In Vitro Production of zero calorie natural sweetener and antioxidant compounds from *Stevia rebaudiana*

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Biotechnology

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2024

DECLARATION

I, hereby declared that the presented work in the thesis entitled "***In Vitro* Production of zero calorie natural sweetener and antioxidant compounds from *Stevia rebaudiana*** " in fulfilment of degree of Doctor of Philosophy (Ph. D.) is outcome of research work carried out by me under the supervision of Dr. Devendra Kumar Pandey working as Professor, in the Department of Botany of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

(Signature of Scholar)

Name of the scholar:

Registration No.:

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Punjab, India

CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled " ***In Vitro*** **Production of zero calorie natural sweetener and antioxidant compounds from** ***Stevia rebaudiana*** " submitted in fulfillment of the requirement for the award of degree of Doctor of Philosophy (Ph.D.) in the Department of biotechnology , is a research work carried out Shahnawaz, 11412651, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

(Signature of Supervisor)

Name of supervisor:

Designation:

Department/school:

University:

(Signature of Co-Supervisor)

Name of Co-Supervisor:

Designation:

Department/school:

University:

ACKNOWLEDGEMENT

With deep sense, respect and obligation from the core of my heart, I wish to thank my Supervisor Dr. Devendra Kumar Pandey, Department of Botany, Lovely Professional University for his endurance, intent supervision, deep attentiveness, valuable time and mentoring abilities, thereby providing me a free and pleasant atmosphere to work against all anomalous circumstances. I am truly very fortunate to have the opportunity to work with him and found his supervision to be enormously valuable.

I would like to express my deep sense of gratitude to Dr.Padmanabh Dwivedi Department of Plant Physiology ,Banaras Hindu University, Varanasi, Dr.R.C. Gupta Department of Botany, Punjabi University, Patiala, Dr.Abhijit Dey Department of Life Sciences, Presidency University and Dr.Amita Pal Plant Molecular & Cellular Genetics, Bose Institute, Kolkata ,Dr.Vijay Kumar Lovely Professional University for there constant encouragement, valuable advises and the freedom of work he provided throughout my research work. I am grateful to Dr. Anshu Bansal, Technical Assistant, Sophisticated Instrumentals Centre (SIC), Punjabi University, Patiala and Bose Institute ,Kolkata for giving the guidance in my experimental work .

I am indebited to my friends namely Dr.Roman Nazir, Kajal Katoch, and for conceding a lot with their comfort only to make me feel comfortable, so I could complete my thesis. I wouldlike to extend my warm thanks to all my research colleagues and friends Dr. Prabjot Kaur, Ms. Ayang, and other friends for their co-operation, and friendly environment in the lab.I am also thankfull to all friends whose name are not written but they helped during the course time .

I would like to show my gratitude to the entire family of Lovely Professional University and School of Bio- Engineering and Biosciences for providing me a suitable research atmosphere to carry out my work.

My deep thanks are also due to many people, whose names are not mentioned, but directly or indirectly helped me to complete my experimentation work and thesis.

Abbreviations

Abbreviations	Descriptions
ANOVA	Analysis of Variance
BA	N^6-Benzyladenine
BBD	Box-Behnken Design
RSM	Response Surface Methodology
DNA	Deoxyribose Nucleic Acid
$HgCl_2$	Mercuric Chloride
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HRE	Heat Reflux Extraction
BA	N^6-Benzyladenine
BAP	6-Benzylaminopurine
BBD	Box-Behnken Design
BM	Basal Medium
IAA	Indole-3-Acetic acid
$CaCl_2$	Calcium Chloride
PGR	Plant Growth Regulators
SA	Salicylic Acid
SD	Standard Deviation
TDZ	Thiadizuron
SG	Steviol Glycosides

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ABSTRACT

India is a biodiversity hotspot with a variety of ecological zones. India is also known as the World's Medicinal Garden. It features a vast range of environmental zones including mountains in the Himalayas, sand in Rajasthan, and mountains and valleys in the north-east, all of which provide a diverse range of climatic conditions for India's rich flora. Plants are a fantastic source for discovering new items with medicinal use. Several different secondary metabolites generated from plants are now major medications that are used to treat severe diseases in several nations. These inexpensive pharmaceuticals are employed as medicines, flavoring agents, and food additives. Many secondary metabolites derived from plants such as taxol, artimisinin, and forscolin, are used to treat ailments such as cancer, malaria, and hypertension respectively.

Stevia rebaudiana (Family: *Asteraceae*) is one of the 154 members of the genus *Stevia* native to Paraguay, whose leaves have been used for centuries as a sweetener. *S. rebaudiana* is a perennial plant that exhibits the habit of both herbs and shrubs. The species *S. rebaudiana*, popularly described as sugar-leaf sweet-leaf and meethipatti. *Stevia* is also antimicrobial and anti-inflammatory in nature. *Stevia* leaf adds no calories and has no harmful side effects and is used as a non-caloric sweetener in several countries. *S. rebaudiana* is a storehouse of various other compounds apart from steviosides like rebaudiosides A and others. The plant has shown tremendous promise as a prime source of natural sweetener for the ever-expanding food industry. In recent years, micropropagation and other in vitro techniques have been used for plants which present particular problems in conventional horticulture and has been widely used to conserve vast genetic resources in crop plants. Tissue culture-mediated propagation of medicinally important plants has now become popular practice for sustainable utilization of many of these plants. *In vitro* propagation is the technique that accelerated mass propagation of *Stevia*. To increase the amount of steviol glycosides (SGs), elicitors and precursors can be used in tissue cultured *S. rebaudiana* plants.

To select and screen the elite variety, the assessment of phytochemical diversity associated with variation in the climatic factors and geographical location play a very significant role on secondary metabolite. In the present study, phytochemical variations in *S. rebaudiana* were observed which were related to the geographical location variation There are variations in the content of stevioside and rebaudioside A based on geographical locations. The highest contents (% dry wt) was found

from climatic zones of semi arid region like Punjab (5.8%) and Highland like Himachal Pradesh (5.2%). The populations of high amount of secondary has been observed in Highlands and Semi arid regions of India. These can be explored for the conservation and cultivation.

S. rebaudiana leaves contains non-caloric sweetening compounds as well as antioxidant compounds. Response surface methodology (RSM) has been employed successfully by solid-liquid reflux extraction method for the optimization of different extraction variables i.e temperature (X_1 : 30–70 °C), extraction time (X_2 : 30–60 min), solvent composition (X_3 : 35–70%), solvent-to-solid ratio (X_4 : 10–20 ml g^{-1}), particle size (X_5 : 0.6–1.2 mm) and extraction steps(1-3). A Plackett-Burman design has been used initially to screen out the three extraction factors i.e mean particle size, solid solvent ratio, and solvent composition on antioxidant activity and yield of Quercetin , chlorogenic acid (CA) and Gallic acid . The three extraction parameters i.e solvent:solid ratio (20:1), mean particle size (0.80 mm) and solvent composition (55% ethanol in ethanol–water mixture) can be considered as significant for better yield.

Tissue culture of *S. rebaudiana* is very useful for Industries. *In-vitro* culture of stevia from nodal explants with different combinations of growth regulators gives better results for *in- vitro* cultures. The effect of benzyl adenine (BA), kinetin (Kin), Indole-3-butyric acid (IBA), Thidiazuron (TDZ), Indole-3-acetic acid (IAA) and mT at various concentrations (0.5–2.5 mg L^{-1}) were compared on the basis of shoot induction, multiplication and root organogenesis. Superior shoot regeneration was attained on MS medium augmented with mT and BA. They can be used for the commercial production for high multiplication rate.

The plant tissue cultures of *S. rebaudiana* were elicited with MeJa, Chitosan and SA as elicitors and sucrose and GA3 as a precursors at different concentrations. The shoot cultures were tested by these elicitors and precursors and the experiment was performed and analysed by Response Surface Methodology. The increases in SGs content were promoted by chitosan, salicylic acid, methyl jasmonate and GA3. These showed the increase in the content of natural sweetners.

CHAPTER-1

INTRODUCTION

CHAPTER 1 : INTRODUCTION

1.1. Medicinal importance of plants

For many years, plants have been used to treat human ailments. Shamans or medicine men and women from Eurasia, America, and India have had a vast understanding of medicinal herbs since prehistoric times. Nearly 1800 medicinal plant species are commercially available in the United States (Muller and Clauson, 1998). They are also employed because of their cultural adaptability, improved bodily compatibility, and lack of adverse effects. They've stood the test of time in terms of safety and efficacy (Kamboj *et al.* 2000). Plants are a fantastic source for discovering new items with medicinal use. Several different secondary metabolites generated from plants are now major medications that are used to treat severe diseases in several nations. These inexpensive pharmaceuticals are employed as medicines, flavoring agents, and food additives. Many of the medications in the market today are simple synthetic adaptations or duplicates of naturally occurring molecules. Taxol, artimisinin, and forscolin, are used to treat ailments such as cancer, malaria, and hypertension respectively. India is a biodiversity hotspot with a variety of ecological zones including mountains in the Himalayas, sand in Rajasthan, and mountains and valleys in the north-east, all of which provide a diverse range of climatic conditions for India's rich flora.

