difficult to root, wherein, only 63% in vitro shoots formed roots on ½ MS + 1 mg/L IAA. This issue could be easily overcome by application of temporary immersion system. It reduces the limitations posed by semi-solid and liquid cultures and aids in largescale cultivation with enhanced growth and survival. Aforesaid, immersion period in temporary immersion system plays a key role. In this context, Melviana et al. (2021) found 30 min immersion every 6 h for 21 d, gave better shoot proliferation, biomass and rate of sucrose consumption, as compared to 15 min immersion time.

6.2. Genome editing/ Genetic engineering prospects

In the course of enhancement of steviol glycosides yield, molecular breeding and sequencing techniques have been enacted. In a KEGG annotation study in *S. rebaudiana* revealed the involvement of 23 pathways, expression of *MYB* and basic helix–loop–helix (*bHLH*) related transcription factors, involved in regulation of secondary metabolism, growth regulator mediated response and cellular morphogenesis. Expression of *KA13H*, *UGT85C2*, *UGT74G1* and *UGT76G1* genes (signifying the commitment to steviol glycosidic pathway) was relatively higher during July end, signifying a higher steviol glycosides content during the vegetative phase. While *KAO*, *GA20O* and *GA3O*, corresponds to gibberellic acid genes and are widely expressed during September and October phase, indicating the reduction of steviol glycosides content during flowering phase of *S. rebaudiana*. The authors concluded *CYPs* and *UGTs* as probable targets for assessing and improving steviol glycosides content in leaves (Singh et al., 2017b). In a recent transcriptomic sequence analysis, gamma-irradiated mutant was generated with prolonged vegetative phase, which had downregulation of *FT* and *LEAFY* floral integrator genes, improved photosynthetic efficiency and carbon assimilation. Downregulation of *MADS*-box (MIKC--type), an important flowering related TF, was noticed in mutant genotype of prolonged vegetative phase. Talking about SG-related genes, mutant showed higher expression of *UGT85C2*, *UGT74G1* and *UGT76G1* in the vegetative phase only as compared to the decreased expression in prolonged vegetative phase (Singh et al., 2020).

In a recent study, role of *SrDXS1* (1-deoxy-D-xylulose-5-phosphate synthase 1) and *SrKAH* (kaurenoic acid hydroxylase) was investigated in production of 57–71% stevioside and 133–200% rebaudioside A content, respectively, in the developed transgenic lines (Zheng et al., 2019). The genes were overexpressed using *Agrobacterium* mediated

Table 4
Scale up strategies of in vitro cultures of *S. rebaudiana* in bioreactor and its impact on steviol glycosides yield.

S. No.	Objective of study	Explant type	Response	Steviol glycoside (s) studies	References
1.	Largescale shoot proliferation in roller bioreactor	In vitro shoots	1.5–2-fold higher shoot length	1.7-fold higher SG content	Bondarev et al. (2002)
2.	Largescale shoot proliferation in roller bioreactor	In vitro shoots	Two-fold increase on double MS + 3% sucrose	10 mg/g DW SG content	Bondarev et al. (2003)
3.	Largescale cultivation of shoot in bubble column bioreactor	Leaves	Elongated shoots (13.3 cm) with higher fresh weight (8.8-fold)	–	Sreedhar et al. (2008)
4.	Establishment of adventitious root system in roller bottle	Root-tip	Increase in biomass by 120-fold in 4-weeks	No traces of stevioside and rebaudioside A was detected	Reis et al. (2011)
5.	Analysing efficiency of TIB for mass propagation	Shoot-tip and nodal segments	Two-fold increase with average shoot length of 9.3 cm	–	Noordin et al. (2012)
6.	Red LED light effect on shoot growth and stevioside yield in TIB	Shoot-tip and nodal segments	Higher biomass under red LED light (1.6-fold per day)	A 1.6-fold higher stevioside (71.0 μg/g)	Alexander and Esyanti (2016)
7.	Micropropagation in TIB	Nodal segments	Elongated shoots of 19.4 cm per explant with an immersion frequency of 2 min, every 8 hr in 20 ml medium	–	Ramírez-Mosqueda et al. (2016)
8.	Comparative analysis of micropropagation methods among semi-solid, liquid and BIT® TIB	Nodal segments	Multiple taller shoots with larger leaves on TIB.	43.4 mg/g DW SG content	Vives et al. (2017)
9.	*Trichoderma asperellum* induced micropropagation in TIB	In vitro shoots	Heightened plants of 20.6 cm were obtained after treatment	15.6 mg/DW leaf of SG content	Villamarín-Gallegos et al. (2020)
10.	Daminozide induced plant growth in TIB	In vitro shoots	20 ppm treatment induced shortening in plants with higher leaf size and biomass	14.6 mg/g DW rebaudioside A and 8.1 mg/g DW stevioside	Saptari et al. (2022)

