WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 4, Issue 2, 1501-1511. Research Article ISSN 2277– 7105

IN-VITRO REGENERATION OF *STEVIA REBAUDIANA* BERTONI AND SCREENING OF BIOACTIVE PRINCIPAL COMPOUNDS

Rashmi Arnold[1] and Seema Tiwari*[2]

[1]Dept of Botany, Govt. Model Science College, Rewa, M.P.

[2]Study Centre for Biochemistry, A.P.S.U., Rewa, M.P.

Article Received on
12 Dec 2014,

Revised on 06 Jan 2015,
Accepted on 31 Jan 2015

***Correspondence for Author**
Dr. Seema Tiwari
Study Centre for Biochemistry, A.P.S.U., Rewa, M.P.

ABSTRACT

Plants are considered as a significant source of new medicines. Bioactive compounds in plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. *Stevia rebaudiana* Bertoni is a small perennial herb with green leaves that belongs to the aster (Asteraceae) or chrysanthemum family of plants. The leaf of this plant is the main attraction for its economic and commercial uses because of its valuable quality of extreme sweetness particularly with the presence of the main active constituents Stevioside and rebaudioside A. Plant tissue culture techniques have been increasingly applied to many medicinal plants in particular for mass propagation, conservation of germplasm, study and production of bioactive compounds, and for genetic improvement. Medicinal plants have a vast genetic diversity, which is a valuable source of agronomic genes of interest for the future. Large-scale plant tissue culture is found to be an attractive alternative approach to the traditional methods of plantations.

KEYWORDS: *Stevia rebaudiana* Bertoni, Stevioside, rebaudioside, Micropropagation etc.

INTRODUCTION

Secondary metabolites are produced within the plants besides the primary biosynthetic and metabolic routes for compounds associated with plant growth and development, and are regarded as products of biochemical “side tracks” in the plant cells which are not needed for the daily functioning of the plant. Several of them are found to hold various types of important functions in the living plants such as protection, attraction or signaling.[1] Most species of plants seem to be capable of producing such compounds. Bioactive compounds in plants are compounds produced by plants having pharmacological or toxicological effects in

man and animals. The typical bioactive compounds in plants are produced as secondary metabolites. Secondary metabolites are produced within the plants besides the primary biosynthetic and metabolic routes of compounds aimed at plant growth and development, such as carbohydrates, amino acids, proteins and lipids. For example, flavonoids can protect against free radicals generated during photosynthesis.[2, 3]

Natural sweeteners that can substitute sucrose have caught great attention due to the growing incidence of obesity and diabetes. Much attention has been placed on Stevioside, a sweet glycoside extracted from *Stevia rebaudiana* Bertoni. Due to the sweetness and supposed therapeutic properties of its leaf, *Stevia rebaudiana* Bertoni has attracted economic and scientific interests too.[4] Japan was the first country in Asia to market Stevioside as a sweetener in food and drug industry. Since then, cultivation of this plant has expanded to several countries in Asia, including China, Malaysia, Singapore, South Korea, Taiwan, and Thailand. It has also been successfully grown in the United States of America, Canada, and Europe and worldwide.[5] Use of these sweetening compounds has increased dramatically due to health concerns related to sucrose usage, such as dental caries, obesity and diabetes.[6]

The glycosides consist of various categories of secondary metabolites bound to amono- or oligosaccharide or to uronic acid. The saccharide or uronic acid part is calledglycone, and the other part the aglycone. The main groups of glycosides are cardiac glycosides, cyanogenic glycosides, glucosinolates, saponins and anthraquinone glycosides.[7] *Stevia rebaudiana* Bertoni contains at least ten different glycosides, the major constituents being Stevioside and Rebaudioside A". Primarily Stevioside, Rebaudioside A and C and Dulcoside A, with minor amounts of Rubausoside, Steviolbioside and Rebausoside B, D, E and F. Steviol Glycosides are obtained by extracting leaves of *Stevia rebaudiana* Bertoni with hot water followed by solvent purification of the water-soluble extract. Ion exchange resins may also be used during the purification process.[8]