1.2. Diabetes and obesity problem

Advancement and stressful life style were cause of serious diseases. Diabetes is one of the most frequent diseases seen all over the world (Kujur *et al.*, 2010). People who are overweight or obese have a substantially higher chance of getting type 2 diabetes than those who maintain a healthy weight. In India, the proportion of obese and overweight people increased from roughly 9% of the population in 1980 to 11% in 2008. Rural locations have a higher prevalence of malnutrition, but

metropolitan areas have a threefold increase in overweight and obesity. This could be attributed to the fact that metropolitan areas have lower levels of physical activity. Diabetes has become a pandemic in India. In India, there are 67 million verified diabetes patients and additional 30 million people with prediabetes (Kujur *et al* 2010). Diabetes type 2, often known as diabetes mellitus, is a category of metabolic illness due to insufficient insulin production in which a person has excessive blood glucose (blood sugar). In diabetes type 1 the body does not generate insulin. Type 1 diabetes accounts for about 10% of all diabetes cases, while type *2* diabetes accounts for 90% of all diabetes cases worldwide.

1.3. **Sweeteners used by Food Industries**

Sucrose and corn syrups were employed as sweetening agents in a variety of items in the culinary and medicinal industries. Excess sugar in the diet has been demonstrated to have negative impact on human health. Controlling blood glucose levels is now one of the most important aims of diabetes therapy. To follow the dietary guidelines, they must select the appropriate food. As they attempt to make healthier food choices, consumers are requesting a wider range of low-calorie items adds sweetness to a food. By producing changed food items, the food business can make a significant contribution to this transition. This has led to the development of zero-calorie and safe alternatives in the food industry. Sugar substitutes come in a variety of low-calorie or zero-calorie varieties, and they can be natural or manufactured. Food manufacturers are replacing high-calorie sugar with artificial and zero-calorie sweeteners. Artificial sweeteners, on the other hand, raise safety and toxological concerns.

1.4. Potential zero and Low calorie sweeteners

The necessity for a safer replacement inspired the food industry to seek out safe sweetener agents with no toxicological or safety concerns. There are many plant based primary and secondary metabolites which can substitute. Proteins including thaumatin (*Thaumatococcus danielli*), miraculin (*Richardella dulnifica*), and monellin (*Dioscoreophyllus cumminsii*) have been extracted from the fruits of West African plants and can be utilized as a substitute for high-calorie sugar in a variety of food. Terpenoids discovered in *Stevia rebaudiana* happen to be a strong natural sweetener source that can be utilized instead of sucrose.

1.5. Current status of zero calorie sweeteners in the world

As a result, these natural sweeteners are used as a health and dietary supplement worldwide aas they are zero calorie. Stevioside and rebidioside (*Stevia rebaudiana*), extracts of *Seratia grovenori* containing mogroside, are increasingly used in soft drinks and health drink shakes in the United States. Locals in Paraguay and Brazil utilize *Stevia rebaudiana* leaves as herbal supplements in their drinks and cuisine. Steviosides are produced from *Stevia rebaudiana* plants and are used in diet coke, diabetic and low-calorie eating diets. Japan is the major consumer of steviosides.

1.6. Need of sustainable production of High Potency (HP) zero calorie natural sweeteners

Consumer demand and developing markets have led to the development of cost-effective technologies of natural sweeteners on a global scale. Sugars are sweet but have adverse effcts on the body.As a result, we may successfully increase sweetening potential while lowering calorie content in foods and herbal goods. The concentration of natural sweeteners in plants is influenced by a variety of environmental and genetic factors. Because these chemicals are secondary metabolites, extrinsic and intrinsic factors influence their composition. There is a need for more

effective extraction of these chemicals from plants. For a better yield of photochemicals from plants, there are numerous extraction methods available, including traditional methods such as reflux and soxhlet, as well as newer approaches such as supercritical fluid and microwave aided extraction. Temperature, particle size, solvent, and pH all have an impact on the extraction effectiveness of these substances. We can identify the elite population of plants and cultivate them for mass production in addition to agronomic variables. *In vitro* propagation is already used to create a number of industrially essential plants. We can also use cell suspension culture with precursors and elicitors to increase production of natural sweeteners.

1.7. Biotechnological approach for production of Secondary Metabolites

By using different approachs and factors like medium composition ,salt,nitrogen,phosphate and other plant growth harmones ,we can enhance the metabolites in the plantlets of tissue culture.so by using biotechnological methods,we can achieve goal of commercial production.

1.8. Production of Secondary Metabolites

These secondary metabolites are also important for pharamacutical industries and so they are extracted from plants for the synthesis of drugs for curing of any disease.They show different properties like anti cancer,anti malaria and also act as antioxidants.Some can also be used in cosmetics for their fragrance.In the present scenario .Medicinal plants are important for different purposes to mankind since a very long time. It thus creates the challenge for both conservation of medicinal plants and their sustainable production. As our needs are increasing, we are consuming more of such plants. Here comes the role of Biotechnology, through which we can conserve, clone and increase the production of compounds by regulation at the genetic level. There are *in vitro* methods for plant production through which we can obtain various important secondary metabolites for industrial purpose.

1.9Tissue culture for production of Secondary metabolites.

In the present era , biotechnology is providing one of the best approaches which is plant tissue culture technique for better production of medicinal plants. It has been used widely for mass propagation of economically important plants, disease free plants, ornamental plants like orchids and for economically important plant that are rich in secondary metabolites (Altpeter *et al.*, 2016). Micropropagation helps in mass propagation of plants which depends on different factors like culture medium & its composition, conditions and plant growth hormone for its successful establishment. It produces a high number of identical plants that are disease free and have an advantage over traditional agriculture. There are many plants for which successful protocol of micropogation has been established like *Alpinia galangal, Cynara scolymus (Comino et al., 2019).* In micropropagation, the optimal nutrient condition is very important for production of bioactive compounds. There are some other methods though which secondary metabolite production can be enhanced like altering environmental conditions like light,alteration in culture medium and applications of elicitors in the medium. There are different types of elicitors like plant cell wall component (pectin) and plant resistant signaling component (methyl jasmonate).In plant cell cultures, simple sugars including glucose, fructose, maltose, and sucrose are frequently used as carbon sources to supply energy. The addition of a carbohydrate to the medium had a significant impact on biomass and metabolite synthesis. (Shatnawi *et al.* 2011, Shatnawi *et al.* 2011). Exogenous growth regulators i.e auxin to cytokinin ratio had a significant impact on biomass growth and product generation (Sridhar *et al.* 2014).

1.10 .Elicitor

When elicitors are induced ,then the metabolits accumulates and enhance the production.these can be of different types like biotic or abiotic .the accumulation may be affected by different

parameters like expouser time ,age of plants etc.we can also reduce the cost by regulating the pathways, and advanced bioprocess techniques.

1.11. Aim and Objectives of the research

The development of an effective method for *Stevia rebaudiana* in vitro cultures could open the way for the mass production of antioxidant compounds and steviol glycosides. to cultivate an elite *Stevia rebaudiana in-vitro* and filter the elite population of the plant based on metabolites that are secondary. In addition to that, development of biotechnological strategies for the better yields of natural sweeteners and antioxidants from the plants.

Hence, objective of this study was the cost-effective production of zero- calorie natural sweeteners which are in immense demand as they are now used in various food products as well as in pharmaceutical formulations. Optimization of factors such as Physical, Chemical, Media, Biotic and Abiotic stress *in vitro* culture condition influencing the secondary metabolites accumulation.

The following are objectives of my Research Proposal:

1. Collection of *Stevia rebaudiana* from different geographical locations to screen the elite variety of *Stevia rebaudiana* on the basis of steviol glycosides (Proportion of Steviosides and Rebediosides A) and antioxidant compounds.

2.Cost effective *in vitro* culture of *Stevia rebaudiana* by optimization of various cultural conditions.

3. Biotechnological Strategies for production of secondary metabolite by using biotic and abiotic elicitors and precursors.

CHAPTER-2

Review of literature

CHAPTER 2 : Review of literature

2.1 Introduction

With the advancing world and modernization, we have been introduced to new eating habits, foods that are heaven in taste but are the reason behind the diseases that are fatal in nature. Out of these diseases, diabetes diseases one of them around the globe (Genco et al. 2020). Obesity brings elevated risk of type 2 diabetes in comparison to the healthy individuals (Ortega et al. 2020). India, presently with 67 million confirmed diabetic cases and 30 million prediabetic patients, is facing diabetes almost as an epidemic. Diabetes mellitus (type 2 diabetes) presents an array of metabolic disorders. Diabetes II represents a number of metabolic disorders in which affected people exhibit high level of blood glucose or blood sugar, either due to insufficient insulin production or due to the unresponsiveness of the body cells to insulin, or both. In case of diabetes I though, the body does not produce insulin (Eizirik et al. 2020). The proportion of diabetes type I and type II is 9:1 (Divers et al. 2020). Sucrose, the natural sweetener from sugarcane and sugar beet, are the most widely used sweetener agents in various foods, health drinks, and pharmaceutical products. The food and pharmaceutical industry used sucrose and corn syrups as a sweetening agent in various products (Ali et al. 2021). It was found that excess sucrose in diet leads to adverse effects on the human health (Johnsonet al. 2020). Today one of the major goals of diabetes management is to control the blood glucose level. They must choose the right food to fulfil the dietary recommendations. Consumers are constantly looking for various low-calorie products owing to their healthier food preferences. A sugar substitute is considered as a food additive that enhances the sweetness without adding any extra calorie (Saharudin et al. 2020). The food industry can considerably contribute to this change by providing modified food products. This has led the industries to discover the alternatives which are zero calorie as well as safe. There are various

sugar substitutes which are of low calorie or zero calorie and are both natural as well as synthetic in origin. Food industries are using various artificial and zero calorie sweeteners, i.e., saccharine, cyclamate, aspartame, and acesulfame K in food products instead of high calorie sugar. But there are safety and toxicological issues regarding the use of artificial sweeteners (Plaza-Diaz et al. 2020).

