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Research Paper

BIOTECHNOLOGY AND METABOLIC ENGINEERING OF *STEVIA REBAUDIANA* (BERT.) BERTONI: PERSPECTIVE AND POSSIBILITIES

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Metabolic engineering has emerged as a powerful tool for higher yields of secondary metabolites of industrial importance. *Stevia* plants are known for providing natural calorie-free sweetener and the sweetness arising from several steviol glycosides and their derivatives residing in the leaf tissue. Steviol glycoside biosynthesis pathway has several steps catalyzed by different enzymes, manipulation of which by either increasing or diverting flux to desired product by introducing genes from other organism can offer an attractive prospect in the future. The steviol glycosides are synthesized and get accumulated in glandular trichomes present on the surface of leaves of *Stevia rebaudiana.* Micropropagation can provide for uniform high quality planting material.

Keywords: Metabolic Engineering, *Stevia rebaudiana,* Kaurene, Rebaudioside

INTRODUCTION

S. rebaudiana produces in its leaves a range of valuable ent-kaurene glycosides. Transgenic *Stevia* plants with altered metabolic pathways may have improved beneficial plant secondary metabolites. As the sweetening properties of *Stevia* resides in its leaf tissues, metabolic engineering can be used to improvise accumulation of steviol glycosides along with enhanced productivity by increasing leaf biomass. Plant tissue culture augmenting metabolic engineering, in the present scenario can be valuable techniques for obtaining valuable products from plants like *Stevia* (Pichersky and Greshezon, 2002).

NEED FOR METABOLIC ENGINEERING AND BIOTECHNOLOGY

Metabolic engineering has paved a way for new opportunities in agriculture, environmental application, production of chemicals and medicines. Terpenoids are commercially important due to their wide application in a vast number of industrial products such as flavoring agents, pharmaceuticals, perfumes, insecticides

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and antimicrobial agents (Martin *et al*., 2003). Metabolic engineering of terpenoids in plants may lead to improvement of considerable number of traits in crops such as enhanced disease resistance, weed control by producing allelopathic crops, better pest management, production of medicinal compounds, increased value of ornamental and fruits and improved pollinations. The plants altered in their profile of terpenoids and precursor pools can make important contribution to fundamental studies of terpenoids biosynthesis and its regulation (Aharoni *et al*., 2003). Moreover, metabolic engineering experiments might reveal undiscovered branches to already known metabolic pathways.

The growing demand in today's market for natural and renewable products has refocused attention on in vitro plants materials as potential factories for secondary physiological products. This has paved the way for new research exploring secondary product expression in *in vitro* plants. The plant tissue culture technique can provide resources for flavor, aromas and fragrances, fuels, plastics, enzymes, preservatives, cosmetics, natural pigments and bioactive compounds (Karuppusamy *et al.,* 2009). The success in metabolic engineering of diterpenoids will led to future studies on biological activities of transgenic plants engineered for diterpenoid pathway. Diterpenoids like steviol glycosides present in leaves of *Stevia rebaudiana* are important due to their applications in industrial products such as commercial sweeteners flavoring agents, pharmaceuticals and antimicrobial agents. They play an important role in plant-environment interaction, plant-plant communication and plant-insect and plant-animal interactions (Pichersky and Gershezon, 2002).

BOTANICAL DESCRIPTION OF THE PLANT

Stevia rebaudiana is a new world species. Its distribution ranges from Southern United States and North eastern Paraguay to South eastern Brazil through Central America (Soejarto *et al*., 1982). It is a member of *Asteraceae* family. The plant grows up to the height of 1 m. It bears 2-3 cm long and elliptical leaves with alternate arrangement. Leaves are sessile, opposite lancoelate in shape and are serrated above the middle (Figure 1). The stem is brittle with an extensive root system. Flowers are white in color with pale purple throat. They are small in size (7-15 mm). The compound inflorescence is a cyme of corymbs differentiating in the apex capitula with five white tubular flowers (Goettemoeller and Ching, 1999; Singh and Rao, 2005; Carneiro, 2007). The seed is the acene with feathery pappus (Robinson, 1930). The reproductive anatomy of male and female gametophytes is typical for angiosperms (Shaffert and Chetobar, 1992; 1994a). *Stevia* is diploid and has 11 chromosome pairs, which is characteristic for most of the South American members of the genus (Frederico *et al*., 1996).

Trichomes present on the leaf surface have been investigated by light and Scanning Electron Microscopy (SEM). Two types of non glandular trichomes are present; larger trichomes with conical shape and length of about 300-500 µm and smaller trichomes vary in length between 80-150 µm. Glandular trichomes are round in shape and are situated in hollows of leaf blades. Their diameter is about 50-60 µm in field grown plants of *S. rebaudiana* (Figure 2). The localization of steviol glycoside biosynthesis and its accumulation and ultrastructure of gland cells

Figure 1: *Stevia rebaudiana* Plant — (a) Vegetative Stage; (b) Flowering Stage; (c) Seeds of *Stevia*; (d) Dehiscing Glandular Trichome Present of Leaf Surface