Artificial sweeteners such as aspartame, saccharine, cyclamate etc. are used for diabetic people. But all of these artificial sweeteners have limitations e.g. aspartame a dipeptide of the amino acids aspartic acid and phenylalanine has been the subject of a vigorous public controversy regarding its safety and the circumstances around its approval because it has negative effects on headaches, brain tumors, brain lesions, and lymphoma. Moreover there is also a positive correlation between saccharin consumption and increased frequency of cancer especially bladder cancer. Since cyclamates appear to affect cells involved in the production

of spermatozoa, the question has also been raised as to whether they may also be capable of damaging male reproductive DNA.[9, 10]

The application of micropropagation techniques provides several benefits like it enhances the rate of rapid multiplication of plants, producing thousands of plantlets in a matter of months. It provides the availability of plants throughout the year.[11] It helps the conservation of genetic resources of threatened plants. Plant improvement can be brought about by regeneration techniques. Healthy plant material is ensured since soil and disease-causing organisms are excluded during the propagation cycle.[12] Micropropagation saves an enormous amount of care usually required by cuttings and seedlings (watering, weeding, spraying etc.). Excess material produced can often be stored over long period.[13]

MATERIAL AND METHODS

Plant preparation

Different parts of the plant were used as explants for the *in-vitro* regeneration of *Stevia rebaudiana* Bertoni. The certified seeds and small plants of the *Stevia rebaudiana* Bertoni were obtained from State Forest research Institute, Jabalpur and Jayanti Kunj Rewa, Madhya Pradesh and they were grown in the garden of Study Center For Biochemistry, Awadhesh Pratap Singh University, Rewa, Madhya Pradesh for Micropropagation study. Leaf, nodal and inter-nodal segments, shoot tips etc were collected as explants which were taken from several days (2-4 months) old plants of *Stevia rebaudiana* Bertoni. The shoot apex of *Stevia rebaudiana* Bertoni can also regenerate shoots when its culture on Murashige and Skoog medium is supplemented with different growth regulators. The explants were cut into small pieces (about 0.1 m long) and then were treated with 1% savlon for 5-6 min with constant shaking and washed thoroughly with distilled water. Then the explants were kept inside under the laminar airflow cabinet and the explants surface was sterilized with a 0.1% mercuric chloride solution for 5 min under aseptic conditions. Then again it was washed four times with sterilized distilled water. The explants were then inoculated aseptically into culture medium with different concentrations and combinations of growth regulators.

Culture Medium

Murashige and Skoog medium is a plant growth medium used in the laboratories for cultivation of plant cell culture. MSM was invented by plant scientists Toshio Murashige and K. Skoog in 1962 during Murashige's search for a new plant growth regulator. It is the most commonly used medium in plant tissue culture experiments in laboratorium. MS medium is

one of the most successful media that was formulated by analyzing the inorganic components in tobacco plants and then adding them to medium in amounts similar to those found in the plants. It has comparatively high salt levels compared to White's medium but lower than B5 medium. The type of tissue culture medium selected depends on the species to be cultured. According to Debergh and Zimmerman, 1991 WPM was the medium that promoted a better establishment of the Prunus insititia cultures, but MS supported higher multiplication rates. In contrast, MS was better than WPM for both explant establishment and multiplication of chokecherry *Prunus virginiana*, and mature wild cherry.

Culture conditions

The cultures were maintained in culture tubes and conical flasks and were kept in the culture room at a temperature of 25±2ºC, relative humidity (RH) of 60-70% and a light intensity of approx. 2500 lux provided by cool, white, fluorescent tubes under a photoperiod of 16/8 hours (light/dark).