There are many natural zero calorie or low calorie sweetening agents from plants which falls into a range of structures . Compounds such as thaumatin (*Thaumatococcus daniellii*), miraculin (*Richardella dulcifica*), monellin (*Dioscoreophyllum cumminsii*), and glycyrrhizin *(Glycyrrhiza glabra)* were isolated from plants and can be an alternative for high calorie sugar used in various food products .Terpenoids such as steviosides present in *Stevia rebaudiana* were found to be potent natural sweetener source and can be used as an alternative for the sucrose (Pradhan et al. 2020).

Thus, these natural sweeteners are gaining popularity worldwide, and besides using as a health and dietary supplements, they are also used as natural sweeteners. In the USA, stevioside and rebaudioside (from *S. rebaudiana*), they have introduced it in food products.. In Paraguay and Brazil, the local people use stevia leaves, liquid extracts, or crystal powder directly to their drinks and cook as herbal supplements. Japan is the largest consumer of steviosides extracted from stevia leaves and is used in diet coke, diabetic, and low calorie food diet (Gantait et al. 2015; Pandey et al. 2015).

Continuing consumer's interest and growing markets have led to develop methods for cost-effective production, product improvement, and quality control of natural sweeteners (Anestis 2012). we can effectively multiply sweetening potential while minimizing the calorie in the food and herbal products. There are many environmental and genetic factors which influence the content of natural sweeteners in plants. As these compounds are secondary metabolites, factor like

climate, temperature and moisture content, and age of plants influences the content of these compounds in plants (Evans 2014). There is need of better extraction efficacy of these compounds from the plants. There are many extraction methods both conventional, i.e., reflux and soxhlet, and modern methods like supercritical fluid, microwave-assisted extraction for better yield of photochemical from plants. Other factors like temperature, particle size, solvent, and pH also influences the extraction efficacy of these compounds (Lemus-Mondaca et al. 2016).

2.2 .Taxonomy and botanical description

Stevia rebaudiana (Family: *Asteraceae*) is from genus *Stevia* endemic to Paraguay and used as sweetener. *S. rebaudiana* is a perennial plant that exhibits the habit of both herbs and shrubs. The plant, when being cultivated, may attain a height up to 1 m or even more. It shows very extensive root system and brittle stems with elliptic, elongated, small, and sessile leaves that are 3–4 cm long, oppositely arranged lanceolate to oblanceolate in shape possessing serrated margin and blunt-tipped lamina. Slight glandular pubescence is noticed on the leaf upper-surface. The woody stem is weak, and at the bottom, it is pubescent. Slightly branched root systems are found in the rhizome. On the leaves two types of trichome are present. The flowers are tiny, light purple, perfect, pentamerous, and composite in small corymbs of 2–6 florets surrounded by an involucre of epicalyx. Corymbs are present in loose panicles. According to some researchers, *Stevia* exhibits self-incompatibility and is pollinated by the insects (Frederico et al. 1996; Skaria et al. 2004; Gantait et al. 2015).

2.3. Distribution and ecology

It belongs to sub-tropical America (Paraguay and Brazil) (Gantait et al. 2015) and area of different altitude ranging from 500m to 3500m and − 6 to + 43 °C temperature. The species *S. rebaudiana*, popularly described as sugar leaf, sweet leaf, and meethipatti, is grown in the wild and is also cultivated for its sweet leaves. *S. rebaudiana* possesses the sweetest essence. Since time immemorial, it has been employed as a tea sweetener, and South America's Guarani Indians have been found to use it (Singh et al. 2014a, b, c). Figure 1 shows worldwide spread of *S. rebaudiana*

Fig. 1

Worldwide distribution of *S. rebaudiana*

2.4.Phytochemistry and pharmacology

The leaves of *S. rebaudiana* contain diterpenoid glycosides viz. stevioside, steviolbioside, dulcoside A, and rebaudiosides A–F owing to its typical sweetening properties. These steviol glycosides are low caloric, non-toxic, and non-mutagenic. Several diterpene glycosides have been isolated from *Stevia rebaudiana* and named including steviosides, steviobiosides, dulcoside A and

rebaudiosides A, B, C, D, E, and F. Besides steviol glycosides, labdene diterpene like sterebins, triterpenoids, sterols, alkaloids, and flavonoids are some of the non-sweet secondary metabolites present in the leaves of *S. rebaudiana* (Chatsudthipong and Muanprasat 2009; Gantait et al. 2015).

Apart from being a natural sweetener, *S. rebaudiana* is also considered to be of great therapeutic value (Kinghorn and Soejarto 2002). *Stevia* has the potential to boost the immune system and prevent free radical-mediated diseases and distinctly possesses good antioxidant activity. Additionally, stevia has antibacterial and anti-inflammatory properties (Pandey et al. 2015). 100–400 times sweeter than glucose are these isolated diterpene glycosides.One teaspoon of the liquid's sweetness is equal to one cup of sugar. Unlike sugar, *Stevia* leaf adds no calories and has no harmful side effects and is used as a non-caloric sweetener in several countries. *S. rebaudiana* is a storehouse of various other compounds like steviol, isosteviol, and dulcoside A, as well as rebaudiosides A, B, C, D, and E, stevioside, along with other compounds, offers various therapeutic advantages. *Stevia* leaves are known to exhibit functional and sensory attributes better than a number of other highly proclaimed sweeteners (Goyal et al. 2010). The plant has shown tremendous promise as a prime source of natural sweetener for the ever-expanding food industry (Gantait et al. 2015). Besides, a number of pharmacological attributes are presented by various compounds such as anti-diabetic (stevioside and rebaudioside A); hypoglycemic, antioxidant, anticancer, and antibacterial; and anti-hyperglycemic and antihypertensive (chlorogenic acid) (Moraes et al. 2013).

2.5. Biosynthesis of steviol glycosides

The biosynthetic pathways of the diterpenoid steviol glycosides possess different common steps in the biosynthesis of gibberellic acid. *S. rebaudiana* produces eight glycosides, which are all

variations of the diterpene steviol tetracyclic. The sixteen different step-by-step enzyme-catalyzed systems comprise the majority of the SG biosynthesis pathway.

2.6 Extraction

The major aim in the process of extraction and purification of steviol glycosides was to develop a cost-effective and simple isolation process via minimizing unit operations and additional taste enhancement. Off late, many strategies have been adopted in plant material extraction. Despite the universal use of water as a potent extracting solvent in many traditional protocols, to exploit the varied solubility of active principles from plants, organic solvents of various polarities are commonly preferred in modern extraction protocols. There are two types of extraction i.e conventional eg microwave,ultrasound and aquous two phase system.these conventional methods include natural compounds derived from microbes followed by purification methods like ion exchange techniques.

Despite the significant progress in extraction as well as purification of natural compounds, it is yet a heavily demanding job to isolate water soluble phytochemicals devoid of impurities. The common methods of stevioside extraction from *S. rebaudiana* leaves represent varied traditional physical and chemical processes including prolonged procedural stages during the steps of isolation and ion exchange purification, thus producing toxic impurities and residues in the end product, interfering with the taste and quality of the sweet glycosides. Efforts have been made to refine the sweetening quality of steviosides with the addition of natural sweeteners like glucose, sucrose, or fructose via glycosylation of the steviosides or via employing UF/NF membrane separation or supercritical fluid extraction to achieve superior steviosides with better taste, purity, and quality (Li and Chase 2010).

2.7. Solvents used for extraction of steviosides

There are many solvens used for th purpose of extraction like chloroform,methanol,and microwave extraction.so for the selection of solvents are also important in the extraction process.but there are also few consideration also to take care like use of minimum organic solvent.

2.8. Methods for mass production of *S. rebaudiana*

In recent years, micropropagation and other in vitro techniques have been used for plants has been widely used to conserve vast genetic resources in crop plants, especially in vegetatively propagated ones or in case of recalcitrant seeds or the germplasms that cannot be kept in standard conditions maintained in the seed banks. In vitro culture has been widely used for propagation of agricultural and horticultural as well as medicinally important crops. Tissue culture-mediated propagation of several medicinally important plants has now become popular practice for sustainable utilization of many of these plants. The propagation by tissue culture methods can be classified into firstly conventional and secondly alternative in vitro culture or temporary immersion culture.