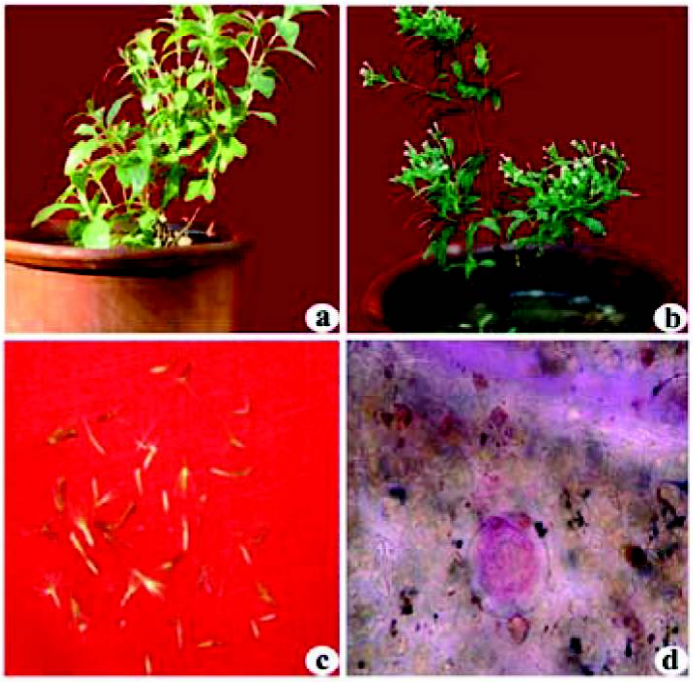
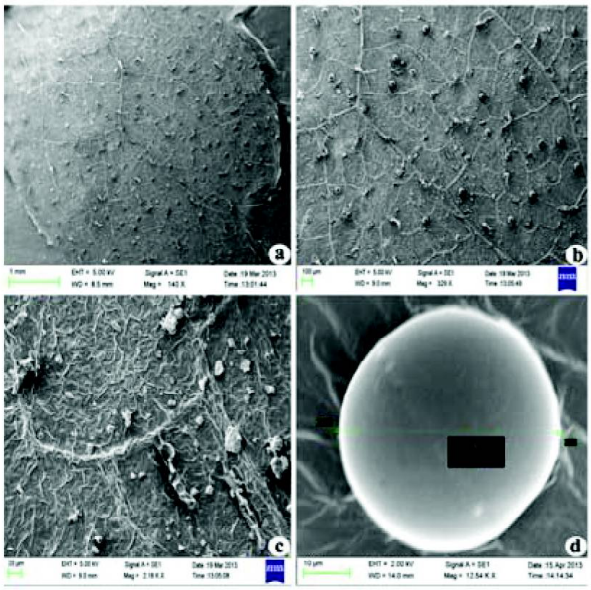


Figure 2: SEM Images of Trichomes Present on Leaf Surface — (a) Leaf surface showing three types of trichomes: large, small and round gland cells (Magnification : 140x Scale bar: 1 mm); (b) Magnified view of the gland cells of diameter 50-60 µm (Magnification: 329 x Scale Bar: 100 µm); (c) Magnified view of the nonglandular trichomes (magnification 2.18 Kx, Scale bar =20 µm); (d) Magnified view of the glandular trichome at 12.54 Kx, Scale bar = 10 µm)



were earlier studied by Transmission Electron Microscopy (TEM) (Bondarev *et al*., 2010). The authors reported that actively functioning gland cells contain a large nucleus, plastid apparatus, mitochondria and other organelles. They were characterized by dense cytoplasm containing small rounded or elongated vacuoles and electron dense inclusions. These granular inclusion bodies were reported to have precursors of diterpenoid biosynthesis (Sukhanova *et al*., 2007; Ladygin *et al*., 2008; Bondarev *et al*., 2010). The active metabolic process occurring in the plastids of gland cells was linked with diterpenoid synthesis (Bondarev *et al*., 2002). Hence a positive correlation exits between the number of glandular trichomes present on the leaf surface and the content of steviol glycosides. Various plant breeding procedures have been used to improve leaf yield and rebaudioside content in the leaves. It was observed that significant genetic variability exits among different cultivars from Japan, Korea and China with respect to leaf yield, rebaudioside content and the ratio of rebaudioside to stevioside (Brandle and Rosa, 1992; Morita, 1987; Shizhen, 1995).

Nakamura and Tamura (1985) reported that major variation in the content of four major glycosides was present in the leaves. Shibata *et al.* (1991) studied correlation among different glycosides. Dulcoside-A, stevioside, rebaudioside-B and C are positively correlated with each other while stevioside and rebaudioside-A, and dulcoside and rebaudioside-C are negatively correlated with each other. These correlations might be due to biosynthetic relationship between the individual glycosides, for e.g., stevioside is the substrate for the synthesis of rebaudioside-A therefore plants with higher content of rebaudioside-A will probably are low in stevioside content.

The genus *Stevia* comprises of 154 species of perennial herbs and shrubs, growing mostly at altitudes of 500-3000 m in semidry mountains (Robinson *et al.,* 1930; Saejarto, 1982; 1983). Among the 154 species in the genus *Stevia*, only the species *Stevia rebaudiana* and *Stevia phelebophylla* produces steviol glycosides (Robinson, 1930, Saejarto, *et al.*, 1982, 1983; Brandle and Telmer, 2007). The plants can also grow in grasslands, scrub forests and sub-alpine areas. It is indigenous to the northern region of South America and grows wild in the highlands of Amambay and near the source of the river Monday (a border area between Brazil and Paraguay) (Katayama *et al*., 1976). Today, its cultivation has also spread to other regions of the world, including Canada, and some parts of Asia and Europe (Amzad-Hossain, 2010; Gardana, 2003). *Stevia* is well known for its high content of sweet diterpenes (4-20%) present in dry-leaf matter (Ghanta *et al*., 2007).

Extensive variability within populations for day length sensitivity has been reported (Valio and Rocha, 1966; Zaidan *et a*l., 1980). Long days resulted into increase in stevioside content up to 50% as compared to plants grown under short days (Metivar and Viana, 1979). The glycoside accumulation takes place just before flowering therefore; long days along with delayed flowering are desirable as it gives more time for steviol glycoside accumulation in the glandular trichomes present on leaf surface. Plants can initiate flowering after a minimum of four true leaves have been produced (Carneiro, 1990). The self incompatibility system is operating in plant thus limiting self pollination between 0-0.5% while

out crossing ranged from 0.7% to 68.7%.(Katayama *et al.*, 1976; Miyagawa, 1986). *Stevia* plants can be propagated vegetatively by stem cuttings as seed germination rates are poor and seedlings are very slow to establish. It is best grown as annual or perennial transplanted crop. In temperate latitudes of northern hemisphere and south western Ontario in Canada, the seedlings are transplanted to the field in mid May. The crops are irrigated and fertilizers are added to the fields. *Stevia* is very slow to establish and grow sluggishly till July and most of the leaf yield is accumulated till September. The plant is harvested and then dried. After drying the leaves are separated from the stem using a thresher, and are stored for further processing.