Establishment of liquid shoot culture

Shoot tips of multiple shoot culture of *Stevia rebaudiana* on MS medium supplemented with BAP (1.5mg/l) and IAA (0.5mg/l) were inoculated into liquid medium of the same composition, but excluding agar. Static liquid shoot culture was initiated by placing shoot tips into 250 ml conical flasks containing 25 ml of MS liquid multiplication medium. The liquid medium was adjusted to pH 5.7 before being sterilized by autoclaving at 15 psi and 121°C for 15 min. All cultures were kept at 25±2°C under a 16 h photoperiod provided by cool white fluorescent lamps. The shoots from static liquid culture were further sub cultured every three weeks and used in the experiments described in the work.

Explants of *Stevia rebaudiana* were incubated in liquid MS medium supplemented with different concentrations of plant growth regulators. The liquid cultures under static conditions were used to determine the effect of liquid media on *Stevia rebaudiana* shoot proliferation.

Thin-Layer Chromatography

Absorbent: - Silica Gel

Solvent System: - Chloroform: Methanol: Water (85: 25: 4)

Visualization: - UV Chamber and Iodine Vapours.

One dimensional Thin Layer Chromatography was performed with chamber saturation.

Method

Thin-layer chromatography consists of a stationary phase immobilized on a glass or plastic plate, and an organic solvent. The sample, either liquid or dissolved in a volatile solvent, is deposited as a spot on the stationary phase. The constituents of a sample can be identified by simultaneously running standards with the unknown. The bottom edge of the plate is placed in a solvent reservoir, and the solvent moves up the plate by capillary action. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. The separated spots are visualized with ultraviolet light or by placing the plate in iodine vapor. The different components in the mixture move up the plate at different speed due to differences in their partioning behavior between the mobile liquid phase and the stationary phase. Different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase, and because of differences in solubility of the solvent. By changing the solvent, or perhaps using a mixture, the separation of components (measured by the R_f value) can be adjusted.

$$R_f \text{ value} = \frac{\text{Distance traveled by solvent from the origin}}{\text{Distance traveled by solute from the origin}}$$

Visualization of compounds

If the compounds are colored, they are easy to see with the naked eye. If not, a UV light is used for visualization. After drying the spots were visualized by spraying with ninhydrin.

RESULTS AND DISCUSSIONS

Callus is dedifferentiated and unorganized mass of parenchyma cells formed by the proliferation of parent tissue. Callus tissue is a good source of genetic variability and adventitious shoot formation in the plant. Apical shoots were cultured on MS medium supplemented with auxins for callus induction. Callus multiplication was optimized on MS medium with plant growth regulators. The callus thus obtained was then used for organogenesis. Leaves from the two month old field grown plants were excised and were used as explants for the purpose of callus induction. Leaf segments (0.5 – 1.0 cm2) which were inoculated on MS medium fortified with different concentrations of auxins (NAA, IAA and 2, 4-D) singly and in combination with cytokinins (BA and Kinetin) gave varied callusing response. The explant failed to produce callus on MS medium lacking growth regulators but the swelling of the explant was observed. Callus that was produced from leaf

segments was dark green, light green, creamish green and brown while segments were dark green, light green, creamish green and brown. They were from nodular to fragile in nature. NAA, 2, 4-D along with BA were observed to be potent hormonal combinations for profuse callus production from leaf explants. In the combination of NAA (1.5 mg/L and 2.0 mg/L) and BA (0.1 mg/L) a profuse yellowish green colored callus was formed. In the lower concentrations of NAA (0.5 mg/L and 1.0 mg/L) with BA (0.1 mg/L) a lesser amount of callus was formed in comparison than that to which was formed in higher concentrations. In the combination of NAA (2.0 mg/L) with kinetin (0.2 mg/L) formed a profuse green colored fragile to nodular callus. 2, 4-D, along with BA was noted to be a potent hormonal combination for stimulating callus induction from leaf explants. Light green fragile callus was formed in the lower concentration of 2, 4-D (0.5 mg/L) along with BA (0.5 mg/L). As the concentration of 2, 4-D increased light brown to dark brown fragile callus was formed even in the presence of BA (0.5 mg/L). IAA at the concentration of 2.0 mg/L along with BA (0.1 mg/L) showed profuse light green colored nodular callus formation (table-1).