2.9. Problem associated with conventional method of propagation of *S. rebaudiana*

In the conventional methods of propagation in *stevia* faces various problems like very low germaination rate.the seed are very small,so it create problems in the germination resulting in less growth of plants.

Because of these constraints, plant cell, tissue, and organ culture are the only substitute for fast and large-scale mass multiplication of *Stevia*. The much reduced germination rate of *Stevia* seeds limited its application for expansive cultivation. In contrast the propogation by seeds generate less homogenous population to produce variation in sweetioning level.The propogation by cutting also produces limited number of plants.

2.10. In vitro propagation

Since,the conventional method is not very effective and there is variation in the plantlet growth and very less germination of seeds resulting in the low growth of plants.Another challenge is the longer duration of the establishment of plants .To overcome these challenge plant tissue culture is the best alternative .It solve both problems of uniformity and the less duration to produce the plants.

The different parameters like culture conditions,variation in medium may give best results in terms of growth.

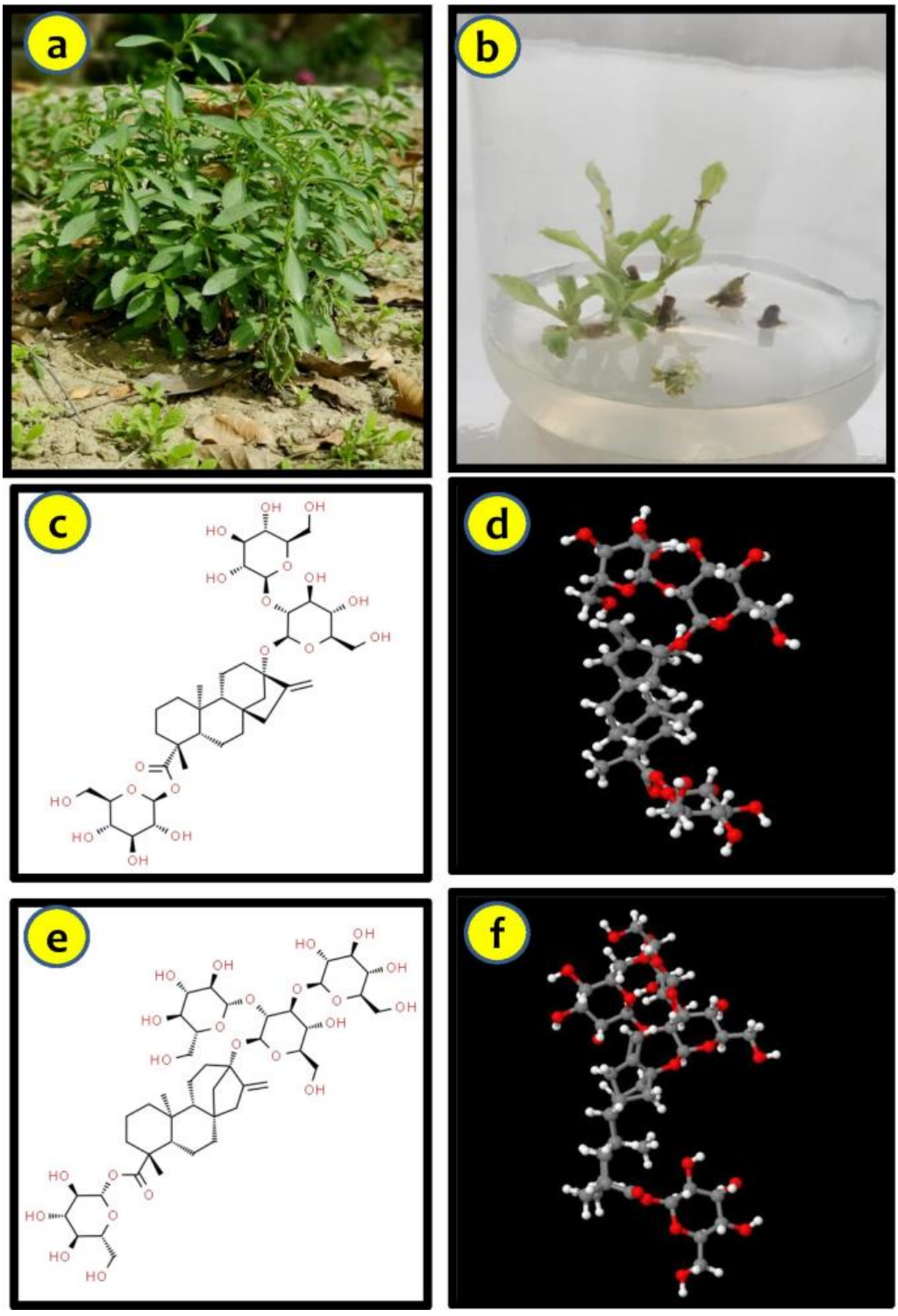


Fig. 2 a Habit of *S. rebaudiana*; **b** Tissue culture regenerated shoots of *Stevia rebaudiana*.;**c,d**-2D and 3D structure of Stevioside ;**e,f**-2D and 3D structure if rebaudioside A respectively.

2.11. Shoot multiplication

In vitro propagation in *Stevia* has been performed from the explants like leaf, nodal, shoot apex, rooting of *Stevia* cuttings ex vitro, and suspension cultures (Ali et al. 2021; Singh and Yadav 2013; Thiyagarajan and Venkatachalam 2012). However, the main constraints observed were generation of fewer shoots and frequency of shoot proliferation. Therefore, in vitro cultures present an exciting alternative technology to raise adequate number of plants in very short span of time.

Nodal parts are considered as excellent explants for micropropagation for having their potent ability for high frequency shoot induction (Singh et al. 2014a, b, c; Thiyagarajan and Venkatachalam 2012; Yücesan et al. 2016). Interestingly, MS medium not supplemented with any plant growth regulator (PGR) was recorded to be extremely potent for direct micropropagation. Enhancing the BAP level from 0.5 to 2 mg L^{-1} resulted in a decrease in shoot length from 2 to 1.3 cm. KIN was noted as more efficacious than BAP in providing better response in shooting devoid of callus generation. Likewise, an enhancement in BAP from 0.5 to 3 mg L^{-1} resulted in a reduced shoot-generation percentage from 85 to 50% and also the shoot quantity/explant (from 2 to 1.25) (Thiyagarajan and Venkatachalam 2012). However, few works have depicted the positive role of higher BAP levels in the production of shoots from various *S. rebaudiana* explants. MS basal media without PGR was found to be more cost-effective in this regard compared to the MS media fortified with PGRs. In a nutshell, it is evident that optimal level and composition of PGRs play a crucial role in shoot induction and multiplication in this plant which in turn may depend on the explant source, cultivation time, genotypes, and so on.

Since callus-mediated shoot organogenesis requires extensive time and efforts for subculturing callus-derived microshoots, it is not directly applied for industry level propagation of medicinal plants.

2.12. Roots

The findings demonstrated faster root proliferation, and the roots were found to be longer in IAA (0.5 mg L^{-1}) compared to IBA. Few experiments demonstrated that the highest quantity of shoot buds was achieved in BAP (0.4 mg/l) and IAA (0.5 mg/l). In another record, the highest shoot formation was reported in MS media fortified with KIN (0.3 mg /l). In addition, the highest number of roots was achieved in MS media fortified with IBA (2 mg L^{-1}). In most of the experiments, the influence of different PGRs were studied separately in two-steps for shoot- or root-production in *Stevia*. Moreover, some studies evaluated the growth of *Stevia* following 5–6 weeks of culture. However, these long intervals along with the generated weak compact shoots were not appropriate for the acclimatization of the plantlets. The diterpenoids, similar to many secondary metabolites, are synthesized in *S. rebaudiana* leaves. However, difficulty in the process of rhizogenesis in this plant is one crucial shortcoming during in vitro regeneration of this plant (Ahmad et al. 2011).

2.13. Somatic embryogenesis and synthetic seed production

Stevia seeds exhibit much reduced level of germination rate and clonal propagation is restricted by fewer quantity of individual plants. Somatic embryogenesis is a strategy in which somatic cells are introduced to produce embryos. The process has been carried out directly using stem and leaf explants and also indirectly from zygotic-embryo-induced callus. Synthetic seed technology or Synseed technology is considered an upgradation in the micropropagation where encapsulation of the somatic embryo or the germplasm is used as an aid to the in vitro conservation strategy for the endangered plants especially those producing non-viable seeds and are hard to propagate by alternative methods. Synthetic seeds also offer genetically identical true-to-type germplasm which is virus-free, easily transportable, can be stored for longer duration, and cost-effective. Table 4

represents the various media constituents and factors responsible for somatic embryogenesis and synthetic seed formation in *S. rebaudiana*.