In tropics, the crop could be transplanted in February or March and seed collected in late summer. Flowering occurs between 54 and 104 days following transplantation depending on the day length sensitivity of the cultivar used for seed production (Katayama *et al.*, 1976). Two types of seeds are reported in *Stevia*, black and tan. Black seeds have more germination potential and higher viability while tan seeds are produced without fertilization and are non viable (Goettemoeller and Ching, 1999). One-thousand seeds of *Stevia* weigh usually between 0.15 and 0.30 g and seed yields up to 8.1 kg/ ha are possible (Carneiro, 1990). Given the aforementioned conditions, seed produced on 1 ha are sufficient for supply of transplants for about 200 ha land production. Seed viability and yield are affected by growing conditions during pollination and seed filling. Excessive rainfall during pollination can affect both seed yield and germination (Carneiro, 1990; Shuping and Shizhen, 1995). Seed is best stored at 0°C, but even under low temperature conditions germination rate declines to 50% over 3 years. Sealing of storage containers or using lower temperatures did not prevent the decrease in germination over time (Shuping and Shizhen, 1995). Poor seed germination percentage is the major limiting factor to large scale cultivation of this species. Two fungal diseases caused by *Septoria steviae* and *Sclerotinia sclerotiorum*, have been reported in Canada (Lovering and Reeleder, 1996; Chang *et al*., 1997).

BIOCHEMICAL PATHWAY AND TARGET GENES

The biochemical pathway of steviol glycosides is closely related to the gibberellins with which they share part of their biosynthetic pathway. In *Stevia* kaurenoic acid, an intermediate in gibberelic acid biosynthesis is converted into the tetracyclic diterpene steviol. Gibberelic acid and steviol are synthesized from the precursor geranyl geranyl diphosphate which is formed by the deoxy xululose -5-phosphate pathway. Two terpene cyclases (Copalyl diphosphate Synthase (CPS) and Kaurene Synthase (KS)) forms of Kaurene which is then oxidized by Kaurene Oxidase (KO) to form Kaurenic acid. Kaurene oxidase belongs to cytochrome P450 family which catalyses the three step oxidation of Kaurene to form Kaurenoic acid. In *Stevia* leaves kaurenoic acid is then hydroxylated to form steviol. (Kim *et al.,* 1996) Steviol is than glycosylated by glycosyltransferase (UGTs). Three of which have been identified have been identified and characterized (UGT 85C2, UGT 74G1, UGT76G1) (Richman *et al.,* 2005). UDP glycosyltransferases catalyze the transfer of a glucose molecule to an acceptor molecule thus altering its solubility, activity, toxicity and

transport. Aglycone steviol is glycosylated differentially by different glycosyltransferases (Shibata *et al.,* 1991, 1995; Richman *et a*l., 2005). Different steviol glycosides have distinctive organoleptic properties. A sugar unit on a carbonyl group at the C19 position or either a sugar or hydroxyl group at C13 position is essential for sweetness (Kasai *et al.,* 1987). Reb-A has both the sugar units thus making it sweeter molecule than stevioside (Du Bois and Stephenson, 1985).

ACCUMULATION OF STEVIOL GLYCOSIDES

The amount of steviol glycosides has been found to decline in the following order: leaves; flower, stems, seeds, roots. The highest content of steviol glycosides is found in upper young actively growing shoot sections. During ontogeny a gradual increase in the steviol glycoside content has been observed in both mature *Stevia* leaves and stems and accumulation process lasted up to the budding phase and the onset of flowering. Leaves of *Stevia* contain a number of sweet Steviol Glycosides (SG) which are low caloric, non toxic and non mutagenic in nature (Lyakhorki *et al.,* 1993, Matsui *et al.,* 1996). In other organ of Stevia (flower, stems and roots), the content is limited (Zaiden *et al.,* 1980, Dues *et al*., 1983, Hsing *et al.,* 1983). In leaves the steviol glycoside proportion changes depending on plant age and the phase of plant development. The steviol glycosides are produced in leaves and are accumulated in glandular trichomes. The density of gland cells (glandular trichomes) and steviol glycosides content is positively correlated.

The level of steviol glycoside accumulation in upper juvenile leaves is 30-170% higher than the lower senescent leaves and the portion of Reb-A also increases as well. During ontogeny the steviol glycoside accumulation takes place in mature leaves and stems. The maximal content of stevioside in leaves is achieved during the formation of flower buds and then it gradually declines (Kang and Lee, 1981).

Hajihashemi *et al.,* (2013) reported the effect of drought stress on transcript level of genes involved in biosynthesis of steviol glycosides. *Stevia rebaudiana* plants were treated with PEG and PBZ and GA .Using quantitative real time PCR the transcript levels of Kaurene Synthase (KS), Kaurene Oxidases (KO) and Kaurenoic Acid Hydroxylase (KAH) and three UDP-dependent glycosyl transferase. UGT85C2, UGT74G1, UGT 76G1 were studied. It was reported that drought stress limits the appearance of leaves and affects steviol glycosides accumulation.

