ABSTRACT

We live in an age of modern convenience and great advancement but mankind’s ongoing battle with health disorders shows no signs of diminishing. These are the results of the fast urban artificial lifestyle, changing diet patterns, lack of exercise, stress etc which are responsible for making many in developing countries susceptible to obesity related health problems like diabetes, dyslipidemia, heart disease, hypertension, stroke etc. The most alarming of them is diabetes. The World Health Organization (WHO) has commented that there is an apparent epidemic of diabetes which is strongly related to lifestyle and economic change.

*Stevia* is a natural sweetener and it is non-caloric sweetener and flavor enhancer. India’s total sugar demand is likely to go up to few million tons this year as against the estimated output. Sugar output was 24.6 million tones for 2012-13 season marginally lower than 26 million tonne produced in the previous year. The recurring shortage scenario for sugar point is needed to focus on *Stevia* cultivation in India in a big way. Of the total demand for sugar in India, around 70% of the sugar is reported to be used for industrial purposes namely soft drinks, chocolates, beverages, ice creams etc. this means that only around 30% of the sugar is used for household consumption.

*Stevia* can certainly be used as substitute for sugar particularly for industrial purpose. One kilogram of *Stevia* is around 300 times sweeter than one kilogram of granular sugar and *Stevia* provides zero calories. The refined extract of *Stevia* contain 85% to 95% of Stevioside which is in liquid or natural creamy off white colour powder form which is 300 times sweetener than sugar.
Stevia has excellent capacities in alleviation, such as diseases blood pressure, blood sugar, diabetes stomach problems, toothache, skin diseases etc. Therefore, Stevia is really a gift and blessing of nature to the mankind.

*Stevia rebaudiana* Bert (Asteraceae) has garnered attention with the rise in demand for low-carbohydrate, low-sugar food alternatives. It has also shown promise in medical research for treating such conditions as obesity and high blood pressure. *Stevia rebaudiana* Bert (Asteraceae) has a negligible effect on blood glucose, even enhancing glucose tolerance, therefore, it is attractive as a natural sweetener to diabetics and others on carbohydrate-controlled diets.

For centuries, worldwide, this 'sweet' desire drove people to seek out sweet foods and ingredients in nature. With the expansion of development beginning in the United States and a number of other countries. Conventional breeding techniques are being pursued to produce varieties of *Stevia rebaudiana* Bert (Asteraceae) that have increased sweet compounds.

The two main glycosides, Stevioside (St) and Rebaudioside A (R-A) confer sweetness. Stevioside is traditionally used in the majority of the sweetener (60-70% of the total) and is assessed as 300 times sweeter than sugar. It is also responsible for the licorice taste. Rebaudioside A (R-A) is usually used in 30 -40% of total sweetener and gives the sweetest, being 180 -400 times sweeter than sugar with no after taste.

Stevioside is the main sweet compound in the leaves of the sweet plant. Stevioside has numerous benefits as a sweetener. It can be used by diabetics, obese persons and patients suffering from phenylketonuria, an illness which requires a strict diet without artificial sweeteners such as aspartame has potential as a treatment for type-2 diabetes.
The unique nature of *Stevia rebaudiana* Bert (Asteraceae) represents a new type of sweet. As such, previous terminology around sweeteners does not accurately describe, the role *Stevia* could play in promoting healthful, tasty diets within energy (calorie) needs. Therefore, it is neither an artificial sweetener nor does it have the caloric properties of sugar. *Stevia* is, therefore, a new kind of sweetener. We propose that the term *Stevia* refer to no calorie, natural sweeteners from the *Stevia rebaudiana* Bert (Asteraceae) plant.

*Stevia rebaudiana* Bert (Asteraceae) is either propagated by seed or by cutting. Seeds of *Stevia rebaudiana* Bert (Asteraceae) show a very low germination percentage [7-10%] and vegetative propagation is affected due to limited availability of mother plants. Production of superior quality trait plants through *in vitro* propagation on a mass scale in a short span holds great potential for this economically important plant.