2.14. Genetic fidelity studies in *S. rebaudiana*

Presently molecular biological tools represent valuable and significant strategies to analyze genetic fidelity of micropropagated plants. These tools are not impacted by environmental conditions and produce authentic and reproducible outcomes. It has been observed that PCR-based methods like RAPD, ISSR, and AFLP are very effective at demonstrating the genetic stability of cultivated and in vitro produced plants. By adopting a combinatorial method using biochemical and molecular markers, Das et al. (2011) published one of the first studies evaluating in vitro increased variability in Stevia. The ISSR banding pattern and peroxidase profile suggested that the regenerated plants should be uniformly clonal. Later Among all the markers, ISSR has been highly preferred and have been successfully employed in the detection of genetic relatedness or variations in in vitro raised plants. ISSR markers are better suited for analysis of genetic variations owing to their ability to amplify various genomic regions. In another record, ISSR banding profile in somaclonal and transgenic plants exhibited higher polymorphic (56.64%) than monomorphic (43.36%) bands (Khan et al. 2014). In addition, confirmation of genetic fidelity in micropropagated *Stevia* was carried out ISSR profiling in which callus-derived in vitro plantlets demonstrated genetic polymorphisms (Singh et al. 2014a, b, c).. Here, the ISSR profiling from the micropropagated plantlets demonstrated monomorphic bands like the mother plants thus confirming the clonal fidelity.

2.15. Development of transgenic *S. rebaudiana*

Presently, many novel strategies are employed to incorporate transgenes into plant-genome in order to raise transgenic-plants. The various types of methods that are primarily used for genetic transformation of plants are mechanical, chemical, and electrical. These techniques deal with particular tools, viz. particle bombardment or gene gun, electroporation, microinjection, and *Agrobacterium*-mediated transformation. These toolkits are commonly accepted and employed that are multifaceted and safe but are considerably lesser efficacious than the viruses. However, even with the recent advancements in this field, very little work has been done in *Stevia*. Producing hairy roots in *S. rebaudiana* is implicated to the industrial-level yield of the valuable metabolites in bioreactors. Cultures of untransformed hairy roots were produced on semi-solid media fortified with high NAA levels, while transformed hairy root cultures were obtained from nodules and incision zone of wounded leaf-parts co-cultivated with *Agrobacterium rhizogenes*. Transformation efficacy was found to be dependent on the bacterial strain, used plant part, and the *S. rebaudiana* clone.

After thirty days of culture ,liquid media showed 1.8 time fast growth on hairy root culture of stevia. (Khan et al. 2014).

2.16. Tissue cultured production of metabolites in *S. rebaudiana*

Toenhance the procuction of metabolites , a variety of microorganisms can be utilised. Mamta et al. (2010) inoculated *Stevia* plants with PSB which induce the production of steviosides and rebaudiosides. Wu et al. (2013) studied the effect of purple bacteria in improvement of steviosides in *Stevia*. Considering their in vitro production, generally liquid cultures are established from the solid cultures using the same culture medium devoid of the gelling agent with some variations. An

enhancement in vitamin level, fortification by MES, glutamine, or ascorbic acid is the major alternatives reported. Off late, researchers have concentrated on obtaining cell cultures showing superior growth and high production of steviol glycosides (SGs). Table 5 represents the varied strategies that have been adopted to facilitate the *in vitro* production of SGs.

However, these ventures are still to get the much needed commercial profit. In the process of scaling up, yield and maintenance of cell biomass and the production of valuable phytochemicals are two major considerations needed to be optimized. A multi-staged protocol involving raising of culture, modulation of media constituents, and addition of appropriate PGRs for morphogenic response, acclimatization or hardening into ex vitro conditions etc). Cell-suspension cultures, however, were not recorded as a profitable strategy for SG accumulation without the interventions of fermentation tools. Earlier, temperature and sunlight were found to influence SG production during various growth seasons. In another study, long day ($\geq$ 14 h) photoperiod enhanced the period of vegetative-growth in *S. rebaudiana* owing to the higher leaf-biomass and superior SG production.

2.17. Feeding and elicitation strategies in *S. rebaudiana*

The prime factors influencing plant biomass production and metabolite accumulation *in vitro* is the supply of sucrose, since the primary carbon and energy during cell metabolic processes is provided by the carbohydrates. Many researchers have discovered that the role of different levels of sucrose in liquid medium subjected to stirring conditions on the dry as well as fresh shoot-biomass production in *Stevia* (Ahmad et al. 2021). However, studies describing the feeding strategies-mediated modulation of SG and antioxidant production in in vitro raised *Stevia* were also attempted (Pandey et al. 2015; Shahnawaz et al. 2018).

Jasmonic acid, salicylates, heavy metals, inorganic salts, fungi, and fungal spores are among the many such elicitors used to upscale the plant secondary metabolite yield. Earlier, in order to enhance the biomass and stevioside production, chlorocholine chloride (CCC), a plant growth retardant, was used in a developed micropropagation strategy. There was an enhancement (1.4 fold) in stevioside production in MS medium supplemented with IBA (3 mg L^{-1}) and CCC (3 mg L^{-1}) (2.9 mgg^{-1} fresh weight) compared to the basal MS media containing only CCC (3 mg l^{-1}) (2.1 mgg^{-1} fresh weight) (Dey et al. 2013a, b). In another investigation, the influence of 4 compounds, viz. polyethylene glycol, proline, Na_2CO_3, and NaCl on growth and SG accumulation in *S. rebaudiana* in vitro raised shoots, was observed.

Kim et al 2015 studied the role of pgr like $Na2CO_3$, mM proline, NaCl and PEG.They were induced in different concentrations .They showed that at higher conc there is decrease in content of metabolites.so it has to be taken care of higher doses of the PGR at some extent. (Kim et al. 2015).

The role of photoperiod was studied by Ceunen et al 2012.the plants were grown at different photoperiod for 8 and 26 hrs.In an other study,SA,BAP CaCl2 and H2O2 were treatd to observe the chilling stress in the plants.this can be used for quality and quantity maintenance of stevia.

The impact of photoperiodism on the synthesis of steviol glycosides was noted by Ceunen et al. (2012). First, plants with topped growth were allowed to sprout new branches every 8 or 16 hours of photoperiod. A long day increased vegetative growth, leaf biomass, and total steviol glycosides, according to an analysis of nine steviol glycosides at different ontogenic stages. Another study reported that chilling stress, in conjunction with pre-exposure to endogenous signaling molecules such as salicylic acid, BAP, CaCl2, and H2O2, plays a stimulatory role in imposing chilling tolerance on the plant. This approach was recommended as a suitable means of preserving both the qualitative and quantitative aspects of steviol glycosides.

2.18. Use of bioreactors

Although cell cultures are reported to produce an array of plant secondary metabolites, but the sufficiency in their level has not always been satisfactorily. The production of such compounds can further be enhanced using precursor feeding, biotic, and abiotic elicitation. Knowing the behavior of the cells in a scale-up protocol is considered as very crucial. Prime constraints associated with the scale-up are DO, gas composition, and shear stress. ‘

The plant cells are more efficient to tolerate the stress in comparison to microbial cell.Some reactors facilitates the emvirenment like airlift and bubble column reactor.Using roller bioreactor has also escalated shoot growth as well as SG production in *Stevia* by around 1.5/2 times superior to the similar methods where shoots are cultured in culture tubes. In one study, composition of the nutrient media was found to impact significantly the shoot growth and SG yield in roller-bioreactor. It was noted that shoot extension and root development were much superior in the media containing glucose or fructose compared to the sucrose-fortified media. However, such conditions reduced the leaf dry mass and SG accumulation. Liquid media was found to be very appropriate to optimize SG production in vitro due to the manageability of the scale-up process, increased nutrient uptake, etc. Moreover, the less SG production in liquid media can be negated via employing elicitors in the culture media.

Conclusion

To increase the quantity of metabolites there are many methods like conventional and by biotechnology methods.There are different techniques like root culture ,shoot culture,clonal propagation and others.These methods can be utilized for the production of metabolites from stevia.Plant tissue culture plays an important role in the production of important compounds used by industry.

CHAPTER-3

CHAPTER : 3

Collection of *Stevia rebaudiana* from different geographical locations to screen the elite variety of *Stevia rebaudiana* on the basis of steviol glycosides

Response surface methodology for optimization of extraction conditions and analysis of Antioxidant compounds.

Stevia (*Stevia rebaudiana* Bertoni: Asteraceae family) is native to the Paraguay region of South America. *S. rebaudiana* leaves consists of SG (Fig.3.1), namely stevioside and rebaudioside A, both of which are diterpenoid glycosides (Hanson et al., 1993). Among the additional components are antioxidants rebaudioside B, C, D, E, and F. (Starratt et al., 2002). *Stevia* leaves can also be utilized as sweeteners, and they have the potential to become a major supply of high-potency sweeteners in the future for the increasing natural food sector (Kahrizi et al., 2017). Diabetes mellitus (Ghaheri et al., 2018; Shivanna et al., 2013), obesity, and other disorders have all been treated using *Stevia* (Rojas et al., 2018). *Stevia* leaves also have antioxidant (Bender et al., 2015), antihypertensive, anti-inflammatory and antihyperglycemic (Bender et al., 2015) properties (Chatsudthipong and Muanprasat, 2009).