These natural products taste intensely sweet and have similar biosynthetic origin to those of Gibberelic acid. In plants, CPS and KS genes code for enzymes that are usually involved in the biosynthesis of the hormone gibberelic acid (Heddin and Kamiya, 1997). The sweet steviol glycosides found in the leaves of *Stevia* are derived from the diterpene steviol which is produced from a branch of the gibberelic acid biosynthetic pathway (Humphrey *et al.,* 2006). Steviol glycosides can accumulate in leaves at concentration as high as 20% of dry wt (Brandle *et al.,* 1998). Its concentration is about 10,000 times higher than the concentration of GA 20 found in Stevia leaves (Alves and Ruddat, 1979).

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medicines. Terpenoids are commercially important due to their wide application in a vast number of industrial products in a vast number of industrial products such as flavoring agents, pharmaceuticals, perfumes, insecticides and antimicrobial agents. (Martin *et al.,* 2003). They play an important role in plant—environment interaction, plant – plant communication, plant insect and plant -animal interaction (Pichersky and Gershezon 2002).

PLANT TISSUE CULTURE OF *STEVIA REBAUDIANA*

Tissue culture raised plants could provide the best planting material for large scale cultivation of *Stevia*. The plants raised from elite germplasm through tissue culture are genetically pure plants, free from all pathogens and have excellent vigor and can be planted throughout the year. In order to meet the demand for large scale cultivation of *Stevia rebaudiana*, extensive research work is carried out in this field.

Axillary buds were used as most preferred explants for *in vitro* regeneration (Deshmukh and Ade, 2012; Singh *et al.*, 2012; and Mehta *et al.*, 2012). Javed *et al.* (2012) applied different concentrations of sucrose, Fe-EDTA and $CuSO_4$ during *in vitro* multiplication of *Stevia rebaudiana.* The concentration of 60 g/L sucrose, 9 mL/l Fe-EDTA and 27.5 µg/L $CuSO_4$ was found to be optimum for shoot bud regeneration in liquid cultures kept on shaker.

Shatnawi *et al.* (2011) reported the use of vitrified crop reserved shoots as explants for micropropagation and liquid nitrogen was used for crop reservation of explants. Prior to cryopreservation the shoot tips were precultured with 0.4 M sorbitol for 2 days followed by 80% PVS-2 treatment for 20 min and then dehydrated with highly concentrated vitrification solution (100%PSV-2) for 60 min at 0ºC . MS medium supplemented with 1.5 mg/L BAP and 0.2 mg/L IBA was used for proliferation. Maximum regrowth (upto 68.8%) was obtained with post thawing at 40ºC for 2 min.

Modi *et al.* (2012) reported the use of hormone free MS liquid medium for micropropagation and proposed use of 100 ppm charcoal in half MS rooting medium in order to increase percent root induction and root length. Shoot bud induction using shoot tips, nodal segments, and axillary bud explants within six days of culture in MS medium supplemented with 2 mg/L Kn was achieved and the clonal fidelity of regenerated plantlets was checked by ISSR markers (Das *et al.*, 2011).

Clonal propagation of *Stevia rebaudiana* was reported using micro shoots for *in vitro* conservation. MS medium supplemented with (1.5 mg/L) BAP and (0.2 mg/L) IAA showed maximum shoot bud proliferation and (0.4 mg/L) IBA/NAA was used as rooting medium. After acclimatization 90% survival rate was achieved, when mixture of soil, perlite and peat was used in ratio of (1:1:1) (Shatnawi *et al*., 2011).

MS medium supplemented with BAP (8.87 µM) with IAA (5.71 µM) was found to be optimum for shoot regeneration. At the hardening phase, cocopeat showed the survival rate upto 70% and GCMS studies showed the presence of highest sweetener content in callus cultures supplemented with above medium (Jagatheeswari and Ranganathan, 2012). Deshmukh and Ade (2012) reported maximum number of shoots (18.3± 0.8) on MS medium supplemented with BAP+ Kn (1.5 + 0.5 mg/L)

and 95.25% rooting was reported with MS medium supplemented with (0.1 mg/L) IAA.

Dube *et al.* (2011) studied the effect of organic manures, biofertilizers and growth regulators alone and in combination for growth of *Stevia* leaves and reported that Farm Yard Manure (FYM) mixed with vermicompost and PSB mixed with Azobacter treatments could induce maximum number of leaf pairs with larger size. *In vitro* propagation of *Stevia rebaudiana* was done using apical and nodal meristerm and callogenesis was reported using leaf, nodal and internodal explants at MS medium supplemented with BAP (1 mg/L). (Ali *et al.*, 2010).

Scherwinski-Pereira *et al*. (2010) reported that *in vitro* shoot cultures stored with temperature range of 18-20ºC on half strength MS medium along with 2% sucrose, reduces growth and enhances the subculture intervals of accessions to fresh medium. Genetic resources of important medicinal plants are conserved and maintained in a pathogen free stage, facitating the distribution and germplasm exchange. The National Genetic Resources and Biotechnology Research Centre (CENARGEN) were created by Brazilian organization for Agriculture and Research (EMBRAPA) for germplasm conservation of medicinal plants.

