

**Studies of somatic embryogenesis of *Stevia rebaudiana* (Bertoni) Bertoni and
clonal fidelity of regenerated plants**

A Dissertation

Submitted in partial fulfilment of the requirement for the award of degree of

Masters of Science

In

Biotechnology



UNDER THE SUPERVISION OF

Dr. ANIL KUMAR

ASSOCIATE PROFESSOR

DEPARTMENT OF BIOTECHNOLOGY

THAPAR UNIVERSITY, PATIALA

SUBMITTED BY

SHIVANI GULERIA

301501016

M. Sc BIOTECHNOLOGY (2015-2017)

DECLARATION

I declare that the thesis entitled "**Studies of Somatic embryogenesis of *Stevia rebaudiana* (Bertoni) Bertoni and Clonal fidelity of regenerated plants**" is an authentic record of my own work, carried out during a period of six months. The work has been completed under the supervision and guidance of **Dr. Anil Kumar**, Associate Professor, Department of Biotechnology, Thapar University (Patiala).

I also declare that matter presented in this thesis has not been submitted by me in any other University / Institute for the award degree.

Place: Patiala

Date: 22.08.17

Shivani
Shivani Gulera
22/8/17

CERTIFICATE

This is to certify that the thesis entitled "Studies of Somatic embryogenesis of *Stevia rebaudiana* (Bertoni) Bertoni and Clonal fidelity of regenerated plants" submitted by Shivani Guleria (Roll no. 301501016) to the Thapar University (Patiala) towards partial fulfillment of the requirements for the award of the degree of Master of Science in Biotechnology. This work has been carried out under my supervision.

It is also certify that this thesis or any other part of this thesis has never been submitted, neither in part nor in full to this university or any other university for the award of any degree.


Dr. Anil Kumar

Supervisor

Department of Biotechnology

Thapar University, Patiala


Shivani Guleria

Roll no: 301501016

M.sc Biotechnology

ACKNOWLEDGEMENT

I thank and praise GOD ALMIGHTY for his immense grace and blessings, which guided me in taking up this research project and gave me the confidence and sustained ability to complete this work successfully.

I am very thankful to my parents whose blessing, continuous support, inspiration helped me a lot.

I have immense pleasure to express my deep sense of gratitude and indebtedness to my supervisor **Dr. Anil Kumar, Associate Professor, Department of Biotechnology, Thapar University (Patiala)** for his valuable advice, constant support, encouragement, unfailing patience and suggestions for completion of my research project. I am very thankful to **Dr. Anil Kumar** for giving me the opportunity and privilege to work in his **Plant Tissue culture lab, TIFAC CORE, Thapar University (Patiala)**.

I thank **Dr. Moushumi Ghosh, Professor and Head, Department of Biotechnology** for giving me the opportunity of doing this project. I thank all the members of Department of biotechnology and TIFAC CORE for their cooperation and support.

Special thanks to **Miss Amanpreet Kaur, Research Scholar, Department of Biotechnology** for her inspiration, encouragement, help and active co-operation for carrying out the present study.

I also thank my friends for their love support and encouragement.

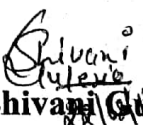

Shivani Guleria

TABLE OF CONTENTS

| Title | Page no |
|------------------------------------|----------------|
| Declaration | i |
| Certificate | ii |
| Acknowledgement | iii |
| Table of contents | iv |
| Abbreviations | v-vi |
| List of figures | vii-viii |
| List of tables | ix |
| Abstract | x |
| Chapter I. Introduction | 1-9 |
| Chapter II. Review of literature | 11-15 |
| Chapter III. Materials and methods | 17-19 |
| Chapter IV. Results | 20-29 |
| Chapter V. Discussion | 30-33 |
| Chapter VI. Conclusion | 34 |
| References | |
| Annexure I | |
| Annexure II | |

ABBREVIATIONS

| S.no | Abbreviations | Full form |
|-------------|--|---|
| 1. | et al | et alia alii (Latin) = and other people |
| 2. | mg/l | mili gram(s) per litre |
| 3. | ml/l | milli litre/litre |
| 4. | mM | mili Mole |
| 5. | μ M | micromollar |
| 6. | $^{\circ}$ C | degree Celsius |
| 7. | μ mol $\text{ms}^{-2} \text{s}^{-1}$ | micromoles per square meter per second |
| 8. | v/v | volume by volume |
| 9. | w/v | weight by volume |
| 10. | nmol | nanomoles |
| 11. | μ l | microliter |
| 12. | Sec | seconds |
| 13. | Min | minutes |
| 14. | Fig | Figure |
| 15. | PGRs | Plant Growth Regulators |
| 16. | MS | Murashige and Skoog medium |
| 17. | TDZ | Thidiazuron |

| | | |
|-----|-------|--|
| 18. | EDTA | Ethylenediaminetetraacetic acid |
| 19. | NAA | Naphthalene acetic acid |
| 20. | IAA | Indole 3-acetic acid |
| 21. | 2,4-D | 2,4 Dichlorophenoxy acetic acid |
| 22. | Kn | Kinetin |
| 23. | BA | 6-benzyl adenine |
| 24. | IBA | Indole 3-butyric acid |
| 25. | dNTPs | deoxynucleotide triphosphates |
| 26. | CTAB | Cetyl trimethylammonium bromide |
| 27. | RFLP | Restriction Fragment Length Polymorphism |
| 28. | RAPD | Random Amplification of Polymorphic DNA |
| 29. | ISSR | Inter-Simple Sequence Repeat |
| 30. | AFLP | Amplified Fragment Length Polymorphism |
| 31. | SNP | Single Nucleotide Polymorphism |
| 32. | SEM | Scanning Electron Microscopy |

LIST OF FIGURES

Figure 1: Morphological features of *Stevia rebaudiana* **A-B** Acclimatized plants showing white colored pentamerous flowers **C** seeds and **D** branched fully developed root system.

Figure 2: Molecular structure of **A** Steviol **B** Stevioside and **C** Rebaudioside A.

Figure 3: 21 days old cultures growing on MS medium. **A** Without silver nitrate (AgNO_3) **B** supplemented with silver nitrate (AgNO_3).

Figure 4: Somatic embryo and shoot bud induction from **A** leaf explant of *Stevia rebaudiana* cultures on MS medium supplemented with BA, 2,4-D and NAA. Callus (**B** Friable, **C** Nodular callus (NC) was observed after 3 weeks on MS medium supplemented with $1\mu\text{M}$ 2,4-D + $5\mu\text{M}$ NAA, $1\mu\text{M}$ BA and $5\mu\text{M}$ 2,4-D. **D** Shoot organogenesis was observed from callus on medium supplemented with $1\mu\text{M}$ BA whereas **E** Somatic embryo (SE) initiated on MS medium supplemented with $1\mu\text{M}$ 2,4-D + $5\mu\text{M}$ NAA. **F** Somatic embryos were transfer to basal medium without incorporation of PGR for germination (GE: Globular embryo; EG: Embryo germination) **G-H** germinated shoots were multiplied on basal medium.

Figure 5: Histological analysis of Somatic embryos showing **A**. active cell division in meristematic pockets (MP) and **B-C** development of early globular embryo (GE). SEM studies showing **D** globular embryo (GE), torpedo shape embryos (TE) and **E** early heart shape embryo (HE). Scale bar: $100\mu\text{m}$.

Figure 6: Amplification profiles of RAPD (A, B) and ISSR (C, D) markers of regenerated plants from somatic embryos. RAPD amplifications (A, B) were carried out using primer RAPD-1 and RAPD 12 and ISSR amplifications (C, D) were carried out using primer ISSR-1 and ISSR-12, Lane M (mother culture); and lane 1–12 plant grown from somatic embryos; L 1-kb molecular-weight markers; Lane N (negative control).

LIST OF TABLES

Table 1: Shoot organogenesis in *Stevia rebaudiana*.

Table 2: Somatic embryogenesis in *Stevia rebaudiana*.

Table 3: The effect of naphthalene acetic acid (NAA) and N⁶ benzyladenine (BA) on shoot organogenesis from leaf explant of *Stevia rebaudiana*.

Table 4: The effect of 2,4-Dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) on induction of Somatic Embryos from leaf explant of *S. rebaudiana*.

Table 5: The effect of N⁶ benzyladenine (BA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) on induction of Somatic Embryos from leaf explant of *S. rebaudiana*.

Table 6: The details of the primers used for Random amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) amplifications from regenerated shoots of *Stevia rebaudiana*.

ABSTRACT

Stevia rebaudiana (family Asteracea) is an important medicinal plant mainly known for its non-caloric natural sweetness. The present study was focused on the studies of somatic embryogenesis of *S. rebaudiana* (Bertoni) Bertoni and testing the clonal fidelity of regenerated plants.

Multiplication of cultures was done by culturing microshoots of *Stevia rebaudiana* on MS medium supplemented with 10.0 μ M Silver nitrate (AgNO_3) for obtaining expanded leaves. Effect of different concentration and combinations of 2,4-D, NAA and BA on shoot organogenesis and somatic embryogenesis was evaluated. MS medium supplemented with PGR concentration (1 μ M 2,4-D and 5 μ M NAA) showed maximum (94.44%) explant differentiating into somatic embryos. Different concentration and combination of BA and NAA was tested for shoot organogenesis and MS medium with 1 μ M BA showed highest (66.67%) percent of shoot organogenesis.

The developed embryo like structure were further analyzed through histological and Scanning Electron microscopic (SEM) studies. Histology studies revealed the epidermis layer with actively dividing meristematic pockets and SEM studies revealed different development stages of embryos like globular, heart, torpedo shape of embryos as well. Regenerated plantlets from somatic embryos were checked for any somaclonal variations using PCR based molecular marker such as RAPD and ISSR. The regenerated shoots were found true to type.

CHAPTER I

Introduction

A small perennial herb called *Stevia rebaudiana* is a member of Asteraceae family. In Sanskrit, *S. rebaudiana* is known as “Madhu Patra” which means sweet leaf. It is also named as honey yerba, candy leaf or honey leaf (Yadav et al. 2010). In 1899, Swiss Botanist, Moises Santiago Bertoni gave botanical classification of *S. rebaudiana* and studied the chemical characteristics of the plant (Bertoni 1905). Scientific Classification of the plant is given below:

| | |
|---------|-------------------|
| Domain | Eukaryota |
| Kingdom | Plantae |
| Order | Asterales |
| Family | Asteraceae |
| Genus | <i>Stevia</i> |
| Species | <i>rebaudiana</i> |

Morphological characteristics of *S. rebaudiana* includes sub ligneous stem (partially woody) usually 65-80 cm in height, with fine hairs and filiform (branched) root system (Fig 1A, D). Leaf arrangement of the plant is alternate and lanceolate (tapering at each end). The plant has white pentamerous flowers of size 10-15 mm (Fig 1B). *S. rebaudiana* requires a short period of light and long period of night for blooming. In southern and northern hemisphere flowers bloom from January to March and September to December respectively. *S. rebaudiana* has spindle shaped achene which contains seeds inside it (Fig 1C). Color difference of the seed regards to fertility and

infertility of seed (Goettemoeller and Ching 1999). Dark colored seeds are usually fertile and pale colored are infertile (Yadav et al. 2010).



Fig 1: Morphological features of *Stevia rebaudiana* **A-B** Acclimatized plants showing white colored pentamerous flowers **C** seeds and **D** branched fully developed root system.

Source: Moraes et al.2013; <https://bonnieplants.com>; <http://foodmysteries.com>; <https://typicalgardener.files>.