Natural sweeteners are economically valuable substances, so proper testing procedures are essential. For the separation and quantification of SG, a variety of analytical methods have been reported, including Orbitrap mass spectrometry (Gardana et al., 2018), HPLC (Kolb et al., 2001), LC-QTOF analysis (Molina-Calle et al., 2017), and High-performance thin layer chromatography (HPTLC) (Rouhani et al., 2019; Morlock et al., 2014). Phytochemical and genetic diversity are important factor.Many researchers have studied about phytochemical and genetic variations in different medicinal plants like *Swertia* sp. (Kaur et al., 2019 a, b, c), *Glycyrrhiza glabra* (Esmaeili et al., 2019), *Gymnema sylvestre* , poppy (Qaderi et al., 2019), *Hedychium coronarium* .

Similarly in *Stevia*, many researchers have studied genetic diversity using RAPD marker (Chester et al., 2013), phytochemical analysis using HPTLC method (Jaitak et al., 2008, Chester et al., 2012, Saifi et al., 2012). Due to its high importance, it is important to study phytochemical diversity of *Stevia rebaudiana*. Due to high demand of natural sweeteners and antioxidant compounds, it is necessary for cultivation and domestication of the plant (Akbari et al., 2017; Ghorbani et al., 2017).

To obtain the elite variety for the cultivation process, it is important to know about phytochemical diversity of *Stevia rebaudiana* from different geographical locations of India. Therefore, in this study, we studied about the phytochemical diversity in Northwestern, central to eastern part of India.

2. Material and Methods

3.2.1 Plant Material

The plant materials of *Stevia rebaudiana* were collected from nine states i.e., Punjab, Jammu, Himachal Pradesh, Uttarakhand, Haryana, Delhi, Uttar Pradesh, Bihar and West Bengal of India (Fig 2). All plant samples collected were in vegetative stage from 32 locations (three individuals from each location) during January –August 2018. The detailed characteristics of the collection sites belong to different climatic zones of India are presented in (Table 3.1). The geographical location were recorded by Global positioning system having latitude, longitude, and altitude (Table 3.1). Voucher specimens of the samples were kept at the herbarium of Lovely Professional University, Punjab.

Table 3.1:Collection of *Stevia rebaudiana* from different geographical locations

Sr. no.	Accession	Agro-climatic zones	States	Place	Altitude (m)	Latitude and Latitude
1.	SrP1	Semi-arid	Punjab	Jalandhar	228 m	31.355778, 75.546944
2.	SrP2	Semi-arid	Punjab	Phagwara	234 m	31.188753, 75.772642
3.	SrP3	Semi-arid	Punjab	Kapurthala	225 m	31.381250, 75.387861
4.	SrP 4	Semi-arid	Punjab	Nawanshahr	355 m	31.138139, 76.105222
5.	SrP 5	Semi-arid	Punjab	Banga	237 m	31.187719, 75.982694
6.	SrP6	Semi-arid	Punjab	Hoshiarpur	296 m	31.530032, 75.933533
7.	SrP7	Semi-arid	Punjab	Ludhiana	262 m	30.899095, 75.842284
8.	SrP8	Semi-arid	Punjab	Amritsar	234m	31.654107, 74.856534

9.	SrP9	Semi-arid	Punjab	Patiala	351 m	30.341829, 76.392417
10.	SrJ10	Highland	J and K	Jammu	327 m	32.689060, 74.877558
11.	SrH11	Highland	Himachal Pradesh	Palampur	1472 m	32.111991, 76.535230
12.	SrH12	Highland	Himachal Pradesh	Kangra	733 m	32.122758, 76.288278
13.	SrH13	Highland	Himachal Pradesh	Solan	1,502 m	30.907046, 77.179633
14.	SrU14	Highland	Uttarakhand	Dehradun	447 m	30.335139, 78.010961
15.	SrP15	SA	Punjab	Chandigarh	321 m	30.719550, 76.823467
16.	SrH16	SA	Haryana	Hisar	215 m	29.147465, 75.757456
17.	SrH17	SA	Haryana	Kurukshetra	260 m	29.947959, 76.834024
18.	SrH18	SA	Haryana	Karnal	252 m	29.678879, 77.025219
19.	SrH19	SA	Haryana	Sonipat	224 m	28.973211, 77.044841
20.	SrD20	SA	Delhi	Delhi	225 m	28.604050, 77.191461
21.	SrU21	Humid subtropical	Uttar Pradesh	Moradabad	198 m	28.903200, 78.658898
22.	SrU22	Humid subtropical	Uttar Pradesh	Allahabad	98 m	25.433554, 81.803664
23.	SrU23	Humid subtropical	Uttar Pradesh	Lucknow	123 m	26.864394, 80.874536
24.	SrU24	Humid subtropical	Uttar Pradesh	Alamnagar	123 m	26.873926, 80.867058
25.	SrU25	Humid subtropical	Uttar Pradesh	Raibareli	123 m	26.212056, 81.249929
26.	SrU26	Humid subtropical	Uttar Pradesh	Varanasi	81 m	25.363648, 82.854467
27.	SrU27	Humid subtropical	Bihar	Siwan	72 m	26.232398, 84.378674

28.	SrU28	Humid subtropical	Bihar	Patna	53m	25.605135, 85.144775
29.	SrW29	Tropical wet & dry	West Bengal	Asansol	111 m	23.678898, 86.978709
30.	SrW30	Tropical wet & dry	West Bengal	Kolkata	9.14 m	22.479375, 88.304777
31.	SrW31	Tropical wet & dry	West Bengal	Kestopur	9.14 m	22.591667, 88.409252
32.	SrW32	Tropical wet & dry	West Bengal	Bardhaman	30 m	23.252043, 87.856122

SA-Semi Arid

3.3. Chemical and Reagents

Stevioside (Fig 1A) and rebaudioside A (Fig 1B), analytical reference compounds with a purity of 98 %, were purchased from Sigma business in Bangalore, India. All chemicals used in the experiment, such as ethanol, acetone, toluene, ethyl acetate, sulphuric acid, and others.Loba Chemicals provided the methanol. In the experiment, HPTLC plates (20cm X 10cm) (E. Merck, Darmastadt, Germany) were used.