Liquid shoot culture of *Stevia rebaudiana* Bertoni in MS medium containing BAP (1.5 mg/l) and IAA (0.5 mg/l) was developed and evaluated in relation to shoot multiplication. An average of 37 new shoots per explants was obtained within 3 weeks. The shoots were regenerated from nodal explants of Stevia through axillary shoot proliferation. The experiment showed that static liquid culture was effective in growth and multiplication of Stevia shoot tips with callus. The shoots were partially submerged in liquid medium and no hyperhydricity symptoms were observed, when the time spent by shoots in multiplication medium took no longer than 3 weeks. Also on the agar-gelled medium with the same growth regulators, Stevia responded with an average number of 3-11 shoots per explant, but the time required for the shoot induction was longer (5 weeks).

Stevia shoots grown in liquid medium were characterized by a higher number of leaves, larger leaf areas and a greater number of multiple shoots were formed in comparison to those cultured on the agar-solidified medium. A decrease in the average shoot length in the liquid medium was also observed. The average length of these shoots was 5.3 cm as against 7.9 cm in agar culture (graph1, 2 and 3). Moreover, shoots taken from or grown in liquid culture were found difficult to root. Only 63 % of the shoots formed roots within 4 weeks on ½ MS agar medium supplemented with IAA (1.0 mg/l), whereas the percentage of shoot grown on agar solidified medium multiplied 90%. Application of liquid medium was associated with

decreased shoot length, and the effect can probably be responsible for the reduced rooting of the shoots. On inhibition of rooting of shoots, which were multiplied in liquid culture has been reported for *Centaurium erythraea, Salvia officinalis* and *Curcuma longa.* Kalpna *et al*., 2009 where similar results were also found.[14]

After the multiplication of the number of plants the plantlet clumps were dissected and the plantlets were transferred in to the rooting medium. The one way factorial design was carried out to study the effect of IBA (auxins) and their interaction on frequency of root number (FRN) and frequency of root length (FRL) at different concentrations then thin layer chromatography was performed for estimation of bioactive principal compounds from in-vitro regenerated plants of *Stevia rebaudiana* Bertoni (graph-3).

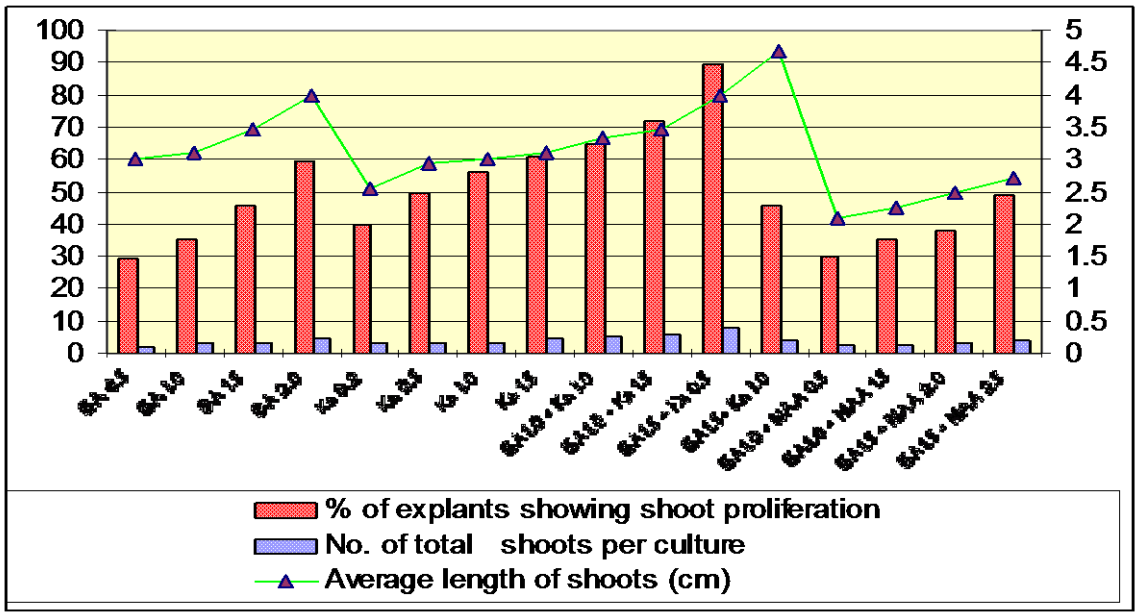
TLC analysis of *Stevia rebaudiana* Bertoni