In the present study Development of somaclonal variants, through organogenesis and somatic embryogenesis. The above obtained variants can be analyzed for increasing of biomass (leaf weight dry/fresh). Estimation of Stevioside percentage in both Micro propagated plant and somatic embryo genetically grown plants. DNA isolation and RAPD Analysis of micro propagated, somatic embryogenetic plants and normally grown plant. Acclamatization of this *in vitro* grown plants to green house and final transfer to the field are the few objectives taken into consideration.

Here in this work A two-pronged experimental strategy was adopted to unravel the possibilities of any sort of somaclonal variations in both the *In vivo* and *In vitro* plants.

1) *In vitro* growth and acclimatization of the plant.

2) Molecular analysis to detect the possibilities for any sort of somaclonal variations
In this due process the mother plant was grown *In vitro* in order to minimize the contamination for further experiments to be carried over. Here standardization of protocols for the surface sterilization, media standardization for different phases of growth of the plant has been the keen priority taken keeping in view the sensitivity of the plant to the atmosphere of Hyderabad (research area).

The first priority was to avoid maximum fungal and bacterial contamination in the explants used for the experiments, in this context surface sterilization of the explants (Nodal region, Shoot apex, Leaf region) was done here the explants were washed with distilled water along with Tween - 20 and they were also washed with a mixture of fungicides (Bavistin+ Streptomycin+ K-Cyclin) followed by 4 to 5 times of regular wash so that the plants are contaminant free later they were shifted to the laminar air flow chamber and inoculated in inoculation media.

A standardized medium was used for the inoculation of *Stevia* using BAP (1.0 mg/l) used and the explants used were the shoot apex, nodal region and leaf segments with in a period of seven days and the formation of first leaf, second leaf formation has been taken into consideration.

Callus formation was observed from both the nodal and leaf explants when the explants were treated with BAP and 2,4-D. Profuse callus was observed. We were successful in observing different developmental stages of somatic embryos using SEM studies. The fine structure of somatic embryos was clearly identified during the following sequential developmental stages: single spherical cell, early globular shape with conspicuous suspensor, typical globular shape, elongated shape, early heart shape, typical heart shape, torpedo shape.
Somatic embryogenesis was seen in the medium supplemented with 2,4-D, 2-ip and the explant source was the callus obtained from the nodal and leaf explants. Plant regeneration was seen from the somatic embryos which was later on acclimatized in the green house.

After rooting the plant is shifted to green house for Hardening. Hardening is taking place in two phases. Initially the invitro plants are placed in Green house for 2-3 days to acclimatize it to new environment. Then the explants are washed with fungicide (Bavistin) thoroughly so that no media particles are left attach to the newly formed roots then the small plantlets are shifted into Plastic Cavities for primary hardening containing potting mixture bearing a composition of Coco peat + vermiculite + neemcake + trico powder in 3:1:5:3 ratio this potting mixture is mixed well and kept 15 days before the usage. After 15 days this mixture is filled in small cavities stand then the stevia plants are washed and shifted in this cavities. Temperature of around 25° -28 °C is maintained inside the plastic sheeted tents. After two weeks good healthy root development has been observed.

Then the whole stevia plant is shifted into polythene bags containing a mixture of Red soil ¾ +potting mixture ¼ here the potting mixture used has a slight variation in the composition. It has Vermicompost + Coir pith + DAP +SSP + Neem cake +Red soil in (4:1:2:2:2:1) ratio this potting mixture has been prepared 10 days before usage by watering it alternate days. After 10 days this potting mixture can be used for second stage of hardening now shift the plantlets from cavities to polythene bags for 2 weeks and keep this polythene bags also under the plastic tunnels and water them daily and after 4 weeks it is shifted to pots also. At this stage the plant is ready for outlet.
The main problem lies with the stevioside content of a stevia plants raised through seeds which varied widely (probably due to gene segregation). To avoid such segregation and also to improve the yield of stevioside it is necessary to propagate a genetically homogenous population from a selected elite plant of desirable character. The available germplasm is limited and also the plant is propagated by means of vegetative propagation, micro propagation, organogenesis and Somatic embryogenesis can serve as an alternative method to conventional techniques which give disease free and resistant plants. This also helps to preserve germplasm by storing the *in vitro* developed propagules under low temperature. Therefore the present study has also been focused on the acclimatization of plants in the green house as it has been observed that after ex-vitro transfer, these plantlets might easily be impaired by sudden changes in environmental conditions. Proper acclimatization procedure has been developed to enhance the survival percentage of plants.