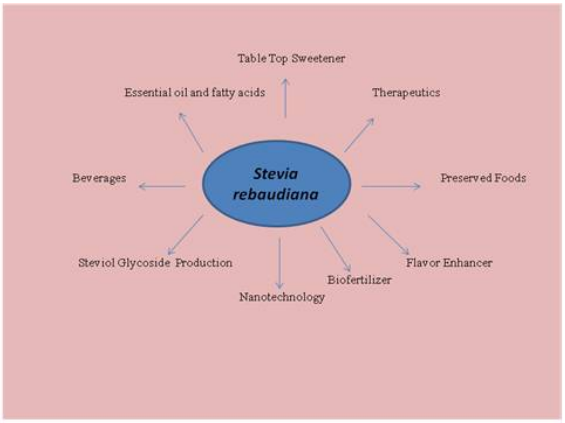
Taware *et al.* (2010) reported that biological activity of various *Stevia* extracts from calli grown cultures and micropropagated plants. The extracts were analyzed *in vitro* by using *Triticum aestivum, sorghum vulgare, Arachis hypogea, Glycine max, Cajanus cajans*, and *Cicer arientinum* as bioassay materials. It was reported that cold and hot water extracts of calli propagated on 2-4D singly and in combination of 2-4D and IBA and 2-4D and GA_3 resulted in significant inhibition for germination of *C. cajans* and *C. arientinum.*

Manjusha and Sathyanarayan (2010) used several hardening media under different environmental conditions to improve post acclimatization survival. With sand as a hardening medium, mist house had higher survival rate than green house. Inclusion of cocopeat 75% or above in the hardening medium increased 60% survival rate of plants as compared to sand as hardening media with only (1.35%) survival rate.

Pratibha *et al.* (2010) reported that nodal explants showed maximum number of shoots (35 shoots/culture) in MS medium supplemented with 4.0 mg/L Kn while Jena *et al*. (2009) had reported the use of BAP (3.0 mg/L) as optimum medium for shoot regeneration in *S. rebaudiana* and Sairkar e*t al.* (2009) reported maximum shoot proliferation from nodal segments at lower concentration of BAP, i.e. (0.1 mg/L). Janartharam *et al*. (2009) reported maximum callus formation from juvenile leaf explants with (4.44 µM) BAP and (1.34 µM) NAA with production of (14.0 ± 1.0) shoots with a length of (5.6 ± 0.1 cm) within 28 days. Sreedhar *et al.* (2008) reported shoot bud induction directly from leaf explants at 8.88 µM BAP and Kn ranging from (4.65-6.98 µM). Same medium was later modified for bioreactor cultivation of regenerated shoots resulted in a high biomass yield of ($50.68\ g\ dm^{-3}$) (m/v) It resulted into formation of 590 microcuttings in 3 weeks. Jain *et al*. (2009) reported improved micropropagation from leaf explants of *Stevia* and

enhancement in biomass and chlorophyll content by using optimal level of micronutrient copper sulphate present in the MS medium. Use of macronutrients present in MS medium for increasing *in vitro* plant propagation was reported by Ibrahim *et al*. (2008) while enhanced *in vitro* plant regeneration of *Stevia* by optimization of micronutrients present in culture medium was reported by Jain *et al.* (2012). Mitra and Pal (2007) developed micropropagation protocol using nodal segments as explants, with maximum proliferation at MS supplemented with (1.0 mg/L) IAA + (10 mg/L) Kn + (30 mg/L) adenine sulphate. Uddin *et al*. (2006) reported *in vitro* propagation of *Stevia* from calli generated using leaf, nodal, and intermodal segments as explants which were cultured on MS medium containing 2, 4-D at 2, 3, 4 and 5 mg/L for callus induction. Inter-nodal segments initiated callus earlier than node and leaf. The highest amount of callus was found in MS medium with 3.0 mg/L 2,4-D and MS medium with 5.0 mg/L 2,4-D gave the poorest callus. Sivaram and Mukundan (2003) achieved micropropagation using shoot apex, nodal and leaf explants of *Stevia* when cultured on MS medium supplemented with BAP (8.87 µM) and IAA (5.71 µM). In earlier reports, *Stevia*, regeneration has been obtained by organogenesis from different explants: leaves (Ferreira and Handro, 1987; Ferreira and Handro, 1988; Yang and Chang, 1979), axillary shoots (Bespalhok *et al.,* 1992); stem tips (Tamura *et al.,* 1984), suspension cultures (Ferreira and Handro, 1988) and anthers (Flachsland *et al.*, 1996). Somatic embryogenesis was reported from leaves (Bespalhok *et al.*, 1993; Wada *et al.*, 1981), stems (Miyagawa *et al.*, 1984) and florets (Filho and Hittari, 1997).

Biotechnology of *Stevia rebaudiana*



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EFFECT AS A SWEETENING AGENT

Stevia has been used for centuries as a bio-sweetener. It also lowers blood glucose level. Its white crystalline compound (stevioside) is the natural herbal sweetener with no calories and is over 100"300 times sweeter than table sugar (Goyal *et al.*, 2010). *Stevia* leaf extracts are commercially available in Japan, Korea, China, South-East Asia and South America, where it has been used for decades to sweeten a variety of foods and beverages (Koyama *et al*., 2003). In USA, powdered *Stevia* leaves and their extracts were used only as a dietary supplement and a skin care product, but not as a sweetener. After 2008, FDA had recognized rebiana (purified rebaudiaside-A) as safe and approved rebiana use to sweeten beverages and food products (FDA GRAS Notice GRN000253 and GRN000252 (Serio, 2010). The steviol glycosides are currently in use as a sweetener in a number of industrial foods, such as soft drinks and juices (Goyal *et al*., 2010; Jayaraman *et al*., 2008; Tadhani and Subhash, 2006a; Wallin, 2007), desserts, cold confectionery, sauces, delicacies, sweet corn,

breads, biscuits, table-top sweetener. They had replaced saccharose, in ready-to-eat cereals (Wallin, 2007), pickles (Koyama *et al.,* 2003), yoghurt (Amzad-Hossain *et al.,* 2010; Tadhani and Subhash, 2006a; Wallin, 2007), candies (Goyal *et al*., 2010; Koyama *et al*., 2003), soju, soy sauce (Amzad-Hossain *et al.*, 2010; Tadhani and Subhash, 2006a) and seafoods (Goyal *et al.,* 2010; Koyama *et al.*, 2003).