S. rebaudiana was first discovered for its sweet properties by Guarani peoples of South America. It was used as “kaa he he” (sweet herb) to sweeten the taste of medicines and tea (Brandle and Rosa 1992). In present scenario, the plant is cultivated all over the world in 32,000 hectares due to its sweetening property. Thailand, Korea, Japan, China, Taiwan Brazil, Malaysia and Paraguay are the commercial cultivators and largest producers of the plant. In India, the plant is successfully cultivated in many states mainly Orissa, Rajasthan, Kerala, Maharashtra (Goyal et al. 2010). Almost 240 species of genus *Stevia* are known till date, out of which species *rebaudiana* is well reputed for its sweet leaf. Apart from the sweet taste sugar extracted from the *S. rebaudiana* have many other properties which make them distinguishable from the table sugar (Gupta et al. 2014). Few of these properties are discussed below:

1.1 Properties of sugar extracted from *S. rebaudiana*:

(a) Sweetness: Sugar extracted from the *S. rebaudiana* has 250-300 times more sweetness than cane sugar. Presence of stevioside and rebaudioside contribute to its sweetness. This property, makes the plant more attractive for food industries.

(b) Zero calorie: Calories act as a body fuel to perform body functions. Being a zero calorie sugar, it helps in weight loss as the body burn fat for energy rather than utilizing energy from carbohydrates.

(c) Non metabolizable: Sugar of this plant is a non metabolizable compound, thus it does not affect blood glucose levels.

(d) Non- fermentable: As it is a non- fermentable sugar, bacteria in the mouth do not ferment the sugar to acids, thus protect tooth from decay.

(e) **Heat Stable:** Sugar of *S. rebaudiana* is heat stable (up to 200⁰C) which results in non-caramelization (no browning of sugar) thus, this property of stevia makes it ideal for baking and cooking purpose.

Steviol glycosides mainly rebaudioside A and stevioside contributes to the sweet taste of the leaf in *S. rebaudiana*. These compounds are present throughout the plant, but usually accumulated in leaves (Kumari et al. 2016). In addition to high sweetener content, leaves are a rich source of carbohydrates, potassium, calcium, protein, fat, sodium, fibers, iron, vitamin, magnesium, vitamin A and C (Banerjee and Sarkar 2009). Due to these properties, *S. rebaudiana* is used as nutrient supplement.

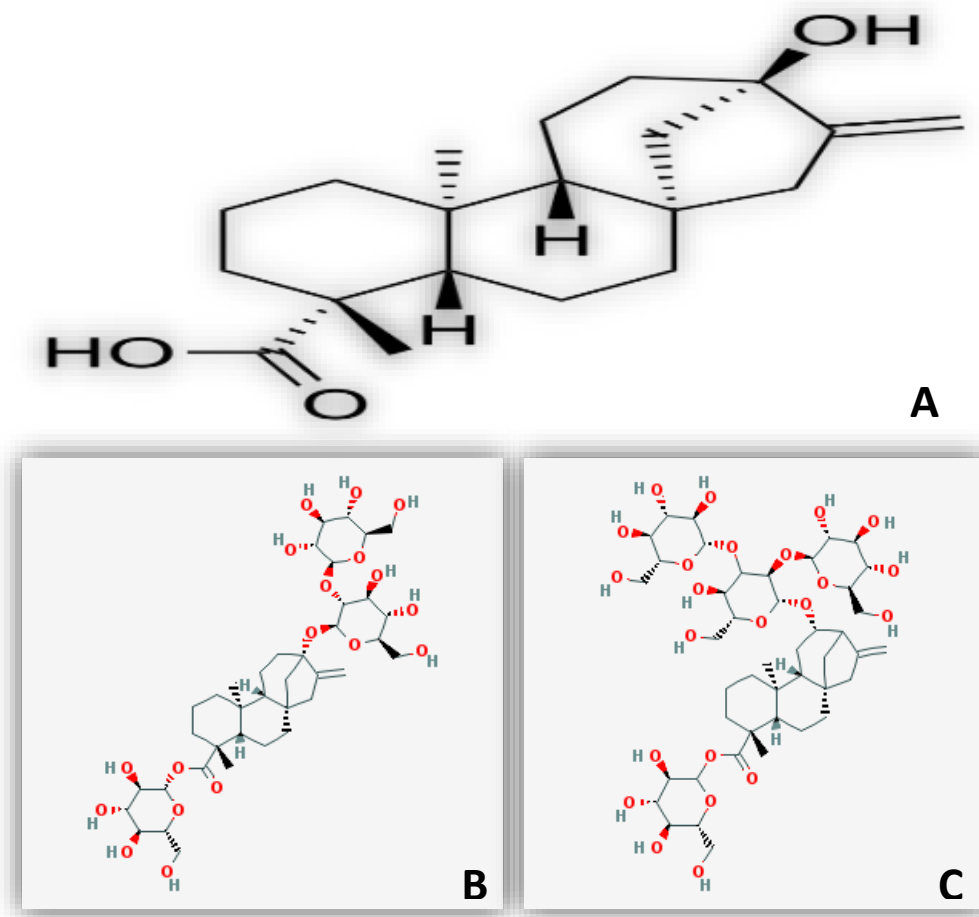


Fig 2: Molecular structure of A Steviol B Stevioside and C Rebaudioside A (Bharamachari et al. 2011; Chester et al. 2012)

Besides sweetness, the plant has many medicinal properties. It is used as anti-diabetic, antimicrobial, anticancerous, antihyperglycemic, antihypertensive, cardiogenic, anti-inflammatory, antidiarrhoeal, anti-oxidant, and immunomodulatory agent. Few of important properties of the plant are explained below:

(a) Anti-diabetic: The presence of stevioside in leaves is responsible for anti-diabetic properties of the plant. In gut, stevioside gets hydrolyzed into glucose and utilized by bacteria rather than being absorbed in the blood. Thus, this is an ideal natural substitute of normal sugar for diabetic patient (Koyama et al. 2003; Wheeler et al. 2008).

(b) Antimicrobial: The bioactive compounds such as tannins, alkaloids, flavonoids, essential oil, niacin, riboflavin present in leaf extract of *S. rebaudiana* have some antimicrobial properties. These compounds basically act as plant defense system. Leaf extract of *S. rebaudiana* is used as an inhibitory agent for wide range of bacteria (*E.coli*, *Klebsiellapneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) yeast (candida species) and fungal (*Penicillium chrysogenum*, *Alternaria solani*, *Aspergillus niger*, *Helminthosporium solani*) species (Jayaraman et al. 2008; Gamboa and Chaves 2012; Siddique et al. 2014).

(c) Anticancerous: The presence of flavonoids, polyphenolic, antioxidant, and phytochemicals in *Stevia* prevents the cancer formation in the body. Some of the anti-cancerous compounds present in *Stevia* includes kaempferol, chlorogenic acid, iso-steviol, caffeic acid, isoquercitrin and quercetin. These compounds prevents mutation of normal healthy cells into cancer cells by eliminating free radicals from the body (Deshmukhan et al. 2014; Yasukawa 2002).

(d) Antihypertensive: Stevioside inhibit Ca^{++} influx in blood vessels. Thus act as a vasodilator. It increases the urination and eliminates sodium from the body. This action of *Stevia* lowers the blood

pressure level. Thus contributing to low pressure on cardiovascular system and prevention of diseases like heart-attacks, strokes and atherosclerosis (Ruiz et al. 2015; Misra et al. 2015).

1.2 Need for study

Due to above mentioned health benefits of *Stevia*, demand of the plant has increased by many folds since 1970 (Gupta et al. 2014). Conventionally, crop is propagated by seed germination and vegetative propagation, but limited span of seed, poor germination rate and non- uniform crop production restrict the use of this method (Singh et al. 2014). Further, vegetative propagation method is labor intensive and number of plants propagated are low (Yadav et al. 2010). Thus techniques involving mass multiplication of the plant through micro propagation is used as alternative to meet the demand of the growers (Singh et al. 2014).

The problem associated with use of *Stevia* as a sugar substitute is presence of low amount of steviol glycosides (Mondaca et al. 2012). It has been reported that glycosides concentration is limited from 2-10 % dry weight in leaves (Yadav et al. 2010). The levels of glycosides is dependent upon the environment as well as growing season (Mondaca et al. 2012). Thus for improvement or enhancement of the compounds in *Stevia*, breeding studies were initiated by many workers. (Lee et al. 1978, 1982; Shu 1989, 1995; Shyu 1994; Suhendi 1989; Weng et al. 1996; Kornienko and Parfenow 1996). But conventional breeding methods possess various problems such as self - incompatibility, limited possibility of improved traits, labor intensive, time consuming (Yadav et al. 2010). Thus, in the age of biotechnology, genetic engineering and transformation of plant is often seen as an alternative. To carry out any trait specific modifications, the optimization of regeneration protocol (Shoot organogenesis or Somatic embryogenesis) is a prerequisite.

1.3 Shoot organogenesis and Somatic embryogenesis: *In vitro* regeneration exploits the ability of the plant cell or tissue to grow and develop into a whole plant. Cellular totipotency is the basic principle and key tool of plant tissue culture particularly micro propagation. The two common pathways of in-vitro regeneration of plants are: shoot organogenesis and somatic embryogenesis.

Shoot organogenesis is defined as a process in which undifferentiated cell mass, tissue develops into organized structures like, root, shoots, buds, and flower by the differentiation ability of the cells. Plant regeneration by organogenesis can be done by two processes: direct shoot organogenesis and indirect shoot organogenesis. Direct shoot organogenesis is the process of regeneration of shoots directly from the explant whereas, the regeneration of shoots indirectly from callus is called indirect shoot organogenesis. Formation of organs is depend upon the concentration of plant growth regulators. The auxin to cytokinin ratio determine the shoot or root regeneration (Bhojwani and Razdan 1996).

(a) High concentration of cytokinin to low auxin concentration ratio develop shoot formation whereas, high auxin concentration to low concentration of cytokinin ratio develop roots

(b) Medium auxin concentration to medium concentration of cytokinin ratio develop both shoot and roots

Like shoot organogenesis, somatic embryogenesis is also dependent on cellular totipotency and is a process of development of non-zygotic embryos from somatic cells. Somatic embryogenesis was first reported in carrot callus cells (Steward et al. 1958). Since then, the process of somatic embryogenesis is used as a potent model for morphogenetic, regulatory and regeneration studies in plants (Zimmerman 1993).

Auxins play a major role in the initiation of somatic embryogenesis. Auxins, especially 2,4-Dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) are most commonly used for initiation of somatic embryogenesis (Dodeman 1997). Other auxins such as picloram, dicamba, indole acetic acid (IAA) and indole butyric acid (IBA) are also used for the initiation of somatic embryogenesis (Arnold et al. 2002). The effect of exogenous auxins depends on the type of explant. Auxins are reported for the formation of embryogenic calli from explant. The embryogenic calli have potential to differentiate somatic embryos in the medium containing lower auxin levels but further development requires medium free of auxins as auxin is known to inhibit somatic embryogenesis.

Developmental stages of somatic embryogenesis:

- (a) Phase 0: Induction and formation of embryogenic calli from a primary explant when cultured on an auxin rich medium.
- (b) Phase I: Proliferation of embryogenic calli on auxin free medium with differentiation of globular embryogenic cells.
- (c) Phase III: Development of globular embryos to Heart, torpedo and cotyledonary shaped embryos.
- (d) Phase IV: Germination and regeneration of cotyledonary embryos into plantlets.

Somatic embryos can develop through direct or indirect embryogenesis. Direct Somatic embryogenesis is a process of formation of somatic embryos directly from the primary explant without undergoing callus phase. Indirect somatic embryogenesis is a process where somatic embryos develop from intermediate callus. Embryos developed directly from pre-embryogenic determined cells (PEDCs) produce a uniform clonal population, whereas embryos developed indirectly from induced embryogenic determined cells (IEDCs) generate somaclonal variants.