In the present study we have determined stevioside in stevia plants grown *invitro* and *in vivo* qualitatively by using Soxhlet extraction method analyzed via HPLC. A standard solution of stevioside was taken for analysis. The separation of stevioside from the leaves was carried out by preparatory HPLC [Waters HPLC purification System]. The HPLC operating conditions and parameters are as follows: The column used was C18, with dimensions of 250 X 4.6mm, 5μ. The mobile phase used was Acetonitrile : water [80:20 v/v] the Wavelength of detection was at 254nm.

Determination of the content of Stevioside in plant material was performed by external standard method. A stock solution of standard Stevioside (Menge Germany) was used and solutions with concentrations of 100, 200, 300, 500 and 800 mg/L were used to
draw calibration curve. Triplicate determinations were carried out and the average taken in
drawing the calibration curve.

Here in this experiment samples used have almost nearby peaks when compared to
the standard (Stevioside) used. Which indicates that the in vivo and invitro samples used
in HPLC are having the more percentage of stevioside and in vitro sample is better than in vivo samples.

The area and retention time of in vivo and for in vitro has been taken into
consideration. So, we conclude here that the tissue culturally grown plants is better option.
Somaclonal variations are not much seen in the given samples.

DNA ANALYSIS/EXTRACTION:

50g of leaves were weighed and cut into fine pieces. Pieces were then take in pistle
and to that 100ml DNA analysis buffer was added and macerated thoroughly to prepare a
homogenate. The homogenate was centrifuged for 10 min at 8000rp. The clear aqueous
upper layers was transferred to new centrifuge tube, and add two volumes of cold
isopropanal and mixed by inversion and placed in -20°C for 30 min. Pellet the genomic
DNA by centrifuging at 10000 rpm for 20 min at 10°C. Wash the pellet with 70% ethanol
twice and air dry the DNA pellet. Dissolve the DNA in T_{10}E_{1} and use 25 – 50 ng/ul DNA
for PCR analysis.
Testing of Clonal fidelity in tissue cultured plants using RAPDs.

Isolation and quantification of genomic DNA has been carried out.

Genomic DNA was isolated from the field grown plant and regenerated plants on MS medium supplemented growth regulators. The quantification of extracted DNA was done by Spectrophotometer and through comparison with defined concentrations of λ DNA. The quantity of DNA in different samples when compared with λ DNA and was found to be ranging from 200-700 ng/l. Four plant samples were taken for variability assessment.

In RAPD analysis the results were scored as patterns of bands obtained from in vitro regenerated plants grown on MS medium supplemented growth regulators. 20 OPA RAPD primers tested produced amplification products that were monomorphic across all regenerants. The size of the monomorphic was detected after amplification by PCR among regenerated plants grown on different hormones. Thus, all regenerated DNA fragments, produced by these primers was shown. For each primer major bands were scored and the size of the amplification products ranged between 500 bp-2.5 kb. A total of 92 bands were scored from PCR amplification of genomic DNA from four Samples. No polymorphism plants showed 100% similarity.

The experimental data clearly point out that, of the two samples (i.e) Normally propagated plant and somatic embryo genetically grown plants showed no polymorphism. The quantity analysis of stevioside component between in vivo and in vitro plants when compared to the standard stevioside compound is almost similar and not much variation
has been reported. The results of the present study brought into light the potential nature of *Stevia*.

*Stevia* sweeteners are zero-calorie and do not have a significant effect on blood glucose levels. Like other artificial sweeteners, *Stevia* can be a good option for people with diabetes who are trying to cut calories and still enjoy a sweet taste. As with anything, do not go overboard when you use artificial sweeteners.

With its overall flavour acceptability and stability, it is believed the use of *Stevia* as a functional food ingredient has a valuable future. It may have taken a long time for many countries to decide, but today, throughout the world, *Stevia* has certainly earned the right to be considered a safe, natural sugar substitute and alternative sweetener. *Stevia* shows concrete rewards in the future treatment of type 2 Diabetes Mellitus. And with ongoing research and experiments, the applications of *Stevia* as a functional ingredient are immeasurable.