Callus of (Solid medium) extract was used for TLC analysis of *Stevia rebaudiana* Bertoni. One dimensional thin layer chromatography was performed first with pure solvents like methanol and chloroform, which did not give any separation and then after that the standard solvent system chloroform: methanol: water (65: 25: 4) was used, but, no separation was observed and the whole of the extract run up- to the solvent front with tailing. To improve separation the polarity of the solvent system was decreased by increasing the ratio of chloroform and the chromatogram with the ratio (85: 25: 4). It showed three spots at R_f 0.15, 0.21 and 0.5, which may be of rebaudioside, Stevioside and other polar diterpene glycosides respectively, while chlorophyll moved up- to the solvent front. A thin layer chromatogram matches the thin layer of chromatography analysis (Pasquel, 2002) which showed a similar banding with an R_f value of 0.692, 0.682 and 0.686 in standard, standardized callus and leaves of mother plant, thus confirming the presence of Stevioside in leaf and callus tissues. Earlier studies indicate the use of water and methanol for Stevioside extraction with comparable efficiency (Vanek *et al*., 2001). The Stevioside content was higher in leaf (19.25 g/kg) than callus (17.63 g/kg respectively 9(Graph-4). The presence of Stevioside which is higher in leaf than in callus has been observed in this study is in agreement with the results of Sivaram and Mukundan, 2003.[15,16]

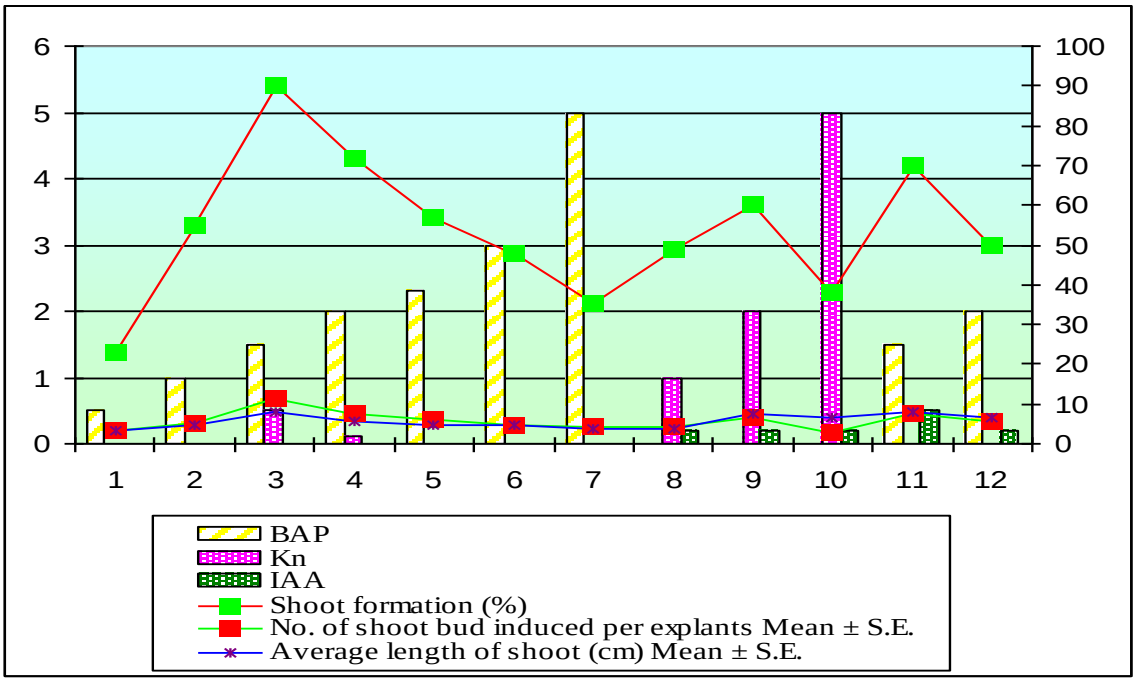
Table1- Effect of different concentrations of auxins on induction of callus