(Bhojwani and Razdan 1996). Somaclonal variations are tested by using many molecular markers such as Random Amplification of Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Inter-Simple Sequence Repeat (ISSR), Amplified Fragment Length Polymorphism (AFLP), and Single Nucleotide Polymorphism (SNP). Among all these molecular markers, Random Amplification of Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) are most commonly used marker as minimal genome information is required for amplification, produce reproducible results, affordable (Moktaduzzamam and Rahman 2009). The present study was focused on shoot organogenesis and somatic embryogenesis from leaf explants of *Stevia rebaudiana*. The clonal uniformity of regenerated plants was also checked by Random Amplification of Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR).

Objectives

1. To induce somatic embryogenesis in *Stevia rebaudiana* using leaf explants.
2. To study the different stages of the somatic embryos by using histological and electron microscopic studies.
3. To study the various factors affecting the conversion of somatic embryos and testing clonal fidelity of the regenerated plants.

CHAPTER II

Review of literature

Stevia rebaudiana (family, Asteraceae) is a medicinal plant (Gupta et al. 2014). Japan is the largest producer of *Stevia* followed by China and Russia. In India, *S. rebaudiana* is grown in 32000 hectares. The importance of herb as sweetening agent has been mentioned since ancient times (Gupta et al.2014). Furthermore, the therapeutics properties of the herb make it an important candidate for pharma industries (Koyama et al. 2003; Wheeler et al. 2008; Jayaraman et al. 2008; Deshmukhan et al. 2014). After the first use of stevia as flavoring and sweetening agent in the food industry of Japan in 1971, the demand of stevia has increased by many folds (Das et al. 2011). The demand of the herb cannot be satisfied by conventional propagation methods such as seed germination and vegetative propagation, thus there is need of intervention of a techniques such as micro propagation. Micro propagation, a rapid multiplication technique has been intensively studied in *Stevia rebaudiana* (Ferreira and Handro 1988; Lata et al. 2013; Pratibha et al. 2010).

In addition to mass propagation, the attempts has also been made to improve sweetening property of the plant (Luwanska et al. 2015; De 2017). Conventionally, plant improvement is carried out through breeding to improve sweetness (Yadav et al. 2010). Varieties like RSIT 94-1306, RSIT 94-751, Synthetic cultivar AC black bird with improved glycoside content and RSIT-95-166-13 with high rebaudioside C content have been developed through breeding (Sys et al.1998; Marsolais et al. 1998; Brandle et al. 1998) but, the limitations of breeding technique like self-incompatibility, limited possibility of improved traits, labor intensiveness and time consumption restrict its use

(Yadav et al. 2010). Thus, biotechnological tools such as genetic engineering and genetic transformation are seen as important alternatives (Pande and Gupta 2013). For successful and effective genetic transformation programme, development of an efficient regeneration protocol is a pre-requisite (Yadav et al. 2010).

Large number of studies on regeneration of *Stevia rebaudiana* have been reported (Yadav et al. 2010; Preethi et al. 2011; Ferreira and Handro 1988; Banerjee and Sarkar 2008). First report on shoot organogenesis from stem tip of *Stevia rebaudiana* was reported by Tamura et al (1984). It was reported that precise concentration of kinetin leads to shoot formation. This was followed by many reports on shoot organogenesis from different explants such as leaf (Preethi et al. 2011; Ferreira and Handro 1988; Banerjee and Sarkar 2008), internodes (Mitra and Pal 2007; Rathi and Arya 2009; Laribi et al. 2012), and shoot tip (Tamura et al. 1984; Sreedhar et al. 2008; Giridhar et al. 2010; Gurucharan and Chinnagounda 2013).

In most of the studies mentioned above, BA in combination with small amount of auxin is described as best PGR combination for shoot bud induction (Rathi and Arya 2009; Laribi et al. 2012). It has been reported that shoot organogenesis is affected by specific ratio of auxin and cytokinin (Luwanska et al. 2015). Any change in this ratio can be detrimental to explants or can result in reduced organogenesis response. In *Stevia rebaudiana*, BA is one of the most commonly used cytokinin (Gupta et al 2010). Usually combination of BA with 2, 4-D or NAA has been used for callus induction, whereas combination of kinetin can result in direct shoot organogenesis (Sreedhar et al. 2008). Few of the important reports on shoot organogenesis are enlisted below (Table 1).

In addition to PGR's, the use of adenine sulphate (Mitra and Pal 2007) and spermine (Gurucharan and Chinnagunda 2013) have also resulted in shoot organogenesis in *Stevia rebaudiana*. Adenine sulphate is known to act as precursor of cytokinin biosynthesis (Mitra and Pal 2007). Shoot organogenesis is also affected by photoperiod (Ferreira and Handro 1988; Okporie and Ekpe 2008; Huang et al 2000). In *Stevia rebaudiana* it has been reported that dark conditions promote plant regeneration from suspension cultures in presence of auxin and cytokinin whereas under light conditions in various plants it has been reported that photoperiod cycle of 14-16 hour lead to maximum regeneration in comparison with below 11 hour of light (Okporie and Ekpe 2008; Zeevaart 1971).

Table 1: Shoot organogenesis in *Stevia rebaudiana*

| S.no | Explant | Medium | Results | References |
|------|---------------------------------------|---|---------------------------------------|------------------------------------|
| 1 | Stem tips | MS medium +10mg/l kinetin | Clonal propagation | Tamura et al. (1984) |
| 2 | Leaf | MS medium +BA (2.0 mg/l) in light or BA (2.0 mg/l) +NAA (2.0 mg/l) in the dark. | Regeneration from suspension cultures | Ferreira and Handro (1988) |
| 3 | Node | MS medium+ IAA (1.0 mg/ l) + Kin (10.0mg/l) + adenine sulphate.30.0 mg/ l | Higher proliferation of shoots | Mitra and Pal (2007) |
| 4 | Leaf | MS medium+ cyanobacteria(5ml/l and 10ml/l | Maximum shoot proliferation | Banerjee and Sarkar (2008) |
| 5 | Shoot tip | MS medium+ BA(8.88 μ M)+Kn (6.98 μ M) | Direct shoot organogenesis | Sreedhar et al. (2008) |
| 6 | Leaf, Internodes Shoot discs | MS medium + BAP 3.0 mg/l +NAA(0.5 mg/l) | Multiple shoots | Rathi and Arya (2009) |
| 7 | Shoot tips | B5 medium+ BA (4.44 μ M)+NAA(0.80 μ M) | Shoot bud formation | Giridhar et al. (2010) |
| 8 | Leaf | MS medium+BA (2.0 mg/L), Kn (0.5 mg/L) and NAA (0.1mg/l) | Maximum number of shoots | Preethi et al. (2011) |
| 9 | Flower explant | MS medium + BAP (2.0mg/l) | Shoot organogenesis | Ahmed et al. (2011) |
| 10 | Nodal explant | MS medium+ BAP(1mg/l)+IBA(0.5mg/l) | Shoot bud initiation | Laribi et al. (2012) |
| 11 | Shoot tip | MSmedium + BAP (1.5mg/l)+ spermine (10mg/l) | Maximum number of shoots | Gurucharan and Chinnagounda (2013) |
| 12 | Nodal segment containing axillary bud | MS medium + TDZ (1.0 μ M) | Maximum multiple shoots | Lata et al. (2013) |

Somatic embryogenesis is considered as an important pathway of cell differentiation in plants (Zimmerman 1993). Somatic embryogenesis is also reported to be influenced by endogenous auxin concentration (Dodeman 1997). There are many reports stating the change in endogenous auxin levels in accordance with exogenous auxin application (Jimenez 2001). In *Stevia rebaudiana* somatic embryogenesis has been reported from leaf, nodal segments, anther, stem tip, shoot tip and floret part (Filho and Hattori 1997; Banerjee and Sarkar 2009; Arellano et al. 2015; Nazneen et al. 2014). In all these reports auxin, especially 2, 4-D has played an indispensable part of medium combination (Jimenez et al. 1993). The work on somatic embryogenesis of *Stevia* has been initiated with a report by Filho et al. (1993). Although in this study somatic embryo induction was reported but embryos failed to germinate into plantlets. In the proceeding study, auxin was reduced to approximately 1/3 and shoot regeneration from somatic embryo were observed (Filho and Hattori 1997). After a long gap of about 20 years, once again research on somatic embryo of *Stevia rebaudiana* was initiated in 2009 (Banerjee and Sarkar 2009). It was reported that presence of 2, 4-D, BA and Thidiazuron (TDZ) lead to embryogenic callus initiation in culture medium. Combination of BA and IBA along with increased sucrose concentration results in organogenesis of embryogenic callus. In 2014, Nazneen et al (2014) studied stages of somatic embryogenesis through Scanning Electron Microscopy (SEM). Niranjo et al. (2015) reported that somatic embryogenesis is affected by the type of genotype. Results showed that genotype SRQ-93 develop the maximum number somatic embryos on a medium supplemented with 2,4-D and 2 ip whereas embryogenic and non- embryogenic callus was observed genotype Bertoni and Morita II.

Table 2: Somatic embryogenesis in *Stevia rebaudiana*

| S.no | Explants | Medium | Results | References |
|-------------|-----------------|--|--|----------------------------|
| 1. | Leaf | MS medium+2,4-D (25 μ M)+BA(1mM) | Somatic embryogenesis, no regeneration | Filho et al. (1993) |
| 2. | Floret | MS medium+ 2,4-D(9.05mM) | Somatic embryogenesis and regeneration | Filho and Hattori (1997) |
| 3. | Leaf and nodes | MS medium+2,4-D(2.0mg/l) +BAP(0.2mg/l +TDZ(0.2mg/l) | Somatic embryogenesis and regeneration | Banerjee and Sarkar (2009) |
| 4. | Leaf | MS medium+2,4-D(0.5-2.0mgL ⁻¹ NAA) + BA (1.0mgL ⁻¹ +500 mg/l CH) | Somatic embryogenesis and regeneration | Arellano et al. (2015) |
| 5. | Leaf and nodes | MS medium+ BAP (0.5mg/l) +Kn(0.4mg/l) | Somatic embryogenesis and SEM | Nazneen et al. (2014) |
| 6. | Leaf | MS medium +2,4-D (18.09 μ M) + 2 ip (7.38 μ M) | Somatic embryogenesis, regeneration and histological studies | Naranjo et al. (2015) |

Gaps in study

Although shoot organogenesis is largely reported, but there are very few reports available on somatic embryogenesis in *S. rebaudiana*. The pathway of embryogenesis was studied through light and Scanning Electron Microscope. As the shoot buds and Somatic embryos differentiated from callus, thus regenerated plantlets were tested for somaclonal variations using molecular marker such as RAPD and ISSR.

CHAPTER III

Materials and Methods

Plant material and Explant preparation: Cultures of *Stevia rebaudiana* maintained at Plant tissue culture laboratory, TIFAC- CORE, Thapar University (Patiala) were used as a source of explants to carry out experiments. Microshoots were maintained and multiplied on MS medium containing sucrose 3% (w/v) and agar 0.7% (w/v) through regular subculture period of 21 days. Leaves from microshoots were observed to have small leaves. Thus, for proper leaf expansion, microshoots were cultured on M1 medium (MS medium containing 10 μ M AgNO₃) (Kaur et al. 2017).

Chemicals and Glassware: All glassware were purchased from Borosil Glass Works Ltd. (Mumbai India). Plant tissue culture bottles purchased from Kasablanka Corporation, (Mumbai India). Plant Growth Regulators (PGR) and tissue culture grade chemicals were purchased from Hi-media Laboratories Pvt. Ltd. Mumbai. Other fine chemicals including Enzymes, dNTPs were purchased from Fermentas (Thermo Scientific India Ltd. Mumbai).