During different processing and storage condition the stability of the natural sweetener stevioside and their interaction with water-soluble vitamin, food relevant organic acids, and other low calorie sweeteners were evaluated. The authors reported that stevioside is very stable up to 120° C. In aqueous solution, stevioside is stable in a pH range 2-10 under thermal treatment up to 80° C. In strong acidic condition, i.e. (pH-1) there was significant decrease in the stevioside concentration (Kroyer *et al.,* 2010). The sweetness of 1.0 g dry *Stevia* leaves in 100 mL water is equivalent to a sucrose solution containing 20 g sucrose (Mishra *et al.,* 2010). The leaf extract of *S. rebaudiana* has promontory effects on physiology of cardiovascular and renal systems and influences hypertension and hyperglycemia (Kodjaiva *et al.*, 2013).

Carbonell-capella *et al.* (2013) studied the impact of High Pressure Processing (HPP) technology on physiological properties (colour, browning index and turbidity index), bioactive compounds (ascorbic acid, total phenolic compounds, total anthocyanin, total carotenoids) and antioxidant capacity of a fruit juice mixture consisting papaya (32.5%v/v), mango (10% v/v) and orange (7.5% v/v) sweetened with *Stevia rebaudiana* Bertoni in different percentage. The author prepared stock solution of 8.33%(w/v) from dried *Stevia* leaves in 100 mL boiling distilled water and the mixture was covered and was infused for 30 min. Then the infusion was vaccum filtered and the filterate obtained was stored at 40°C. Different volumes of *Stevia* stock solution (3 and 6 mL) were added to 14 mL of fruit juice mixture to obtain the *Stevia* concentrated solution 1.25% and 2.5% respectively. The maximum *Stevia* concentration (2.5%) was considered the best and was used taking into account the sucrose concentration of commercial fruit juices and sweeteners equivalence *Stevia*/sucrose (Savita *et al.*, 2004). Shah *et al* (2010) reported the use of sucrose-free milk chocolate sweetened with *Stevia* and having different types of commercial inulin or polydextrose as bulking agent were studied for their physicochemical, rheological and sensory properties (Shah *et al.*, 2010). Jane (2010) reported that when *Stevia* extract (0.5% w/v) was blended with inulin and polydextrose as bulking agent, it resulted into darker chocolate with similar physicochemical and sensory characteristics in comparison to sucrose sweetened milk chocolate. Rebiana, a high purity rebaudioside A was commercialized by Cargill Incorporated and The Coca-Cola Company in foods and beverages (Prakash *et al.,* 2008). Rebiana has been successfully formulated into cereals and cereal-based foods (Prakash and Du-Bios, 2007d), nutraceuticals (Prakash and Du-Bois, 2007e, f, g, h), pharmaceuticals (Prakash and Du-Bois, 2007i); edible gels (Prakash and Du-Bois, 2007j) and confectionary products (Prakash and DuBois, 2007k). Rebiana is more stable then aspartame and neotame at low and high pH applications. It was reported that leaves of *Stevia* have functional and sensory properties superior to other commercial high-potency sweeteners and is likely

to become major source of high potency sweetener for upcoming natural food market

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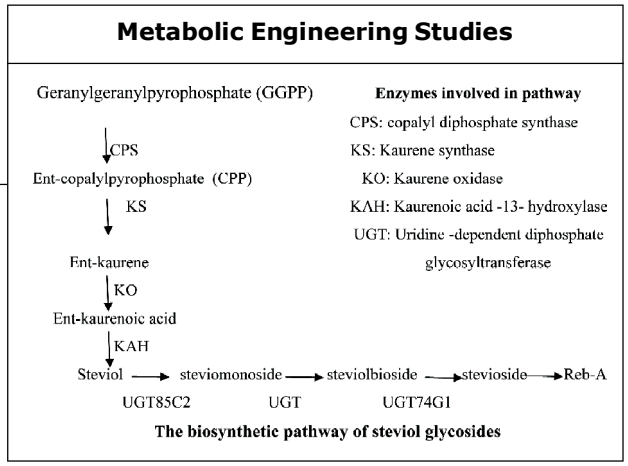

(Goyal *et al.*, 2010).

The spatial organization of the two pathways including sub cellular compartmentation provided important insight for the metabolic engineering of steviol glycosides as well as other secondary metabolites present in plants (Humprey *et al*., 2006). The subcellular location of Kaurene Oxidase (KO) and three of the UDP-dependent glycosyltransferases (UGTs) involved in steviol glycoside biosynthesis was investigated by expression of GFP fusions and cell fractionation which revealed KO to be associated with the endoplasmic reticulum and the UGTs in the cytoplasm. It has also been shown by expressing the *Stevia* UGTs in *Arabidopsis* that the pathway can be partially reconstituted by recruitment of a native *Arabidopsis* glucosyltransferase (Humphrey *et al.*, 2006).

The effect of varying concentration of sucrose (1.3 and 5%) on expression of various genes involved in stevioside biosynthesis pathway and on stevioside content was analyzed by Guleria *et al.* (2011). There was increase in expression of pathway specific genes like CDPS, KO, KS, UGT85C2, and UGT 76 G 1 in 5% sucrose treated plants as compared to plants treated with 1% and 3% sucrose. Quantitative analysis of steviol glycosides revealed about 4.5 times increase in accumulation of steviol glycosides occurred in 5% sucrose treated plants as compared 1 and 3% of sucrose. The authors suggested that sucrose might be acting as transcriptional trigger to the genes of steviol biosynthesis pathway (Guleria *et al.*, 2011). Glycosylation is thought to be one of the most important modification reactions towards plant secondary metabolites, and plays a key role in maintaining cell homeostasis, in the regulation of plant growth and development and in defense responses towards stress environment. The role of Glycosyltransferases as key players involved in modification of plant secondary metabolites was reported by Wang and Hou (2009). Six conserved mi RNAs namely miR169, miR319, miR414, miR164, miR167 and miR398 were identified using stem-loop RT-PCR analysis. The expression analysis of these miRNAs documented their roles in growth and development of *Stevia*. The detected miRNAs were found to target genes involved in plant growth, development, metabolism and signal transduction (Guleria *et al.,* 2011). Treatments of PBZ and PEG given to regenerated plants resulted into reduced plant growth while addition of gibberelic acid enhanced the growth of plants, as reported by Hajihashemi *et al.* (2012). The transcript levels of Kaurene Synthase (KS), Kaurene Oxidase (KO), Kaurenoic Acid Hydroxylase (KAH) and three UDP-dependent glycosyltransferases, UGT85C2, UGT74G1, and UGT76G1 were measured after treatment of PBZ