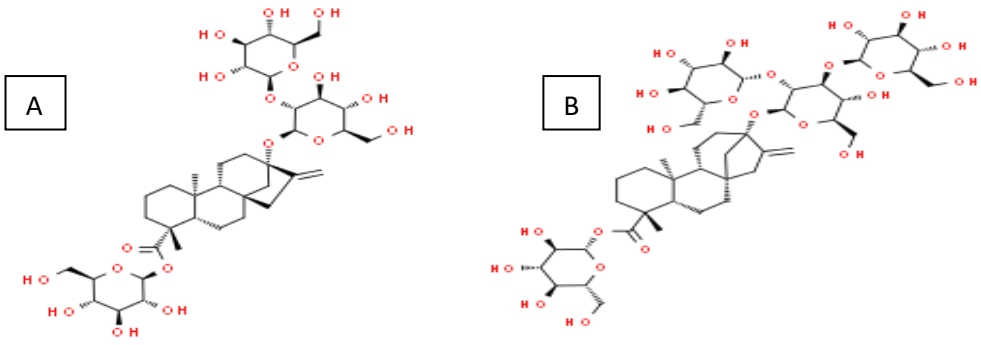


Figure 3.1: Chemical strucures of (A) Stevioside B) Rebaudioside A

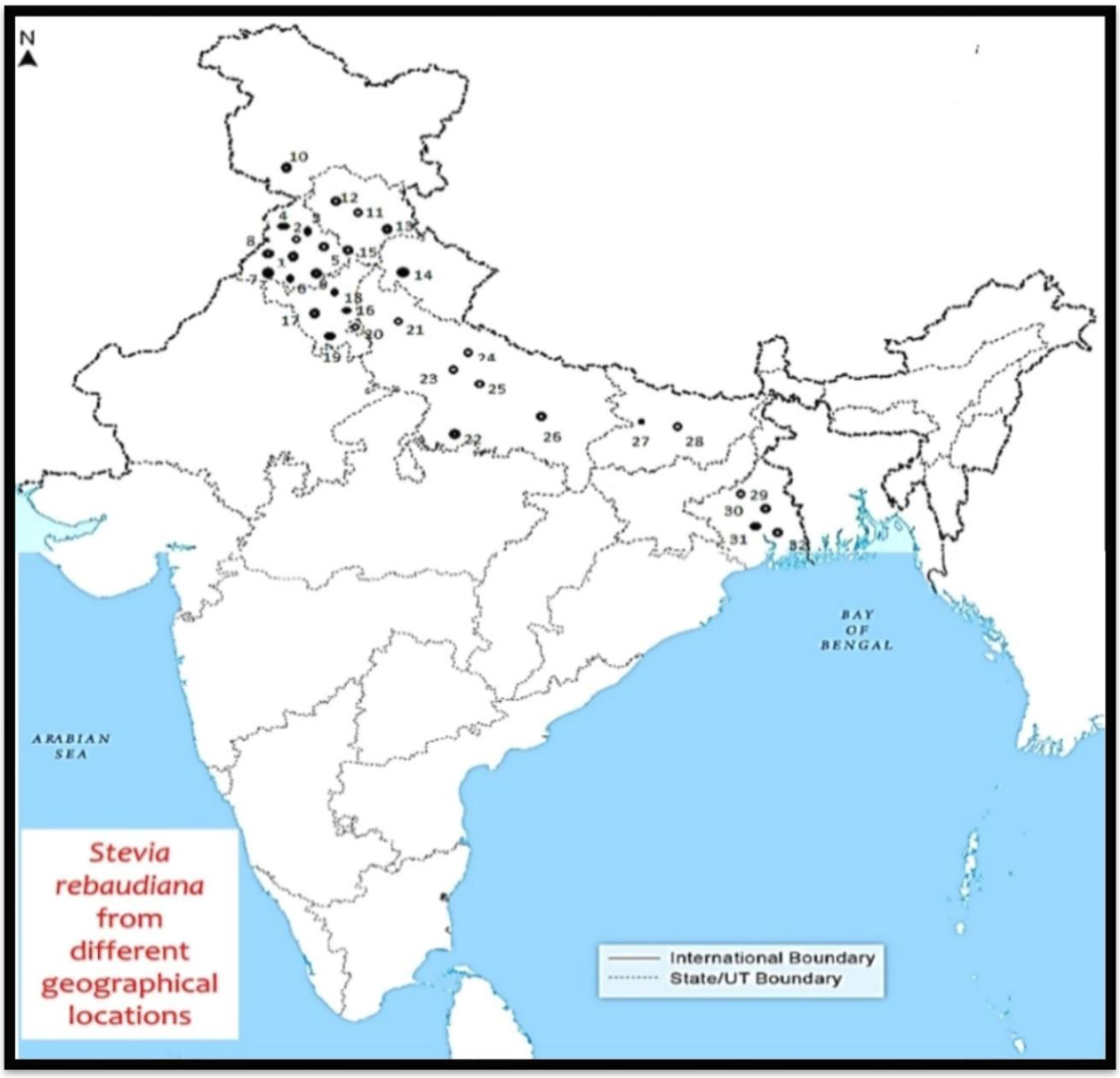


Figure 3.2: Collection of plants from different geographical locations.

(Locations for Collection:1- Jalandhar,2- Phagwara,3- Kapurthala,4- Nawanshahr,5- Banga,6- Hoshiarpur,7- Ludhiana,8- Amritsar,9- Patiala,10- Jammu,11- Palampur,12- Kangra,13- Solan,14- Dehradun,15- Chandigarh,16- Hisar,17-Kurukshetra,18- Karnal,19- Sonipat,20- Delhi,21- Moradabad,22- Allahabad,23- Lucknow,24- Alamnagar,25- Raibareli ,26- Varanasi,27-Siwan,28- Patna,29- Asansol,30- Kolkata,31- Kestopur,32-Bardhaman.).

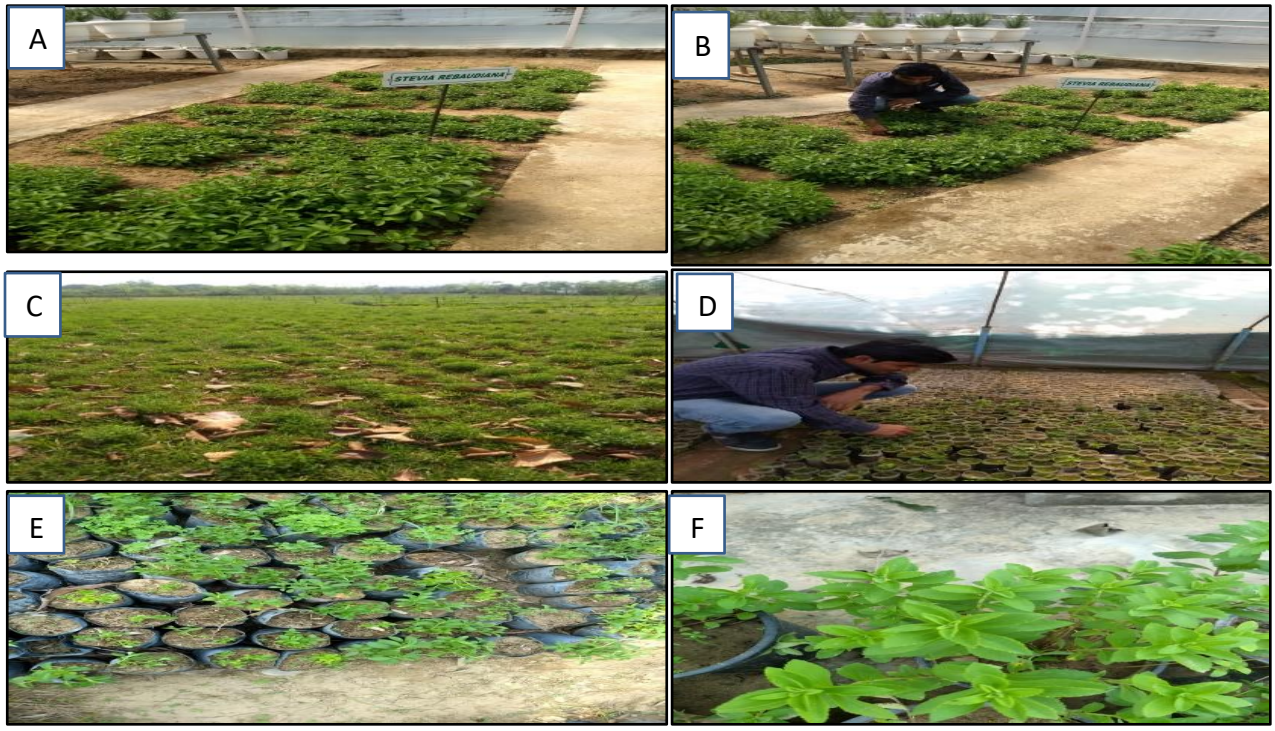


Figure 3.3: Collection of plants from different geographical locations:A, B:Palampur,C:Dehradun:D,Jalandhar E:Varanasi F:Nawashar

3.3.1. Preparation of standard solutions and extracts

Plants with fresh matured green leaves were collected, washed and dried in the shade for ten days. Using a food processor, dried leaves were finely pulverized. Microwave assisted extraction (MAE) was used to extract powdered *Stevia rebaudiana* plant material by following Ameer et al (2017) method. Microwave assisted extraction was processed in closed vessel units of 150ml containing 1g of fine powdered plant material in 100ml of ethanol (75%). MAE was done by using 160W microwave power for 4 minutes of time. The extracts were filtered by filter paper and dried in rotatory evaporator.final conc of 1mg/ml is made through dissolving ten mg in ten ml of methanol/the prepared samples were kept at 4 degree Celcius for HPTLC Analysis.

3.3.2.Chromatographic conditions

Samples were analyzed with HPTLC by Using a Linomat5 applicator (150 nL/s dosing speed) equipped with a 100 mL Hamilton syringe, 5 mL of each plant samples were applied on a pre-coated silica gel 60 F254 HPTLC plate. The plates were kept in the mobile phase (water-etanol-ethyl acetate:12-20-80(v/v).Anisaldehyde sulphuric acid is used for 10 s for post derivatization. The plate was then heated for 5 minutes and exposed at 530 nm to obtain bands.

3.3.3. Preparation of Standard curve

Thr standard stock solution was prepared in range of 12,10,8,6,4,2 microliter to get linerarity range 2-12 microliter and the standard was prepared by preparing the stock solutions in 0.1mg per ml.the area of peek and the equation of regression is used to calculate the yield.

3.3.4. Validation of method

The parameters like LOD,LOQ,specificity,repeatability ,peak purity and recovery percentage was used for the method of validation.(Table 2).

3.3.5.Precision

The instruments was checked by spotting ST 400ng/spot and Reb A 600ng/spot with n=6.the methods intraday repeatability and interday reprocubility were tested on same day using reference compound at 3 different concentrations and after 3 day also.these were repeated seven times .

3.3.6. Specificity

The peak area,and similarity to the reference chemical compound was compared . The well resolved separated marker compounds were compared with the overlay spectra and Rf of the separated bands of the plant samples.

3.3.7.Statistical analysis

Each experiment consisted of ten samples and three replicates, which were repeated three times in total. Fisher's least significant difference test was used to assess the significance of mean differences after the mean values were computed as mean SE. The results are presented as mean SE after the calculations were performed three times using mean values and standard error (Pagano et al., 2000).

3.4. Results

3.4.1. HPTLC quantification of ST and Reb A for screening of elite chemotypes

Documentation of phytochemical diversity may provide very useful information for the identification of useful accessions which could be used as cultivars and for industrial purpose. In the present study, the extracts of *Stevia rebaudiana* samples collected from different geographical

locations were used to quantify biomarker compounds by using HPTLC method. The two biomarker compounds stevioside and rebaudioside A has been quantified in all samples of *Stevia* (Table 3.3).

The accessions from Punjab and Himachal Pradesh were grouped together having highest stevioside (5.8 %) and rebaudioside A (1.9 %) content in Punjab region followed by Himachal (5.2 %) and (1.8 %). The lowest amount of ST and REB A content was found in the samples from West Bengal region. Similar results have been found in which the reports shows that the content of stevioside and rebaudioside A is high in the highland regions (Jaitak et al., 2008).