Plant growth regulator (mg/L)					Intensity of callus formation	Nature of callus
NAA	2,4-D	IAA	BA	Kn		
-	-	-	-	-	-	Swelling of explants
0.5	-		0.1	-	C^{++}	Light green, nodular
1.0	-	-	0.1	-	C^{++}	Light green, fragile
1.5	-	-	0.1	-	C^{+++}	Yellowish green, compact
2.0	-	-	0.1	-	C^{+++}	Yellowish green, compact
0.5	-	-	-	0.2	C^{+}	Creamish green, fragile
1.0	-	-	-	0.2	C^{++}	Light green, nodular
1.5	-	-	-	0.2	C^{++}	Light green, fragile
2.0	-	-	-	0.2	C^{+++}	Dark green, nodular
-	-	-	0.5	-	C^{++}	Light green, fragile
-	0.5	-	0.5	-	C^{+++}	Light brown, fragile
-	1.0	-	0.5	-	C^{++}	Dark brown, fragile
-	2.0	-	-	0.5	C^{++}	Light brown, fragile
-	1.0	-	-	0.5	C^{++}	Light brown, fragile
-	2.0	-	0.1	-	C^{++}	Light green, fragile
-	-	2.0	0.1	-	C^{+++}	Light green, nodular
-	-	2.0	-	0.1	C^{+}	Light green, nodular
-	-	1.0	-	0.1	C^{++}	Dark green, compact

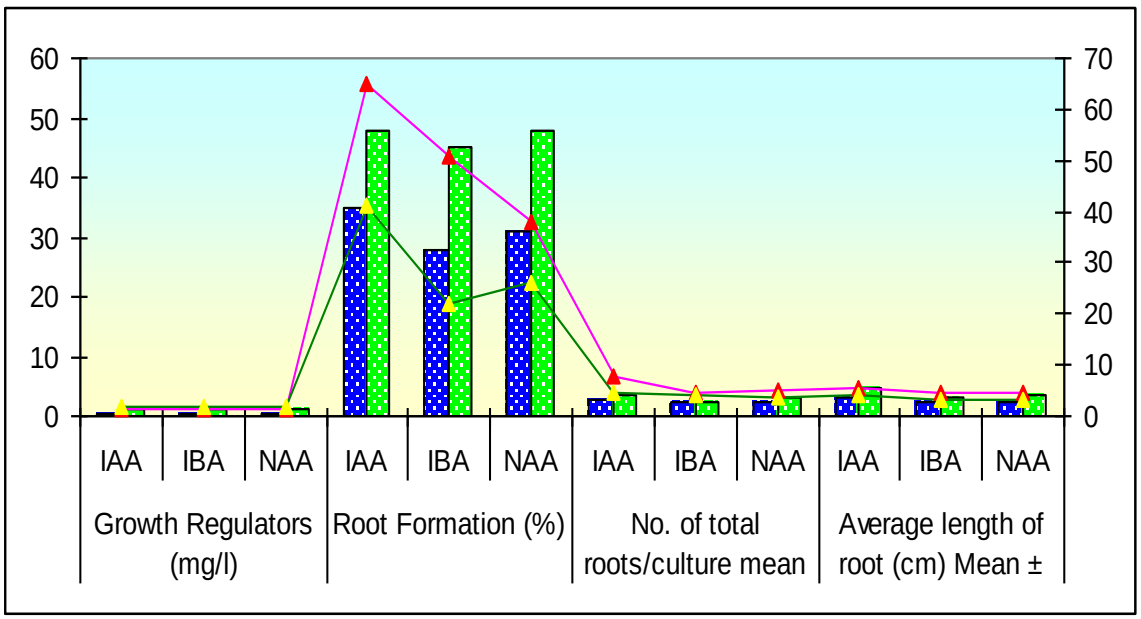
Observation: After 4 weeks; C+-Poor callus; C++ - Moderate callus C+++ - Profuse callus