TIB: Temporary immersion bioreactor; SG: Steviol glycosides

transformation. *DXS* is the first-rate limiting step of steviol glycoside/gibberellic acid pathway and is located in the chloroplast region of cell, while *KAH* is the first committed step towards steviol glycoside synthesis. It leads to the formation of ent-kaurenoic acid, to which it shared its maximum affinity, as revealed via the docking studies. Moreover, it possesses higher number of α-helices and belongs to cytochrome P450 family (Guleria and Yadav, 2013). Among the crucial genes involved in the biosynthesis of steviol glycosides lies *UGT76G1*, which converts stevioside to rebaudioside A and is also responsible for generation of rebaudioside D and M, altogether leading to more sweetened plant with least bitter licorice after taste. Similarly, in a recent study, the authors observed that *UGT76G1* is mostly found in leaves and nodes of the plant and its expression was downregulated by *WRKY* transcription factors, ultimately reducing the content of rebaudioside A in the plant (Zhang et al., 2020). Moreover, the gene was found to be positively correlated by presence of *Acinetobacter* and *Methylobacterium* endophytes in the plant while *Sphingomonas* and *Salinibacterium* upregulates the synthesis of stevioside in the plant, respectively. On the other way round, the productivity could be enhanced by gene editing/silencing approach of steviol glycosides biosynthetic pathway. Specific miRNA genes, which are generally considered as fold-back dsRNA, have been revealed in understanding the gene regulation in steviol glycoside biosynthesis. Further, Table 5 enlists other major genes/miRNA, posing direct or indirect effect on the biosynthesis of target glycosides. Apart from this, Guleria and Yadav (2011) reported differential expression of miRNAs via in silico computational approach and stem-loop reverse transcriptase PCR. Expression of *miR169*, *miR319*, *miR414* and *miR164* was found higher in young leaves of *S. rebaudiana*, while, *mi167* and *mi398* was expressed more in the older leaves, respectively.

CRISPR-cas9 based genome editing have been adopted as robust, accurate and reproducible methodology for gene modification studies and specific trait improvement. In case of *S. rebaudiana* and steviol glycosides production, various parameters have been discussed in this entire review. Primarily, steviol glycosides production is observed maximum during the vegetative phase of the plant, therefore, gene(s) involved in these cascades of events need to be channelized using gene editing approach. Particularly, increasing the vegetative phase and delaying the flower development in *S. rebaudiana*, would lead to higher steviol glycosides yield. Based on this, transcriptome profiling of *S. rebaudiana,* revealed seven genes involved in flower development, few of them included, pyrabactin resistance-like protein 8 (PLY8), late elongated hypocotyl (LHY), pheophorbide A oxygenase (PAO), eukaryotic translation initiation factor 3 subunit E (TIF3E1) and jasmonate ZIM domain-containing protein 1 (JAZ1). Secondly, production of specific steviol glycosides such as rebaudioside A, D and M can specifically enhanced. Precise gRNA (guide RNA) with least off-target effects can be predicted by in silico studies and designed accordingly. Tissue culture-based system presents a suitable model for application of such molecular-based routes. Precisely, polycistronic tRNA-gRNA (*PTG*) was found better as compared to single guide RNA, in displaying 100% mutation rate among the 19 regenerated Cavendish cultivars of banana (Naim et al., 2018). Similarly, PTG/Cas9 system displayed 10-fold efficiency than conventional CRISPR/Cas9 system in targeting phytoene desaturase (*PDS*) gene in callus-derived kiwifruit plantlets (Wang et al., 2018). As a whole, genome editing has been utilized most recently, in several fruit crops as well, providing better insights for plant development and trait improvement strategy (Zhou et al., 2020).