please chk table1 not mentioned in the text

Table 1: List of Some *Stevia* Based Beverages Available in Market

Stevia Sweetened Beverages	Stevia Brand Sweetener	Company
Sprite green, Sprite select,	Truvia	Coke
Zevia (Zero calori diet Soda)	Truvia	Zevia LLC
Fruit Juices	Truvia	Del Monte (UK)
SoBe Life Water with Coconut Water	Purevia	Pepsi
Virgil diet Soda, Virgil Zero (diet root Beer) Virgil Coca and crème Soda	Purevia	Pepsi
Blue Sky Free Soda	Truvia	Blue Sky Beverage company
Thomas Kemper natural diet soda (Zero all natural soda line)	Stevia sweetener	Thomas Kemper Beverage company
Steviva Blend Syrup designed to replace(HFCS) i.e. high Fructose corn Syrup in Beverages	Steviva Blend Syrup	Steviva Brand Inc
Organic tea, Stevia bankable blends, Juices	Stevia based table top sweetener	Pyure Brand
Glacéau vitamin water	Stevia sweetener	Coca Cola

and PEG. The transcription of entKS-1, ent-KAH and UGT74G1 was stable under different treatments while the transcription of ent-KO, UGT85C2 and UGT76G1 was significantly decreased. Both PBZ and PEG resulted into negative effect on genes transcription, which could not be reversed by GA treatment (Hajihashemi *et al.*, 2013).

BEVERAGE INDUSTRY

Stevia has made significant contribution in the beverage industry, specifically in sports beverages, functional beverages and sodas. Extracts and isolated diterpene glycosides are currently used in the food industry as sugar substitutes and sweeteners, because of their unique organoleptic properties, namely their sweetness and the virtual absence of bitterness and adventitious flavors, as well as their extremely low calorific value (Kroyer, 1999).

The soft drink manufacturers have introduced various health drinks and food supplementary beverages for the diabetics emphasizing on the need for fiber and protein content. The addition of dried *Stevia* leaves in powdered form helps in enhancing natural sweetness and also helps in rejuvenating the pancreatic gland. Moreover, *Stevia* is the rich source of proteins, Ca, P, antioxidants, flavonoids and trace elements desired in energy drinks and functional food preparation (Barathi *et al.*, 2003).

Rebiana, a high purity rebaudioside-A is more stable than aspartame and neotame at low and high pH application. In heat-processed beverages, as flavored ice-tea, juices, sport drinks, flavored milk and yogurt and non acidified teas, the sweetener show good stability during high temperature-short time heat processing and on subsequent product storage (Prakash *et al.*, 2007-b). Stability of *Stevia* sweetener in food and

interaction studies in hot beverages like tea and coffee was analyzed and was reported that at 80ºC. The stevioside-sweetened coffee and tea beverages were stable for 4 h and only minimal losses of stevioside upto 5% after 4 h warm keeping of tea and coffee was observed (Kroyer *et al*., 2010). Prakash *et al.* (2012) evaluated the stability of Reb-A in mock beverage solutions by stimulating formulations used in commercial cola soft drinks at pH 2.8 and pH 3.2, lemon-lime soft drink at pH 3.8 and root beer soft drinks at pH 4.2 and at various temperature conditions (5, 20, 30 and 40 C) for 26 weeks at each pH. It was reported that the rate and extent of degradation product formation is increased under acidic conditions (lower pH) and at higher temperatures.

Pfister *et al.* (2012) invented a method of preparing naturally sweetened orally administered products, having natural sweetener composition. A highly purified extract of (Reb-A) of about 80% to 99% purity and other steviol glycoside of about 3% or less along with and one or more bulking agents was used as a ingredients in orally administered pharmaceuticals, food and beverages and other orally administered products, for human and animals. Pyure brand has recently signed in as the *Stevia* supplier for a number of up-coming dairy-based brands. Amongst them are FitPro, the first all-natural milk-based protein shake to be endorsed by the REAL® Dairy seal, and Yoatz, a category-blending yogurt and oat-based functional super snack. Following is the list of some *Stevia* sweetened beverages available today in market.

MILK PRODUCTS

Stevia rebaudiana is a natural herb, which has enormous sweetening power and is safe for consumption. Various food preparations like milk, milk shake, curd, lemon water, *lapsi* (sweet *dalia*), custard, *halwa, kheer*, *carrot halwa*, tea, coffee prepared by using *Stevia* extract were found at par or even superior for some characteristics than table sugar (Mogra and Dashora, 2009). Stevioside is non fermenting and non browning when cooked. It can be used in baking industry for increasing the quality and safety of usage with longer shelf life period. Breads prepared with *Stevia* for diabetics show improvement in the texture and softness of the bread and increase in shelf life. *Stevia* leaf powder is required in very small quantities as compared to table sugar, i.e., 50 g of Stevia leaves can replace 1000 g of cane sugar and also had added advantage against tooth decay due to its anti microbial property (Barathi *et al.*, 2003).