Effect of Plant Growth Regulators (PGRs) on shoot organogenesis: For shoot organogenesis, leaf explants were excised from microshoots growing on M1 medium for atleast 3 weeks and cultured on MS medium fortified with N⁶- benzyladenine (BA; 1-5 μ M) and naphthaleneacetic acid (NAA; 1-25 μ M). Cultures were incubated at 25 \pm 1⁰C under white fluorescent light (50 μ mol m⁻²

s⁻¹) and photoperiod regime of 16 hour light and 8 hour dark cycle. Observations were made after 6-8 weeks.

Effect of Plant Growth Regulators (PGRs) on somatic embryogenesis: For somatic embryogenesis excised leaf explants of microshoots growing on M1 medium were cultured with adaxial side on MS medium fortified with different cytokinin (BA) and auxin (NAA and 2,4-D). Cultures were incubated at $25 \pm 1^{\circ}\text{C}$ under white fluorescent light ($50\mu\text{mol m}^{-2} \text{s}^{-1}$) and photoperiod regime of 16 hour light and 8 hour dark cycle. Observations were made after 6-8 weeks. Explants showing embryo like structures were transferred to basal PGR free MS medium for embryo germination.

Histological studies: Following the procedure of Johansen (1940) fixing of samples in formalin, glacial acetic acid and 50% aqueous ethanol (FAA) in a ratio of (5:5:90 v/v) was done for 1 week .After 1 week material was kept in 70% ethanol until used. After fixation samples were dehydrated using TBA (t-butyl alcohol series (Annexure III). Glass vial containing samples and TBA was kept in an oven at 60°C and pellets of wax were added to it after short intervals. Glass vial was kept in the oven till the smell of the TBA disappeared. Samples in proper orientation was embedded in filtered wax and block was made. Block was placed on the holder. Sections of $10\mu\text{m}$ were cut with the help of microtome blade (short ribbons were made). Short ribbons of sections were placed on slide and stretched by adding few drops of water on slide placed on hot plate at 60°C . Sections were dried for about 5-6 days. According to the method describe by Sharma and Millam (2004) staining of sections was done with Toluidine blue (2% w/v) .Sections were mount on a DPX (distyrene, a plasticizer and xylene) solution. Slide was observed under light microscopy.

Scanning Electron Microscopy (SEM) studies: Samples were fixed in 70% (w/v) aqueous alcohol for 2 days. Ethanol series (70, 90, 100% v/v) were used for dehydration of the samples. Two sided carbon tape was used to mount the samples on metal stab. Using automated fine coater (JFC-1600, JEOL Limited, Japan) surface of the sample was coated with gold under vacuum created by ULVAC (GHD030) vacuum pump (J B Systems, Inc, GA). After gold coating samples were observed at 20.0kV under Scanning Electron Microscope (JSM-6510LV, JEOL Limited, Japan) (Roemhildt et al. 2012).

Clonal fidelity of regenerated plants: Regenerated plants for any somaclonal variations were tested by PCR based molecular markers Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeats (ISSR) The microshoots regenerated from somatic embryogenesis were transferred to M1 medium for multiplication and considered as lines for testing clonal fidelity (Kaur et al. 2017). DNA was isolated from the regenerated shoots and mother plant by CTAB method (Doyle and Doyle 1990). PCR amplification was performed by 20 RAPD and ISSR primers each using Verti-96 well thermal cycler (Applied Biosystems) programmed for 41 cycles. The PCR reaction mixture contained primer (10 nmol), dNTP mixture (100 μ M), 1.0 unit Taq DNA polymerase, 2.0 μ l reaction buffer (10 \times) and water for volume make up to 20 μ l. PCR amplification was done at 94 $^{\circ}$ C for 4min (initial denaturation) followed by 41 cycles of denaturation for 1 min at 94 $^{\circ}$ C, annealing was done at 55 $^{\circ}$ C for 45 sec for ISSR and 40 $^{\circ}$ C for 45 sec for RAPD, primer extension at 72 $^{\circ}$ C for 1.30 min. Final extension was done at 72 $^{\circ}$ C for 5 min. Amplified DNA fragments were separated on 1.2% (w/v) agarose gel containing ethidium bromide and visualised under UV trans-illuminator (Gel Doc Mega; Biosystematica, USA).

CHAPTER IV

Results

Multiplication of cultures: The shoot cultures of *Stevia rebaudiana* maintained on Murashige and Skoog (1962) medium were having slow shoot growth with small leaves. Thus, microshoots were transferred to MS 1 medium (Kaur et al. 2017). These microshoots showed improved growth with expansion of leaflets within two weeks of cultures. The leaf explants excised from microshoots growing on M1 medium were then used for further experimentation.

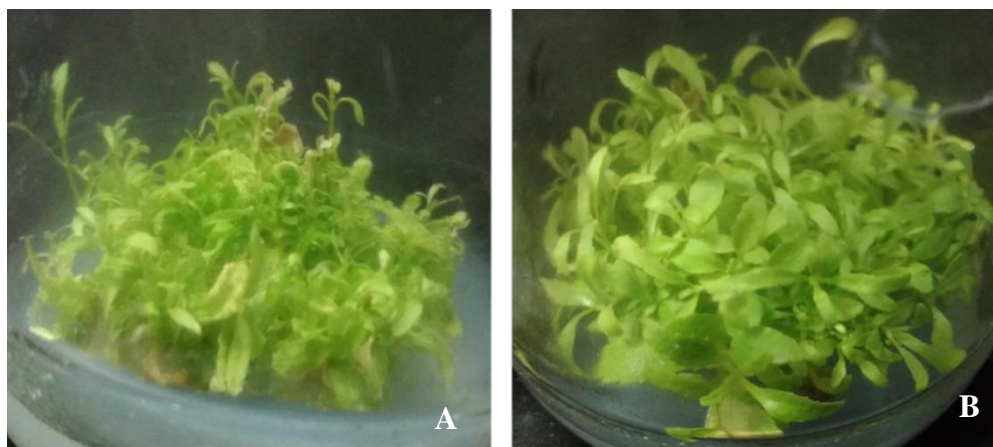


Fig 3: 21 days old cultures growing on MS medium. **A** Without silver nitrate (AgNO_3) **B** supplemented with silver nitrate (AgNO_3)

Effect of Plant Growth Regulators (PGRs) on shoot organogenesis: The presence of (BA) in the medium led to formation of friable calli (Fig 4B) whereas when a combination of NAA was used, nodular compact calli was observed after 3 weeks of initial culture (Fig 4C). However, shoot bud initiation was observed from friable callus after six weeks of culture. Maximum explants (66.67%) showed shoot organogenesis on medium in absence of auxin. Combination of NAA with BA tend to decrease shoot bud initiation from callus (Table 3). It was interesting to note that presence of NAA (1-25 μ M) led to shoot bud induction (upto38.89%), whereas NAA in combination with BA (1-5 μ M) resulted in no shoot organogenesis. It was further observed that BA (above 1 μ M) concentration had adverse effect on shoot organogenesis. However, increase in BA (1-2.5 μ M) concentration results in increased number (5.94) of shoots per explant. Whereas, increase in BA (above 2.5 μ M) showed decrease in number of shoots per explant. Further NAA in combination with BA showed decrease in number of shoots. Moreover, increased concentration of BA results in development of fragile callus.

Table 3: The effect of naphthalene acetic acid (NAA) and N⁶ benzyladenine (BA) on shoot organogenesis from leaf explant of *S. rebaudiana*

| PGR Combinations (µM) | | Callus formation | Percentage explant showing shoot organogenesis | Mean number of shoots per explant |
|-----------------------|-----|------------------|--|-----------------------------------|
| BA | NAA | | | |
| 0 | 0 | + | 5.54 ^{cd} | 0.34 ^e |
| 0 | 1 | + | 53.32 ^{ab} | 2.5 ^{bcd} |
| 0 | 5 | + | 33.34 ^{abcd} | 2 ^{bcd} |
| 0 | 25 | + | 38.89 ^{abcd} | 1.83 ^{bcd} |
| 1 | 0 | + | 66.67 ^a | 3.11 ^b |
| 1 | 1 | + | 28.89 ^{bcd} | 2.83 ^{bc} |
| 1 | 5 | + | 0 ^d | 0 ^e |
| 1 | 25 | + | 0 ^d | 0 ^e |
| 2.5 | 0 | + | 50.00 ^{abc} | 5.94 ^a |
| 2.5 | 1 | + | 44.44 ^{abcd} | 0.67 ^{de} |
| 2.5 | 5 | + | 20.00 ^{cd} | 1.5 ^{bcd} |
| 2.5 | 25 | + | 0 ^d | 0 ^e |
| 5 | 0 | + | 16.67 ^{cd} | 0.87 ^{cde} |
| 5 | 1 | + | 0 ^d | 0 ^d |
| 5 | 5 | + | 0 ^d | 0 ^d |
| 5 | 25 | + | 0 ^d | 0 ^d |

Data was observed after 8 weeks of culture and analysed by ANOVA. Means were compared using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. Values followed by same lowercase letters are not significant at $P < 0.05$

Effect of Plant Growth Regulators (PGRs) on somatic embryogenesis: Effect of PGR (2,4-D, NAA, BA) either singly or in combination with each other to induce somatic embryogenesis in leaf explants of *Stevia rebaudiana* was evaluated. Callus induction (Nodular and friable) was observed from cut ends of leaf explants after 3-4 weeks of initial culture. Callus differentiated into somatic embryos after 6 weeks and globular embryos were visible within 6-8 weeks. It was observed that presence of NAA in the medium in combination with/without 2, 4 -D can increase somatic embryo induction in *S. rebaudiana*. Maximum (94.44%) explants differentiated somatic embryos on MS medium fortified with 2,4-D (1 μ M) and NAA (5 μ M). It was observed number of embryos increases per explant with increase in concentration of 2,4-D and NAA. Further it was observed that high 2,4-D (5 μ M) concentration in combination high concentration of NAA (25 μ M) do not form embryos and form friable callus whereas both NAA and 2,4-D combination results in formation of nodular callus (Table 4).

MS medium fortified with 2,4-D resulted formation of green colored nodular callus whereas BA in combination with 2,4-D or alone develop friable callus. Medium fortified with 2,4-D (5 μ M) resulted in highest percent (86.11%) of somatic embryos. Low differentiation of explants in somatic embryos was found in 2,4-D in association with BA. Increase in 2,4-D concentration results in maximum number (4.67) of somatic embryos per explants, where high concentration of BA (5 μ M) in combination with NAA (1-25 μ M) does not induce embryo formation (Table 5).