7. Present scenario

The myriad therapeutic features of steviol glycosides present in the leaves of the plant behold industrial applications. The safety concerns of the compounds have proved them to be non-carcinogenic, non-cariogenic, non-teratogenic, non-mutagenic and non-toxic to human consumption (Abbas Momtazi-Borojeni et al., 2017). In addition, the crude leaf extract can also be utilized as potential sweetening source accompanied with several other phytochemicals that contribute their presence in the medicinal benefits such as phenolics, alkaloids, terpenoids, carotenoids, chlorophyll, flavonoids, lipids, proteins and essential oils, amino acids and trace elements. In view of enrichment of leaf content with high rebaudioside A, enzymatic bioconversion was performed using fermented cyclodextrin glycosyltransferase from *Bacillus licheniformis* DSM13. This led to 70–80% conversion of stevioside to rebaudioside A (Czinkóczky and Németh, 2022). In 1994, US FDA approved the intake of steviol glycosides in the form of purified *Stevia* leaf extract, as dietary supplements with adequate daily amount of 7.9 mg/kg body weight in humans of which, the major components should be stevioside and rebaudioside A. Further, EFSA and JECFA in 2010 permitted them to be food additives with daily intake rate as 4 mg/kg body weight (EFSA 2010).

Integrated as food additive, the stability of steviol glycosides is of key concern. According to the literatures, stevioside is degraded at temperatures above 140 °C. It is stable over a pH range from 2 to 8, while it is quite stable in organic acid at 80 °C as well. Moreover, stevioside recovery in diverse food products was found to be between 96% and 103%. As compared to stevioside, rebaudioside A showed better stability rate. However, after oral intake, 100% degradation of stevioside was observed within 2 days, in in vitro tests with rat intestinal microflora, while rebaudioside A stabilized till 6 days. On treatment with human microflora under anaerobic conditions, stevioside was degenerated after 10 h followed by rebaudioside A in 24 h duration (González et al., 2014). Therefore, considerable amount of safety and stability studies have proven steviol glycosides as food additives and drug. However, the most important aspect of industrial use is the extraction of these metabolites with maximum yield and with no artefacts. With respect to large scale extraction, there have been several reports discussing various possibilities of extraction. Authors have utilized microwave assisted (Jaitak et al., 2009; Ameer et al., 2017), rapid solid-liquid dynamic extraction (Gallo et al., 2017), pressurized liquid (Kovačević et al.,

Table 5
Major genes/miRNAs involved in direct/indirect effect on the biosynthesis of target steviol glycosides.

S. No	Gene of study	Significance	Modification in SG content	Remarks	Reference
1.	*RG1*	Beta -glucosidase gene	Decreases	Enhances steviol only and effect of silencing constructs was found negligible.	Yang et al. (2020)
2.	*miRStv_11* *miR319g*	*KAH* ↑ *KO, UGT85C2, KS* ↓	Increases Decreases	Co-expression anti-miR319g + *miRStv_11* resulted in 51% and 24.5% increase in rebaudioside A and stevioside content.	Saifi et al. (2019)
3.	*UGT76G1*	Stevioside → R_A	R_A/S ratio increases from 0.3 to 1.6	–	Kim et al. (2018)
4.	*ent-KO, UGT85C2, UGT76G1*	Involved in steviol biosynthesis	PBZ and PEG decreased the synthesis, which was reversed by GA_3 treatment	Effect on *ent-KS1–1*, *ent-KAH*, *UGT74G1* was stable in all the treatments	Hajihashemi et al. (2013)
5.	*KAH, UGT85C2, UGT74G1, UGT76G1*	Involved in steviol biosynthesis	The silenced constructs decreased the content	GA_3 content was enhanced	Guleria and Yadav (2013)