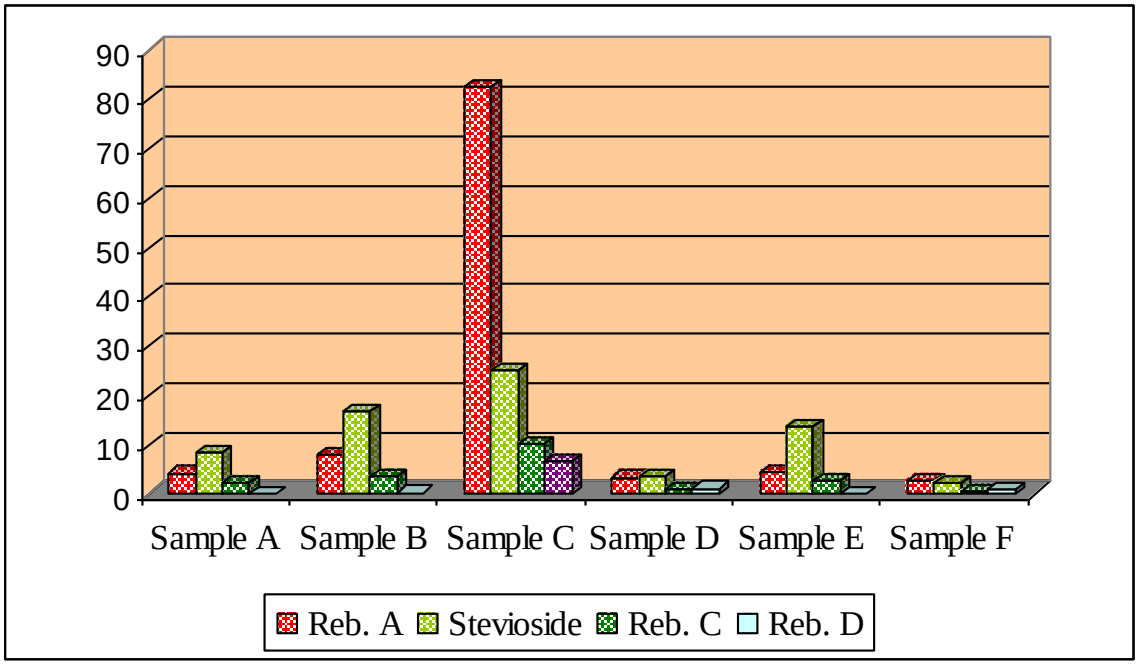
Graph-1 Effect of BAP and IAA in MS liquid medium for shoots proliferation



Graph-2 Effect of BAP, Kn and IAA on MS medium for regeneration of shoot from nodal explants of *Stevia rebaudiana*



Graph-3 Effect of IAA, IBA and NAA in MS medium for root formation



Graph-4 TLC analysis graph of *in-vitro* regenerated *Stevia rebaudiana* Bertoni

CONCLUSION

The present work is an attempt evolves an efficient system for mass multiplication of this important medicinal plant *Stevia rebaudiana* Bertoni within a short period of time and to investigate the protocol for the screening of *in-vitro* regenerated plant. It indicates the use of plant tissue culture techniques to grow it everywhere. It would be helpful in making thousands identical copies of mother plant with in short time. Plant tissue culture will beneficial to improve quality and quantity of plant and to make disease free plant.

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