Table 4: The effect of 2,4-Dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) on induction of somatic embryos from leaf explant of *S. rebaudiana*

| PGR Combinations (µM) | | Callus Formation | Callus Type | Percentage explant differentiating into somatic embryos | Mean number of embryos per explant |
|-----------------------|-----|------------------|---|---|------------------------------------|
| 2,4-D | NAA | | | | |
| 0 | 0 | + | Friable callus | 0 ^c | 0 ^d |
| 0 | 1 | + | Nodular friable callus | 47.78 ^b | 4.22 ^{bc} |
| 0 | 5 | + | Nodular and friable callus | 44.43 ^b | 4.19 ^{bc} |
| 0 | 25 | + | Explants showed necrosis | 66.66 ^{ab} | 7.44 ^a |
| 1 | 1 | + | Yellow Nodular callus | 77.78 ^a | 7.16 ^a |
| 1 | 5 | + | Green and Yellow colored Nodular callus | 94.44^a | 5.58 ^b |
| 1 | 25 | + | Friable callus | 0 ^c | 0 ^d |
| 2.5 | 1 | + | Nodular yellow colored callus | 44.44 ^b | 3.75 ^c |
| 2.5 | 5 | + | Green colored nodular callus | 79.36 ^a | 5.89 ^b |
| 2.5 | 25 | + | Green and yellow colored nodular callus | 61.11 ^{ab} | 5.30 ^b |
| 5 | 1 | + | Compact nodular callus | 65.55 ^{ab} | 4.34 ^{bc} |
| 5 | 5 | + | Yellow colored nodular callus | 42.22 ^b | 3.67 ^c |
| 5 | 25 | + | Friable callus | 0 ^c | 0 ^d |

Data was observed after 8 weeks of culture and analysed by ANOVA. Means were compared using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. Values followed by same lowercase letters are not significant at $P < 0.05$

Table 5: The effect of N⁶benzyladenine (BA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) on induction of somatic embryos from leaf explant of *S. rebaudiana*

| PGR Combinations (µM) | | Callus Formation | Callus Type | Percentage explant differentiating into somatic embryos | Mean number of embryos per explant |
|-----------------------|-------|------------------|---|---|------------------------------------|
| BA | 2,4-D | | | | |
| 0 | 0 | + | Friable callus | 0 ^d | 0 ^c |
| 0 | 1 | + | Yellow colored callus | 22.21 ^{cd} | 4.67 ^a |
| 0 | 5 | + | Yellow and green colored nodular callus | 86.11^a | 4.49 ^a |
| 0 | 25 | + | Compact , nodular callus | 66.65 ^{ab} | 3.44 ^{ab} |
| 1 | 0 | + | Friable callus | 0 ^d | 0 ^c |
| 1 | 1 | + | Friable callus | 0 ^d | 0 ^c |
| 1 | 5 | + | Friable callus | 0 ^d | 0 ^c |
| 1 | 25 | + | Yellow colored callus | 46.65 ^{bc} | 3.67 ^{ab} |
| 2.5 | 0 | + | Friable callus | 0 ^d | 0 ^c |
| 2.5 | 1 | + | Friable callus | 0 ^d | 0 ^c |
| 2.5 | 5 | + | Yellow colored callus | 27.78 ^{cd} | 1.43 ^{bc} |
| 2.5 | 25 | + | Yellow colored callus | 35.56 ^{cd} | 2.39 ^{abc} |
| 5 | 0 | + | Friable callus | 0 ^d | 0 ^c |
| 5 | 1 | + | Friable callus | 0 ^d | 0 ^c |
| 5 | 5 | + | Friable callus | 0 ^d | 0 ^c |
| 5 | 25 | + | Friable callus | 0 ^d | 0 ^c |

Data was observed after 8 weeks of culture and was analysed by ANOVA. Means were compared using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. Values followed by same lowercase letters are not significant at $P < 0.05$

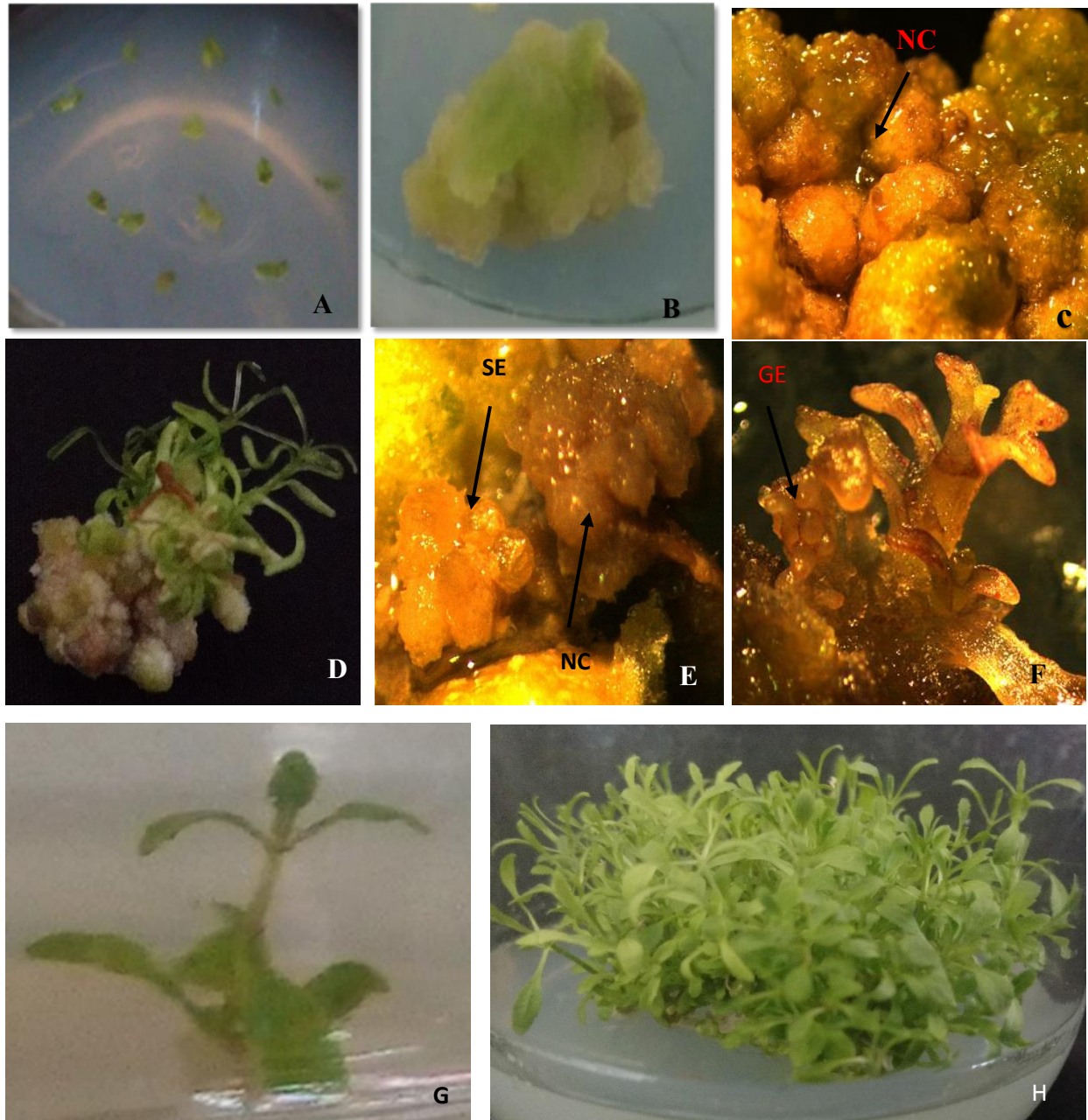


Fig 3: Somatic embryo and shoot bud induction from **A** leaf explant of *Stevia rebaudiana* cultures on MS medium supplemented with BA, 2,4-D and NAA. Callus (**B** Friable, **C** Nodular callus (NC) was observed after 3 weeks on MS medium supplemented with 1 μ M 2,4-D +5 μ M NAA, 1 μ M BA and 5 μ M 2,4-D. **D** Shoot organogenesis was observed from callus on medium supplemented with 1 μ M BA whereas **E** Somatic embryo (SE) initiated on MS medium supplemented with 1 μ M 2,4-D +5 μ M NAA. **F** Somatic embryos were transferred to basal medium without incorporation of PGR for germination (GE: Globular embryo; EG: Embryo germination) **G-H** Germinated shoots were multiplied on basal medium.

Histological studies and Scanning Electron Microscopy (SEM) studies: Histological sections of embryo like structure revealed the defined epidermis layer with actively dividing meristematic pockets. There was no vascular connection of the embryo like structure with maternal tissue.

SEM studies revealed the development stages of embryos emerging from the callus surface. Three developmental stages of the embryo were observed mainly Globular, early heart and torpedo shaped embryos

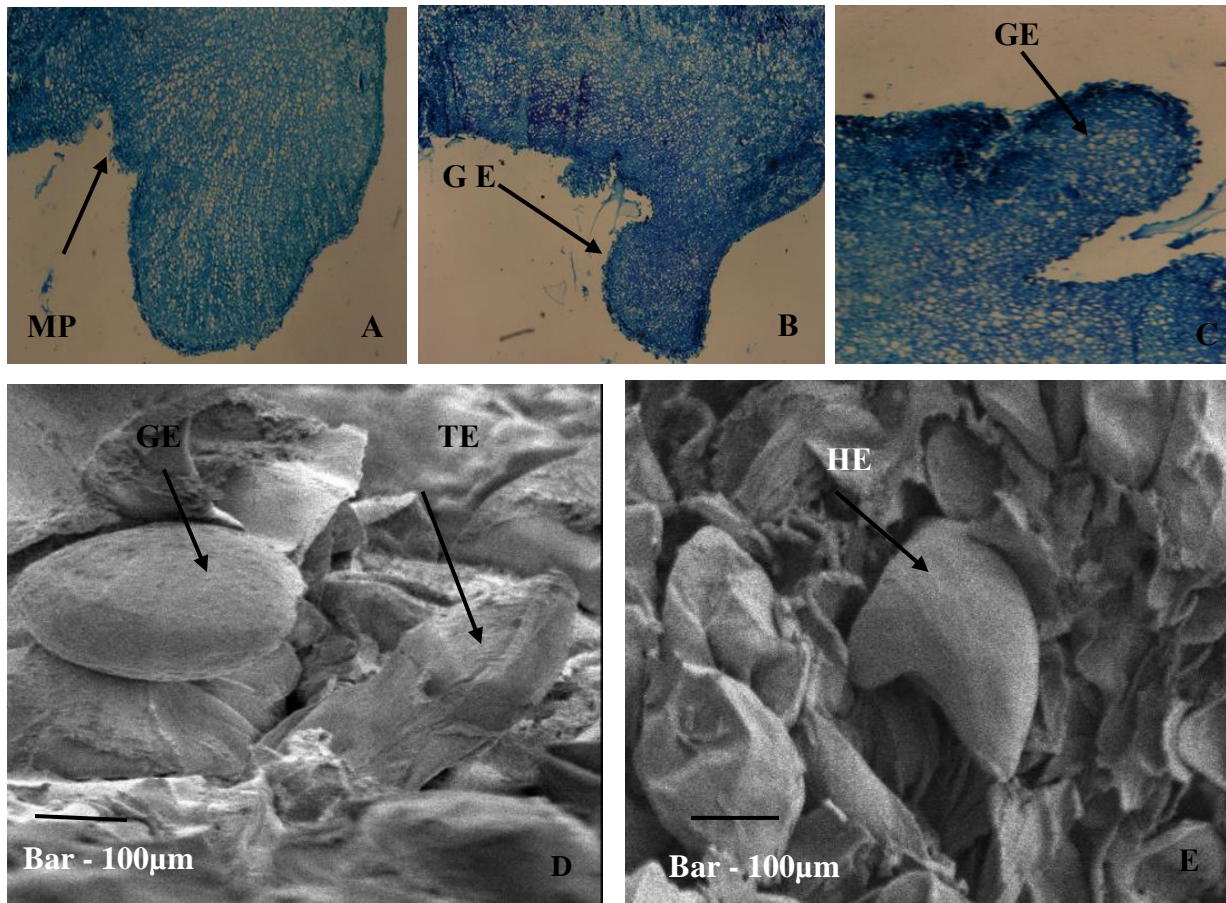


Fig 3: Histological analysis of Somatic embryos showing **A.** active cell division in meristematic pockets (MP) and **B-C** development of early globular embryo (GE). SEM studies showing **D** globular embryo (GE), torpedo shape embryos (TE) and **E** early heart shape embryo (HE). Scale bar : 100µm

Clonal fidelity: The shoots regenerated from the somatic embryos were checked for somaclonal variations. Out of 40 primers (20 RAPD and ISSR each); 18 RAPD and 10 ISSR primers showed the amplification (table 6). Total number of 75 bands were scored (43 for RAPD, 32 for ISSR). Size of the amplified fragments was in range of 250-3000bp. All bands were found to be monomorphic thus, the regenerated plants from the somatic embryos were found to true-to type.