Among the artificial sweeteners: 5 g of sugar, 500 mg of sugar free, 500 mg of nature, 40 mg of saccharine, 650 mg of equals and 1.50 mL of *Stevia* extract in 100 mL of water were found to be equal in sweetness. Recipes prepared by using *Stevia* extract were found to be superior to the other sweeteners while saccharine as a sweetener recorded minimum acceptability (Mogra and Dashora, 2009). Agarwal *et al*. (2009) reported that *Stevia* can be successfully incorporated upto 75 mg in place of sugar in the sweet cereal preparations and it provides good taste and low calories. It serves as a flavor enhancer and remains stable when combined with acidic foods. High temperature does not destroy its sweetening properties. It neither ferments, nor does it discolor. This makes *Stevia* suitable for hot dishes also (Sahelian and Gates,

1999). Several studies on diabetic patients showed that use of one gram of *Stevia* daily for a long period reduces blood glucose level and has no side effects (Hore *et al.,* 2002). Agarwal et al., (2010) did the organoleptic evaluation to select the most acceptable level of *Stevia* in all the recipes and it was reported that *Stevia* can be successfully incorporated upto 25 mg in place of sugar in the sweet milk preparations and it provides a good taste. Sucrose-free milk chocolate sweetened with *Stevia* and having different types of commercial inulin or polydextrose as bulking agent were studied for their physicochemical, rheological and sensory properties (Shah *et al.,* 2010). Jane, (2010) reported that *Stevia* extract (0.5% w/v) was blended with inulin and polydextrose as bulking agent resulted into darker chocolate with similar physic-chemical and sensory characteristics in comparison to sucrose sweetened milk chocolate.

THERAPEUTICS

The presence of macro and microelements in the food products is important for development and maintenance of vital body functions. They are involved in all aspects of growth, health and reproduction, participating in the formation of cells, tissues and organs (Szefer and Nriagu, 2007). *Stevia* contain substantial amount of these important nutrients and is loaded with minerals needed to protect the body and to regulate and maintain various metabolic processes (Mondaca *et al.,* 2012). Minerals which are beneficial to human health like Potassium, Calcium, Magnesium, Zinc and Iron are present in abundance in leaves of *Stevia* (Abou -Arab *et al*., 2010).

Zinc can act as a non enzymatic antioxidant and would prevent oxidative damage in the cells. The main biological function of Iron is to transport oxygen to various organ of body and lack of this mineral in the diet leads to anemia. Presence of high amount of Iron in *Stevia* leaves is therefore helpful in maintaining the normal hemoglobin level in the body. The sweet preparations from *Stevia* leaves can be used commercially to combat Iron deficiency caused due to in anemia which is the major nutritional disorder in developing countries. (Abou -Arab *et al*., 2010).

Kim *et al.* (2011) studied the amount of water soluble vitamins in *Stevia* leaves and callus extracts. They determined that the content of the folic acid, vitamin C, and the vitamin B-2 in the leaf extracts was significantly higher than those of the callus extracts. In the leaf extract folic acid was found to be major compound followed be vitamin C while in the callus extract, vitamin C was the major compound followed by vitamin B.

ESSENTIAL OIL AND FATTY ACIDS

Presence of essential oil in *Stevia* plant extracts are useful in preparation of perfumes, drugs and are added in food products. (Tepe *et al*., 2005). Essential fatty acids are required in the synthesis of many cellular structures and several biologically important compounds. Palmitic, Palmitoleic, stearic, oleic, linoleic and linolenic acids were identified in *Stevia* leaf oil. Among the identified fatty acids, palmitic acid content was found to be highest, whereas stearic acid content was least. *Stevia* leaf oil proves to be a rich source of linolenic acid also. This high value of linolenic acid may help in maintaining an ideal fatty acid ratio in human diet. (Mondaca *et al*., 2012).

NANOTECHNOLOGY

Mishra *et al*. (2010) reported the synthesis of gold within 20 min by reducing gold chloride ($HAuCl_4$) with dried powdered leaves of *Stevia*. The nanoparticles were characterized and investigated by (uv-vis spectroscopy, (HR-TEM) high –resolution transmission electron microscopy, energy-dispersive-X-ray (EDX) spectroscopy, and X-Ray Diffraction (XRD). UV-Vis spectrum of an aqueous medium containing gold nanoparticles showed a peak at 539 nm, corresponding to the Surface Plasmon Resonance band (SPR) of gold nanoparticles. HR-TEM micrograph showed the formation of well dispersed octahedral gold nanoparticles of size 8-20 nm. Yilmaz *et al*. (2011) synthesized silver nanoparticles employing a shadow- dried *Stevia* leaf extracts in $AgNO_3$ solution. TEM and X-ray diffraction indicated that nanoparticles are spherical and polydispersed with diameter ranging 2-50 nm.

CURRENT STATUS

Stevia is the latest innovation in the food industry, quenching the long quest for an alternative to sucrose. Previously *Stevia* was used as a "Natural Health Product" which is defined as a natural substance used to restore or maintain good health (e.g., herbal remedies, vitamins, minerals and probiotics). After FDA approval in (2008), its market share grew exponentially in USA and Europe. Canadian and US market has embraced Stevia as a healthy and effective sugar substitute. Once relegated to natural and organic markets as a niche product, *Stevia* has gained prominence on the shelves of more traditional grocers and malls in different parts of world.

FUTURE PROSPECTS

Since December 2008, it has become a major player in the sweetener industry. Zenith International estimates that the global *Stevia* industry could be worth $1 bn by 2014, and the World Health Organization (WHO) estimates *Stevia* could eventually replace 20 to 30% of all dietary sweeteners.

CONCLUSION

The conclusion drawn from the extensive research done in varied areas of Pharma and Biotech sectors highlight the potential of medicinal herb, *Stevia rebaudiana* to become a sweetener of the millennium in the upcoming era.

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