↑ Upregulated; ↓ Downregulated; PBZ: paclobutrazol; PEG: polyethylene glycol; GA3: gibberellic acid; RA: Rebaudioside A; S: steviosid

2018), supercritical-fluid (Erkucuk et al., 2009), ultrasonic-assisted (Liu et al., 2010; Šic Žlabur et al., 2015), pulse electric field assisted (Barba et al., 2015), enzyme assisted (Puri et al., 2012), cold-plasma assisted (Kujundžić et al., 2017) and high pressure assisted (Kovačević et al., 2018). The ultimate aim of these extractions was maximum productivity of the steviol glycosides.

8. Future prospects

Another significant prospect of *S. rebaudiana* and its in vitro derived cultures is their use in edible food packaging. It is considered as bio-based natural edible films which is biodegradable in nature. The plant is highly antioxidant in nature because of presence of secondary metabolites, particularly, steviol glycosides. Phenolic compounds are generally meant to scavenge free radicals in our body, because of hydroxyl groups (OH). The metabolite donates hydrogen atom to the reactive free radical, making it inactive. Hence, formation of less reactive and a stable phenoxyl radical (Ph-O$^{\cdot}$) takes place (Milenković et al., 2017). Our laboratory explored the free radical scavenging effects of rebaudioside A and stevioside, individually. These diterpene molecules consists of hydroxyl groups, neutralizing the reactive free radicals. Additionally, the crude extracts of ex vivo and in vitro grown plant of *Stevia,* also contributed well to the antioxidant effects. In a report, Karagöz and Demirdöven (2019) obtained high polyphenoloxidase (PPO) activity, antioxidant and antimicrobial effects of coating made up of chitosan and 2.5% *Stevia* extract. This coating was developed for storing freshly cut apple slices/cubes. In another study, Puscaselu et al. (2019) fabricated *Stevia* enriched bio-polymer based edible film. The material was highly soluble, homogenous, thinner, flexible, elastic and strong. It exhibited no microbial growth and low water activity index.

9. Conclusion

The present review provides a comprehensive outlook for commercial production of elite lines of *S. rebaudiana*, which enhances to multitude through plant tissue culture technology. The technique suits best when all the chemical and physical parameters are synced for improved plant growth. It would also give an insight of the factors affecting the biosynthetic pathway of steviol glycosides, thereby, escalating their production. *Stevia* is one of the most-researched models for obtaining non-caloric glycosides. For this purpose, genome editing is the most recent and rationale study in analysis of known and unknown genes or microRNAs involved, and their manipulations. This also omits the generation of transgenic plant varieties and related biosafety concerns. Moreover, the process of production of in vitro plants with ease extraction of compounds, can be tuned economically by application of bioprocess engineering at pilot and industrial scale. For future studies, the present study suggest media optimization approach using statistical methods such as Placket-Burman Design, Response Surface Methodology and Artificial Neural Network for analysing the aforesaid factors and their interaction studies, as well.

Key message

The review encircles around largescale propagation of S. rebaudiana, with emphasis on individual and cumulative effects of significant factors involved in regeneration of the plant and steviol glycoside content. Elaborative details on the genome editing and scale-up studies provide a deeper understanding for obtaining high steviol glycoside content.

CRediT authorship contribution statement

Vartika Srivastava: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Visualization. **Rakhi Chaturvedi:** Conceptualization, Data curation, Writing – review & editing, Visualization, Validation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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