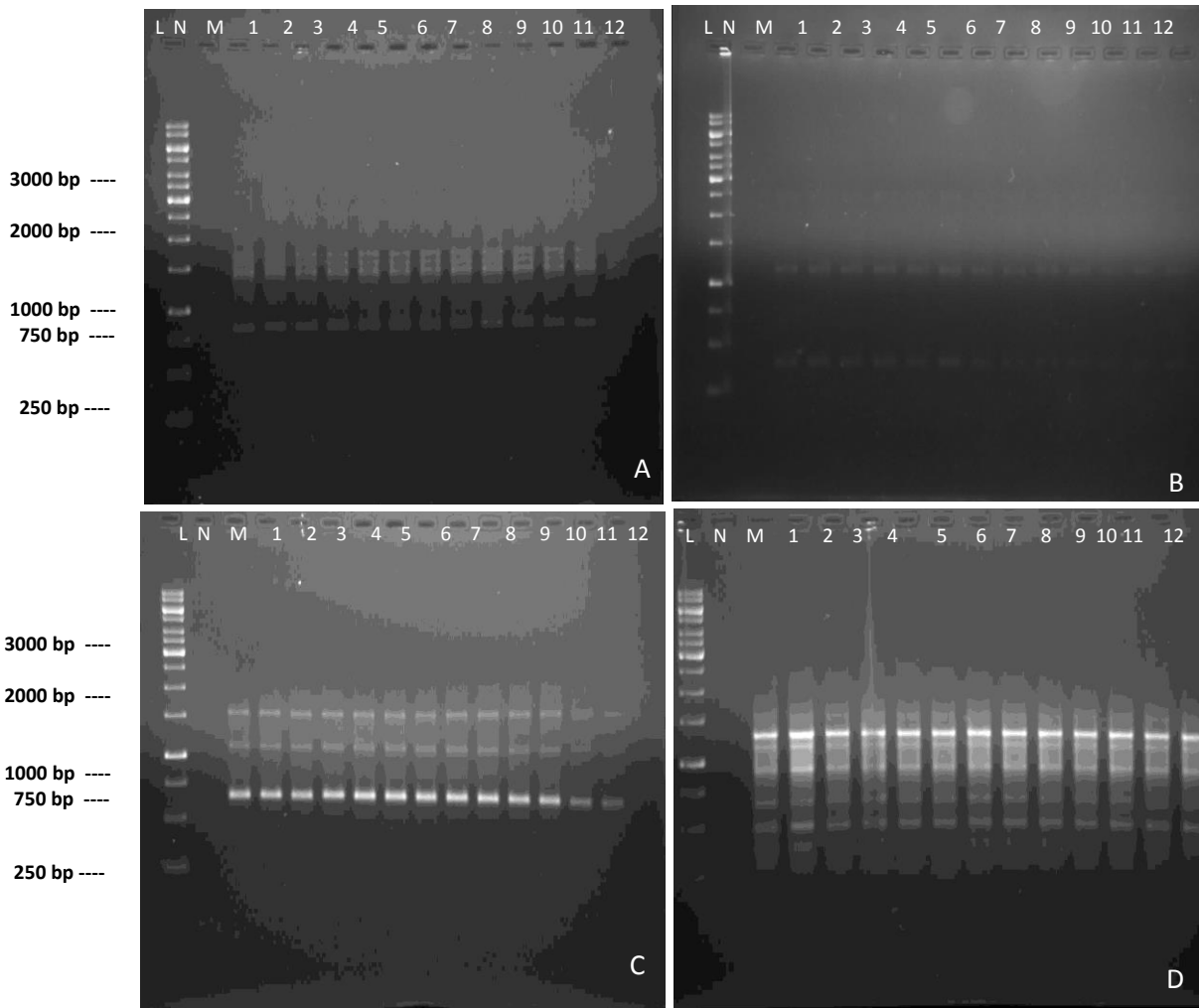


Fig. 4 Amplification profiles of RAPD (A, B) and ISSR (C, D) markers of regenerated plants from somatic embryos. RAPD amplifications (A, B) were carried out using primer RAPD-1 and RAPD 12 and ISSR amplifications (C, D) were carried out using primer ISSR-1 and ISSR-12, Lane M (mother culture); and lane 1–12 plant grown from somatic embryos; L 1-kb molecular-weight markers; Lane N (negative control).

Table 6: The details of the primers used for Random amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) amplifications from regenerated shoots of *Stevia rebaudiana*

| Primer | Sequence | No.of Bands | Size range (bp) | Primer | Sequence | No.of Bands | Size range (bp) |
|-------------|------------|-------------|-----------------|----------------------------|------------------------|-------------|-----------------|
| RAPD | | | | | | | |
| RAPD1 | AGCGCCATTG | 3 | 300- 2500 | RAPD18 | GAGAGCCAAC | 2 | 1000-2500 |
| RAPD2 | CTTCCCAAG | 5 | 300-2500 | RAPD19 | CTGGGGACTT | 1 | 1500 |
| RAPD3 | AGGGCGTAAG | 3 | 750-2500 | Total number of bands = 43 | | | |
| RAPD4 | CTGGGGGACT | 4 | 500-1500 | | | | |
| RAPD5 | ACCGCGAAGG | 4 | 750-2500 | ISSR | | | |
| RAPD6 | GGACCCAACC | 2 | 1000-1500 | ISSR1 | (AC) ₈ CG | 1 | 700 |
| RAPD7 | GTCGCCGTCA | 4 | 250-2500 | ISSR2 | (GA) ₈ CG | 5 | 300-2000 |
| RAPD8 | TCTGGTGAGG | 1 | 300 | ISSR3 | (GA) ₈ TC | 3 | 600-1200 |
| RAPD9 | TGAGCGGACA | 2 | 750-1500 | ISSR4 | (AC) ₈ (GC) | 4 | 200-1000 |
| RAPD10 | ACCTGAACGG | 2 | 1000-2500 | ISSR6 | (CA) ₈ TG | 1 | 300 |
| RAPD11 | TTGCACGGG | 1 | 2000 | ISSR7 | (CA) ₈ GC | 4 | 300-750 |
| RAPD12 | GTGTGCCCCA | 4 | 1000-2500 | ISSR8 | (GA) ₈ TA | 4 | 500-1000 |
| RAPD13 | CTCTGGAGAC | 1 | 2500 | ISSR12 | (CT) ₈ G | 3 | 250-2000 |
| RAPD14 | CACCGTATCC | 1 | 2500 | ISSR13 | (CT) ₈ A | 3 | 250-750 |
| RAPD15 | GGGGTGACGA | 2 | 750-2500 | ISSR14 | (CT) ₈ AG | 4 | 200-3000 |
| RAPD16 | CATCCGTGCT | 1 | 1500 | Total number of bands = 32 | | | |

NOTE: DNA was isolated from regenerated shoots multiplied on MS medium

CHAPTER V

Discussion

Somatic embryogenesis is an important pathway adopted by cells to redifferentiate into whole plant (Dodeman et al. 1997). Apart from its use in propagation, somatic embryogenesis is a role model for studying various developmental pathways undergoing in the plant (Arnold et al. 2002; Sharma and Millam 2004; Khilwani et al. 2016). After the first report in carrot, many researchers have reported the process of somatic embryogenesis in many plants (Sharma and Millam 2004; Khilwani et al. 2016; Banerjee and Sarkar 2009; Jain et al. 1995; Ho and Vasil 1983). Although somatic embryogenesis is reported in *Stevia rebaudiana* but the work is very limited and many factors need to be worked out. (Nazneen et al. 2014; Naranjo et al. 2015). Thus the current study was focused on development of an efficient regeneration protocol (shoot organogenesis and somatic embryogenesis) in *Stevia rebaudiana*. For shoot bud induction and formation of somatic embryos effect of Plant Growth Regulators (PGRs) was evaluated using various combinations of 2, 4-D, BA, NAA.

Microshoots of *Stevia rebaudiana* grown on basal MS medium were having slow shoot growth with small leaves. Thus it was difficult to get leaf explants for experimentation. This problem has been reported and probably due to acquisition of ethylene in many plants in cultural vessels (Kaur et al. 2017; Saltveit 2005), Thus, the microshoots were transferred to previously optimized MS medium supplemented with AgNO₃ for obtaining fully expanded leaves in potato. The effect of AgNO₃ on leaf expansion have been reported in other plants (Kaur et al. 2017; Tamini et al. 2015; Saltveit 2005; Sirisom and Te-chato 2012; Soo Cheon Chae and Sang Un Park 2012)

For somatic embryogenesis in leaf explants the effect of 2, 4-D with NAA was also evaluated. Results showed more number of embryos per explant and highest percent of somatic embryo differentiation with increase in NAA and decrease in 2, 4-D concentration. However, somatic embryogenesis is inhibited by high concentration of auxin. These results were in line with many reports where it has been reported that 2, 4-D plays major role in induction of somatic embryos but its high concentration inhibits the process during later stages of somatic embryogenesis (Cooke et al 1993; Liu et al. 1993)

Auxins play major role in the somatic embryogenesis. Most commonly used auxins are 2, 4-D and NAA. Auxins are potent signal molecule for somatic cells to perform cell polarization and asymmetric cell division (Stuart and McCall 1992).

In addition to auxins, a combination of cytokinin was also evaluated for its role in inducing somatic embryos in *Stevia rebaudiana*. It was observed that association of BA with 2, 4-D leads to decrease in explant differentiation into somatic embryos with low number of embryos per explant and increased concentration of BA and 2, 4-D in MS medium leads to callus formation

In addition to auxins, cytokinins play a potent role in cell division and development of shoot buds, inhibit root formation and retard senescence (D'onofrio and Morini 2006). Cytokinins helps in cell proliferation when used in association with auxin. It has been reported that cytokinins inhibit induction of somatic embryogenesis by auxin when added in culture medium (Lakshmanan and Taji 2000). However in other reports it has been reported that cytokinins helps in initiation of somatic embryogenesis in species *Zoysia japonica*, *Begonia gracilis* and *Oncidium sp.* (Chen & Chang 2001; Asano et al. 1996; Castillo and Smith 1997).

Developed somatic embryos were transferred to auxin free medium to germinate. Usually, 2,4-D is required for initiation of somatic embryogenesis but there are many reports which show that maturation and germination of embryos does not require auxin in the medium. Thus in present study embryo induced in auxin rich medium were transferred to auxin free medium. It was observed that 66.67 % embryos germinated to develop plantlets. These results are in line with many reports (Sharma and Millam 2004; Ahloowalia et al. 1983; Remakanthan et al. 2014). Embryo like structures are not clearly differentiable from callus. So cellular level study is mandatory. There are many reports which clearly show that embryos developed from globular to heart shape followed by torpedo and cotyledonary shape. During present study, the ontogenic stages of somatic embryogenesis were studied by histological techniques. Various stages of somatic embryos were also confirmed though Scanning Electron Microscope (SEM). These techniques were earlier used to study the developmental stages of somatic embryogenesis (Nazneen et al. 2014; Naranjo et al; Khilwani et al. 2014).

In *Stevia rebaudiana* shoot organogenesis is widely reported from leaf, internode, floret, shoot tip, and shoot apex explants (Tamura et al. 1984; Gurucharan and Chinnagounda 2013; Giridhar et al. 2010; Majumder and Rahman 2016). It has been reported that addition of cytokinin in the medium resulted in development of shoots from calli. Similar results were earlier observed in *Stevia rebaudiana* In present study, results showed that presence of only cytokinin (BA) in the medium results in maximum shoot organogenesis whereas, BA in association with NAA results in less percent of shoot induction. Differentiation of callus to organized structures such as shoot or somatic embryos led to somaclonal variations (Kaur et al. 2017).The use of molecular marker such as RAPD, ISSR, SSR,SNPs etc. for clonal fidelity studies is widely reported in many plants(Kaur et al. 2017; Aggarwal et al. 2010; Bansal et al. 2014).

Though, both shoot organogenesis and somatic embryogenesis develop from callus, thus the testing of clonal fidelity was mandatory. In the present study, molecular marker such as RAPD and ISSR were used to test clonal uniformity. Total 75 number of markers were scored (43 for RAPD and 32 for ISSR). All the bands were monomorphic, thus indicating the clonal uniformity of regenerated plants.

CHAPTER VI

Conclusion

Stevia rebaudiana is an important perennial medicinal plant. From the current study it was found that the plant growth regulators play a major role in the induction of shoot bud and somatic embryos

1. *Stevia rebaudiana* cultures were multiplied on MS medium fortified with 10.0 μ M silver nitrate for normal growth and expanded leaves.
2. MS medium fortified with 1 μ M 2,4-D and 5 μ M NAA was found to be best medium for initiation of somatic embryos.
3. Histological sections of embryo like structure revealed the cell division in the pockets of epidermis layer which later on give rise to various stages (globular, early heart shape and torpedo shape) of somatic embryogenesis. These stages were also confirmed using Scanning Electron Microscope
4. Regenerated plantlets from somatic embryos were tested for clonal uniformity and all the bands were found to be monomorphic thus, indicates the clonal uniformity of regenerated plants

References

1. Aggarwal D, Kumar A, Reddy MS (2010) Shoot organogenesis in elite clones of *Eucalyptus tereticornis*. *Plant Cell Tissue Organ Culture* 102:45–52
2. Ahloowalia BS, Maretzki A (1983) Plant regeneration via somatic embryogenesis in sugarcane. *Plant Cell Report* 5:21-25
3. Ahmad N, Fazal H, Zamir R, Khalil SA, Abbasi BH (2011) Callogenesis and shoot organogenesis from flowers of *Stevia rebaudiana* (Bert.). *Sugar Tech* 13:174-177
4. Arellano M, Dhir S, Albino NC, Santiago A, Morris T, Dhir SK (2015) Somatic embryogenesis And Plantlet Regeneration from Protoplast culture of *Stevia rebaudiana*. *British Biotechnology Journal* 5:1-12
5. Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. *Plant Cell, Tissue and Organ culture* 69:233-249
6. Asano Y, Katsumoto H, Inocuma C, Kaneko S, Ito Y and Fuji L (1996) Cytokinin and thiamine requirements and stimulative effects of riboflavin and α -ketoglutaric acid on embryogenic callus induction from seeds of *Zoysia japonica* sp. *Journal of Plant Physiology* 149:413-417
7. Banerjee M and Sarkar P (2008) *In vitro* callusing in *Stevia rebaudiana* Bertoni using cyanobacterial media -a novel approach to tissue culture. *International Journal of Integrative Biology* 3:163-168

8. Banerjee M and Sarkar P (2009) Effect of different concentration of growth hormones on somatic embryogenesis in *Stevia rebaudiana*. International Journal of Plant Sciences 5:284-289
9. Bansal M, Kumar A, Reddy MS (2014) Diversity among wild accessions of *Bacopa monnieri* (L.) Wettst. and their morphogenetic potential. Acta Physiol Plant 36:1177–1186
10. Bertoni MS (1905) Le Kaa He-e Sa nature et ses propriétés. Ancient Paraguayos 1:1-14
11. Bhojwani SS and Razdan MK (1996) Plant tissue culture: Theory and Practice. Elsevier Publications, Amsterdam pp. 125-151
12. Brahmachari G, Mandal LC, Roy R, Mondal S, and Brahmachari AK (2011) Stevioside and Related Compounds – Molecules of Pharmaceutical Promise: A Critical Overview. Archiv der Pharmazie – Chemistry in Life Sciences 1:5-9
13. Brandle JE and Rosa N (1992) Heritability for yield, leaf-stem ratio and stevioside content estimated from a landrace cultivar of *Stevia rebaudiana*. Canadian Journal of Plant sciences 72:1263–1266
14. Brandle JE, Starratt AN and Gijzen M (1998) *Stevia rebaudiana*: its agricultural, biological, and chemical properties. Canadian Journal of Plant Sciences 78:527-536
15. Castilo B and Smith MAL (1997) Direct somatic embryogenesis from *Begonia gracilis* explants. Plant Cell Reports 16:385-388
16. Chen JT and Chang WC (2001) Effects of auxins and cytokinins on direct somatic embryogenesis on leaf explants of *Oncidium 'Gower Ramsey*. Plant Growth Regulation 34:229-232

17. Chester K, Tamboli E, Singh M and Ahmad S (2012) Simultaneous quantification of stevioside and rebaudioside A in different *stevia* samples collected from the Indian subcontinent. *Journal of Pharmacy and Bioallied Science* 4:76-81
18. Cooke TJ, Racusen RH, Cohen JD (1993) The role of auxin in plant embryogenesis. *Plant Cell* 5:1494–1495
19. Das A, Gantait S and Mandal N (2011) Micropropagation of elite Medicinal plant: *Stevia rebaudiana* Bertoni. *International Journal of Agricultural Research* 6:40-48
20. De LC (2017) Breeding of medicinal and aromatic plants –an overview. *International Journal of Botany and Research* 7:25-34
21. Deshmukhan YRK, Sirsat A, Hande P, Zele SS, More KD (2014) Preparation of ice cream using natural sweetener *Stevia*. *Food Science Research Journal* 5:30-33
22. Dodeman VL, Ducreux G and Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. *Journal of Experimental Botany* 48:1493-1509
23. D'Onofrio C and Morini S (2006) Effects of different cytokinins on somatic embryogenesis, caulogenesis and rhizogenesis in *in vitro*-cultured quince (*Cydonia oblonga* Mill.) leaves. *Advances in Horticultural Science* 20:215-219
24. Doyle JJ and Doyle JL (1990) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19:11-15
25. Ferreira CM and Handro W (1988) Production, maintenance and plant regeneration from cell suspension cultures of *Stevia rebaudiana* (Bert.) Bertoni. *Plant Cell Reports* 7:123-126
26. Filho JC and Hattori K (1997) Embryogenic callus formation and histological studies from *Stevia rebaudiana* (Bert) bertoni floret explants. *The Revista Brasileira de Fisiologia Vegetal* 9:185-188

27. Filho JC, Hashimoto JM and Vieira LGE (1993) Induction of somatic embryogenesis from leaf explants of *Stevia rebaudiana*. The Revista Brasileira de Fisiologia Vegetal 5:51-53
28. Gamboa F and Chaves M (2012) Antimicrobial Potential of Extracts from *Stevia rebaudiana* leaves against bacteria of importance in dental caries. Acta Odontol Latinoam 25:171-175
29. Giridhar P, Sowmya SK, Ramakrishna A, Ravishankar GA (2010) Rapid Clonal Propagation and Stevioside Profiles of *Stevia rebaudiana* Bertoni. International Journal of Plant Developmental Biology 4:47-52
30. Goettemoeller J and Ching A (1999) Seed germination in *Stevia rebaudiana*. In: JanickJ (ed.) Perspectives on new crops and new uses. ASHS Press, Alexandria, VA: pp 510-511
31. Goyal SK, Samsher and Goyal RK (2010) *Stevia (Stevia rebaudiana)* a bio-sweetener: a review. International Journal of Food Sciences and Nutrition 61:1-10
32. Gupta P, Sharma S and Saxena S (2010) Micropropagation of *Stevia Rebaudiana* (natural sweetener) using kinetin for steviol glycoside production. Research Journal of Biotech 5: 63-67
33. Gupta R, Yadav V, Rastogi MA (2014) review on importance of natural sweetener, a zero calorie plant – *Stevia* -having medicinal and commercial importance. International Journal of Food and Nutritional Sciences 3:90–94
34. Gurucharan V and Chinnagounder S (2013) Effect of Polyamines on *In-Vitro* Organogenesis using Shoot tip Explants of *S. rebaudiana* Bert. International Journal of Current Biotechnology 1:16-18

35. Ho WJ and Vasil IK (1983) Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.) I. The morphology and physiology of callus formation and the ontogeny of somatic embryos. Florida Agriculture Experiment Station Journal 118:169-182
36. http://foodmysteries.com/wp-content/uploads/2014/06/Stevia_rebaudiana_flowers.jpg
37. <https://bonnieplants.com/growing/growing-stevia/>
38. <https://typicalgardener.files.wordpress.com/2010/05/stevia-seeds.jpg>
39. Huang JZ, Shrestha A, Tollenaar M, Deen W, Rahimian H and Swanton CJ (2000) Effects of photoperiod on the phenological development of red root pigweed (*Amaranthus retroflexus* L.). Canadian Journal of Plant Science 80:929-938
40. Jain SM, Gupta PK, and Newton RJ (1995) Somatic embryogenesis in Woody Plants. Vol 3. Gymnosperms. Kluwer Academic Publishers, Dordrecht.
41. Jayaraman S, Manoharan MS and Illanchezian S (2008) *In-vitro* Antimicrobial and Antitumor Activities of *Stevia Rebaudiana* (Asteraceae) Leaf Extracts. Tropical Journal of Pharmaceutical Research 7:1143-1149
42. Jimenez V (2001) Regulation of in vitro somatic embryogenesis with emphasis on the role of endogenous hormones. The Revista Brasileira de *Fisiologia Vegetal* 13:196-223
43. Jimenez-Florez R, Klipfel NJ, Tobias J (1993) Ice cream and frozen desserts. In: Hui YH (ed) Dairy science and technology handbook.2. Product manufacturing. VCH Publishers, New York, pp. 57
44. Johansen D (1940) Plant microtechnique. McGraw Hill, London
45. Kaur A, Reddy MS and Kumar A (2014) Efficient, one step and cultivar independent shoot organogenesis of potato. *Physiology and Molecular Biology of Plants* 23:461-469

46. Khilwani B, Kaur A, Ranjan R and Kumar A (2014) Direct somatic embryogenesis and encapsulation of somatic embryos for *in vitro* conservation of *Bacopa monnieri* (L.) Wettst. Plant Cell, Tissue and Organ Culture 127:433-442
47. Kornienko AV and Parfenov AM (1996) Some results of work at the All-Russian Sugar beet and Sugar Institute. Sakharnaya Svekla 5:6-7
48. Koyama E, Kitazawa K, Ohori Y, Izawa O, Kakegawa K, Fujino A, Ui M (2003) *In vitro* metabolism of the glycosidic sweeteners, Stevia mixture and enzymatically modified Stevia in human intestinal microflora. Food and Chemical Toxicology 41:359–374
49. Kumari K, Abeysinghe DC and Dharmadasa RM (2016) Distribution of Phytochemicals and Bioactivity in Different Parts and Leaf Positions of *Stevia rebaudiana* (Bertoni)-a Non caloric, Natural Sweetener. World Journal of Agricultural Research 4:162-165
50. Lakshmanan P, Taji A (2000) Somatic Embryogenesis in Leguminous Plants. Plant Biology 2:136–148
51. Laribi B, Rouatbi N, Kouki K, Bettaieb T (2012) *In vitro* propagation of *Stevia rebaudiana*(Bert.)- A non-caloric sweetener and antidiabetic medicinal plant. International Journal of Medicinal and Aromatic Plants 2:333-339
52. Lata H, Chandra S, Techen N, Wang Y and Khan IA (2013) Molecular analysis of genetic fidelity in micropropagated plants of *Stevia rebaudiana* bert. using ISSR marker. American Journal of Plant Sciences 4:964-971
53. Lee JI, Kang KH and Ham YS (1978) The high yielding *Stevia* variety Suweon 2. South Korea, Office of Rural Development. Annual Research Report 21:21
54. Lee JI, Kang KK, and Park HW (1982) New high rebaudioside A stevia variety ‘Suweon11’. Research Reports of the Office of Rural Development Crops 24:186-188

55. Liu C, Xu ZH, Chua NH (1993) Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. *Plant Cell* 5:621–630
56. Luwanska A, Perz A, Mankowska G and Wielgus K (2015) Application of in vitro *Stevia* (*Stevia rebaudiana Bertoni*) cultures in obtaining steviol glycoside rich material. *Herba Polonica* 61:51-63
57. Majumder S and Rahman M (2016) Micropropagation of *Stevia rebaudiana Bertoni* through direct and indirect organogenesis. *Journal of Innovations in Pharamaceuticals and Biological Sciences* 3:47-56
58. Marsolais AA, Brandle J and Sys EA (1998) *Stevia* plant named 'RSIT 94-751' United States Patent PP10564
59. Misra H, Mehta D, Mehta BK and Jain DC (2015) Antihypertensive (blood pressure lowering) effects of stevioside, from *Stevia rebaudiana Bertoni*, on rats, dogs and Humans –A short review. *India Journal of Drugs* 3:102-108
60. Mitra A and Pal A (2007) *In Vitro* Regeneration of *Stevia rebaudiana (Bert)* from the Nodal Explant. *Journal of Plant Biochemistry and Biotechnology* 16:59-62
61. Moktaduzzaman MD and Rahman SMM (2009) Regeneration of *Stevia rebaudiana* and analysis of somaclonal variation by RAPD. *Biotechnology* 8:449-455
62. Mondaca R, Galvez A, Bravo Land Hen K (2012) *Stevia rebaudiana Bertoni*, source of a high potency natural sweetener: A comprehensive review on the biochemical, nutritional, functional aspects. *Food Chemistry* 132:1121-1132
63. Moraes RM, Donega MA, Cantrell CL, Mello SC, McChesney JD (2013) Effect of harvest timing on leaf production and yield of diterpene glycosides in *Stevia rebaudiana Bert*: A specialty perennial crop for Mississippi. *Industrial Crops and Products* 51:385-389.

64. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
65. Naranjo EJ, Fernandez Betin O, Urrea Trujillo AI (2015) Effect of Genotype on the *in vitro* regeneration of *Stevia rebaudiana* via somatic embryogenesis. *Acta Biologica Colombiana* 21:87-98
66. Nazneen H, Sridhar S and Reddy SK (2014). Studies on Somatic embryogenesis of *Stevia rebaudiana* (Bertoni). By Scanning Electron Microscope (SEM). *World Journal of Pharmaceutical Research* 4:918-924
67. Okporie EO and Ekpe II (2008) Effect of photoperiod on the growth of bulbing of Tropical Onion (*Allium cepa L*) Varieties. *World journal of Agricultural Sciences* 4:36-39
68. Pande SS and Gupta P (2013) Plant tissue culture of *Stevia rebaudiana* (Bertoni): A review. *Journal of Pharmacognosy and Phytotherapy* 5:26-33
69. Pratibha G, Satyawati S and Sanjay S (2010) Micropropagation of *Stevia rebaudiana* (natural sweetener) using kinetin for steviol glycoside production. *Research Journal of Biotechnology* 5:63-67
70. Preethi D, Sridhar TM and Naidu CV (2011) Direct shoot organogenesis from leaf explant of *Stevia rebaudiana*. *Journal of Pathology* 3:69-73
71. Rathi N and Arya S (2009) *In vitro* regeneration through callus culture of medicinally important plant *Stevia rebaudiana* (Bert.) Bertoni. *International journal of Plant Sciences*. 4:559-563
72. Remakanthan A, Menon TG and Soniya EV (2014) Somatic embryogenesis in banana (*Musa acuminata* AAA cv. *Grand Naine*): effect of explant and culture conditions. *In Vitro Cellular & Developmental Biology - Plant* 50:127-136

73. Roemhildt ML, Beynon BD, Gardener MM (2012) Mineralization of articular cartilage in the Sprague–Dawley rat: characterization and mechanical analysis. *Osteoarthritis Cartilage* 20:796–800
74. Ruiz P, Maldonado P, Hidalgo V, Sauma D, Roseblatt M and Bono MS (2015) Alloreactive Regulatory T cells allow the generation of mixed chimerism and Transplant Tolerance. *Frontiers in immunology* 6:1-9
75. Saltveit ME (2005) Aminoethoxyvinylglycine (AVG) reduces ethylene and protein biosynthesis in excised disc of mature-green tomato pericarp tissue. *Postharvest Biology and Technology* 35:183–190
76. Sharma SK, Millam S (2004) Somatic embryogenesis in *Solanum tuberosum* L.: a histological examination of key development stages. *Plant Cell Report* 23:115–119
77. Shu S (1989) *Stevia rebaudiana* variety trials. *Zuowu Pinzhong Ziyuan* 1:17-18
78. Shu S (1995) A study on good variety selection in *Stevia rebaudiana*. *Scientia Agricultura Sinica* 28:37-42
79. Shyu YT (1994) Effects of harvesting dates on the characteristics, yield, and sweet. *Journal of Agricultural Research in China* 43:29-39
80. Siddique AB, Rahman SMM, Hossain MA and Rashid MA (2014) Phytochemical screening and comparative antimicrobial potential of different extracts of *Stevia rebaudiana* Bertoni leaves. *Asian Pacific Journal of Tropical Disease* 4:275–280
81. Singh B, Singh J and Kaur A (2014) Agro-production, Processing and Utilization of *Stevia rebaudiana* as Natural Sweetener. *Journal of Agricultural Engineering and Food Technology* 1:28-31

82. Sirisom Y and Te- chato (2012) The effect of peptone and silver nitrate on In vitro shoot formation in *Hevea brasiliensis* Muell Arg. Journal of Agricultural Technology 8:1509-1516
83. Soo Cheon Chae and Sanh Un Park (2012) Improved shoot organogenesis of *Echinacea angustifolia* DC treated with ethylene inhibitors. Life Science Journal 9:1725-1728
84. Sreedhar RV, Venkatachalam L, Thimmaraju R, Bhagyalakshmi N, Narayan MS and Ravishankar GA (2008) Direct organogenesis from leaf explants of *Stevia rebaudiana* and cultivation in bioreactor. Biologia Plantarum 52:355-360
85. Steward FC, Mapes MO and Mears K (1958) Growth and organized development of cultured cells. II: Organization in cultures grown from freely suspended cells. American Journal of Botany 45:705-800
86. Stuart DA and McCall CM (1992) Induction of somatic embryogenesis using side chain and ring modified forms of phenoxy Acid growth regulators. Plant Physiology 99:111-118
87. Suhendi D (1989) Mass selection of *Stevia rebaudiana* Bertoni. Menara Perkebunan 56:93-95
88. Sys EA, Marsolais AA, and Brandle J (1998) Stevia plant named 'RSIT 94-1306' United States Patent PP10562
89. Tamimi SM (2015) Effects of ethylene inhibitors, silver nitrate (AgNO_3), cobalt chloride (CoCl_2) and aminooxyacetic acid (AOA), on *in vitro* shoot induction and rooting of banana (*Musa acuminata* L.). African Journal of Biotechnology 14:2510-2516
90. Tamura V, Nakamura S, Fukui H and Tabata M (1984) Clonal propagation of *Stevia rebaudiana* Bertoni by stem-tip culture. Plant Cell Reports 3:183-185

91. Weng XY, Sun JY and Zang RC (1996) Study on the growth and physiological characteristics of *Stevia rebaudiana* SM4. Journal of Zhejiang Agricultural University 22: 538-540
92. Wheeler A, Boileau AC, Winkler PC, Compton JC, Prakash I, Jiang X, Mandarino DA (2008) Pharmacokinetics of rebaudioside A and stevioside after single oral dose in healthy men. Food and Chemical Toxicology 46(Suppl. 7):S54–S60
93. Yadav AK, Singh S, Dhyani D and Ahuja PS (2010) Review on the improvement of [*Stevia rebaudiana* (Bertoni)]. Canadian Journal of Plant Science 91:1-27
94. Yasukawa K, Kitanaka S and Seo S (2002) Inhibitory effect of stevioside on tumour promotion by 12-*O*-Tetradecanoylphorbol-13 acetate in Two-Stage Carcinogenesis in Mouse Skin. Biological and Pharmaceutical Bulletin 25:1488-1490
95. Zeevart JAD (1971) (+)-Abscisic Acid Content of Spinach in Relation to Photoperiod and Water Stress. Plant Physiology 48: 86-90
96. Zimmerman JL (1993) Somatic embryogenesis: A Model for Early Development in Higher Plants. The Plant Cell 5:1411-1423

Annexure I

Table 4: Composition and concentration of macronutrients, micronutrients and vitamins present in the Murashige and Skoog (1962) medium.

| Components | Amount(mg/L) |
|---------------------------------------|---------------------|
| Macronutrients | |
| NH ₄ NO ₃ | 1650 |
| KNO ₃ | 1900 |
| CaCl ₂ .2H ₂ O | 440 |
| MgSO ₄ .7H ₂ O | 370 |
| KH ₂ PO ₄ | 170 |
| Micronutrients | |
| H ₃ BO ₃ | 6.2 |
| MnSO ₄ .4H ₂ O | 16.9 |
| ZnSO ₄ .H ₂ O | 8.6 |
| KI | 0.83 |
| NaMO ₄ .2 H ₂ O | 0.25 |
| CuSO ₄ .5H ₂ O | 0.025 |
| CoCl ₂ .6H ₂ O | 0.025 |
| Vitamins | |
| Nicotinic acid | 0.5 |
| Pyridoxine HCl | 0.5 |
| Thiamine HCl | 0.1 |
| Myo-inositol | 100 |
| Other | |
| Glycine | 2 |
| Sucrose | 3% (w/v) |
| Agar | 0.7% (w/v) |

NOTE - Stocks of ferric EDTA, myo-inositol and sucrose were freshly prepared before use.

Annexure II

Table 4: Stock solution preparation of Growth Regulators

| S.no | Growth Regulator | Amount (mg) | Solvent | Volume make up (100ml) |
|------|-------------------|-------------|---------|------------------------|
| 1. | 2.5 μ M BAP | 56.315 | HCL | d H ₂ O |
| 2. | 2.5 μ M NAA | 46 | KOH | d H ₂ O |
| 3 | 2.5 μ M 2,4-D | 55.26 | - | d H ₂ O |

Table 5: TBA series for dehydration of samples fixed in FAA (Formalin Acetic Acid)

| S.No | Rectified alcohol (ml) | Tertiary butyl alcohol (ml) | Water (ml) | Time (hr) |
|------|---------------------------|--------------------------------|---------------|--------------|
| A | 30 | 20 | 50 | 3-4 |
| B | 50 | 20 | 50 | 3-4 |
| C | 50 | 35 | 15 | 3-4 |
| D | 45 | 55 | - | 3-4 |
| E | 25 | 75 | - | 3-4 |
| F | - | 100 | - | 3-4 |

Grade E can be kept for longer period (24hr)