There are variations in the content of stevioside and rebaudioside A based on geographical locations. The highest ST and REB A contents were found from the climatic zones of semi-arid region like Punjab (5.88 % and 1.95 % respectively) and highland like Himachal Pradesh (5.28 % and 1.89 %). In humid subtropical region like Uttar Pradesh, the contents of ST and REB A were intermediate and the lowest content was from tropical wet and dry region like West Bengal. The HPTLC fingerprints are shown in Fig. 3.4 and the densitometry chromatograms are presented in Fig. 3.5 and 3.6.

Dendrogram was prepared based up on the complete linkage, Euclidean distance, and similarity level (Fig 3.7). The dendrogram of the samples collected from 32 locations broadly separated into four main clusters. Cluster 1 contains two sub clusters in which contains 5 accessions out of which three accession are from Punjab and two are from Himachal Pradesh. Cluster 2 contains 11 accessions divided into two sub clusters in which six accessions are from Punjab, one accession from Himachal Pradesh, one accession is from Jammu and Kashmir, one accession is from Uttarakhand, one accession is from Delhi and one accession is from Uttar Pradesh. Cluster 3 contains 11 accessions in which six accessions are from Uttar Pradesh, four accessions are from Haryana and one from Punjab. Cluster 4 contains five accessions in which one accession is from Uttar Pradesh and four accessions are from West Bengal. These HPTLC fingerprints retrieved from all investigated *Stevia* accessions demonstrated significant phytochemical diversity among them.

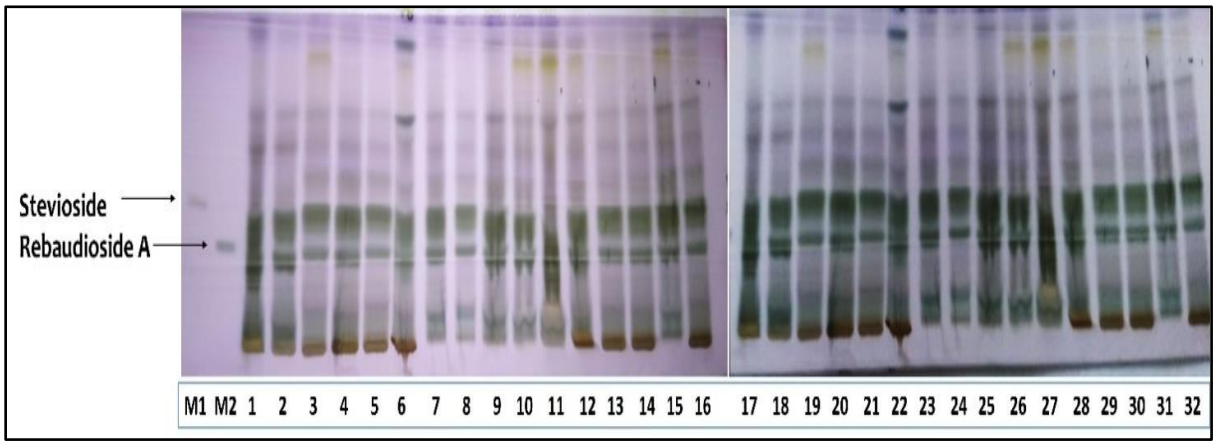


Figure 3.4: HPTLC fingerprints of Stevia rebaudiana where M1 and M2 represent standard compound: stevioside and rebaudioside A; whereas 1-32 tracks are samples from different locations(1. Jalandhar,4. Nawanshahr,11, Palampur,14, Dehradun,21. Delhi,23, Lucknow,26. Varanasi,30. Kolkata,32. Bardhaman)

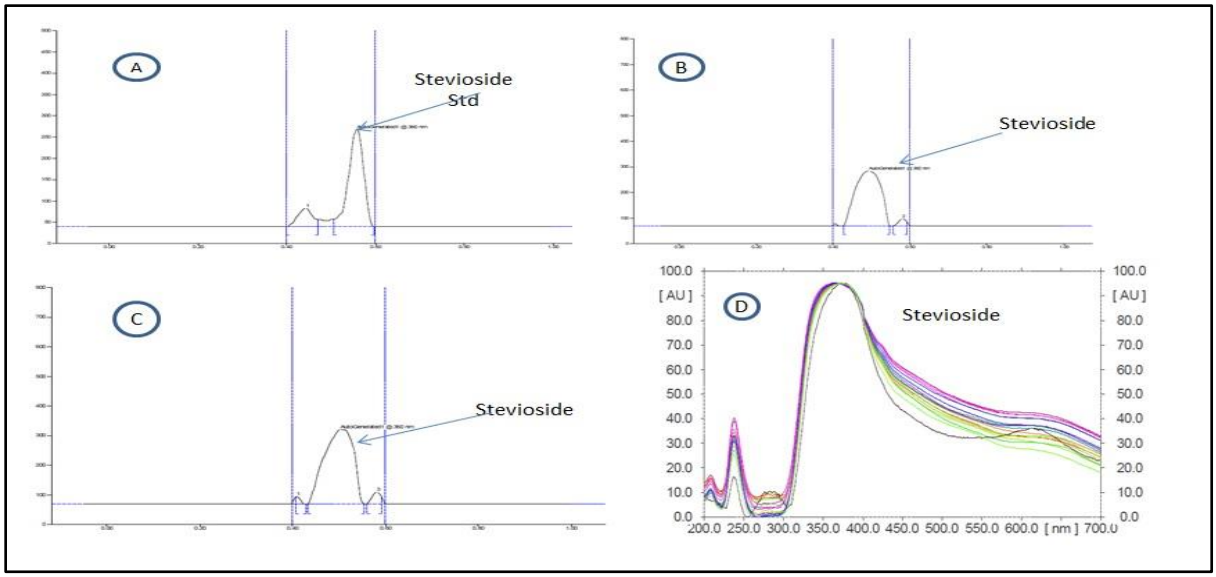


Figure 3.5: Densitometric chromatograms obtained from standard compound of stevioside (A), *Stevia* crude samples with isolated peaks(Sample1,15) (B, C), and overlay spectra of standard compound stevioside with *Stevia* samples(Sample 1-32).

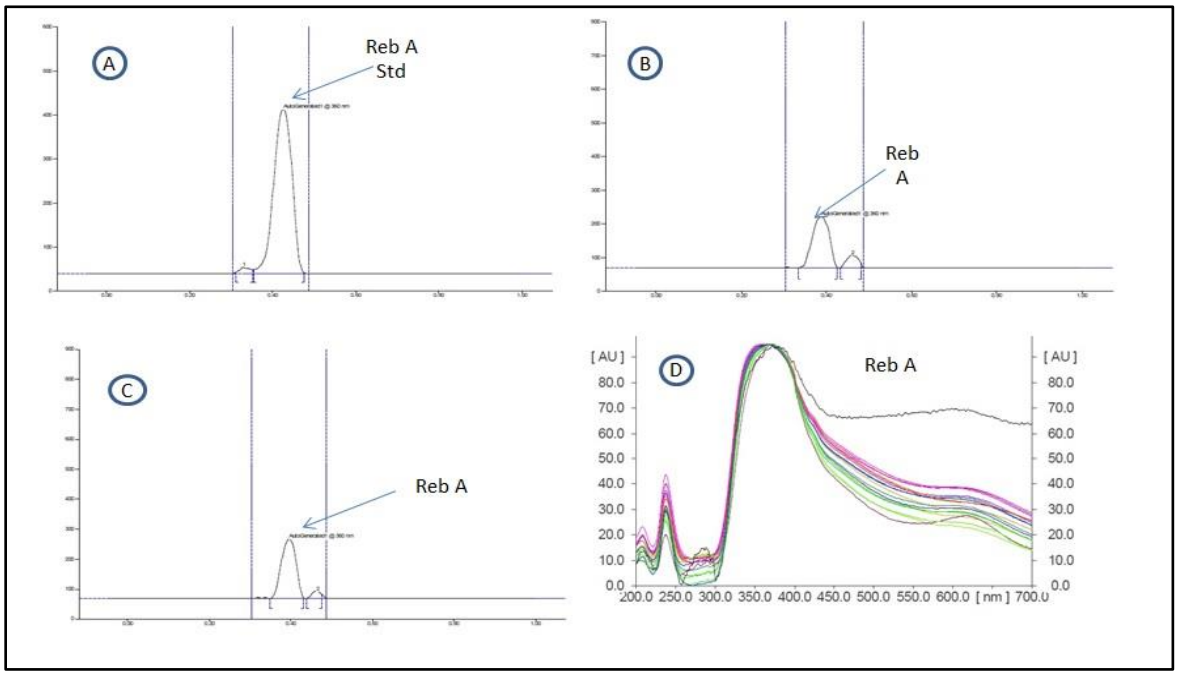


Figure3.6: Densitometric chromatograms obtained from standard compound of rebaudioside A (A), *Stevia* crude samples with isolated peaks(Sample 1-15) (B, C) and overlay spectra of standard compound rebaudioside A with *Stevia* samples.

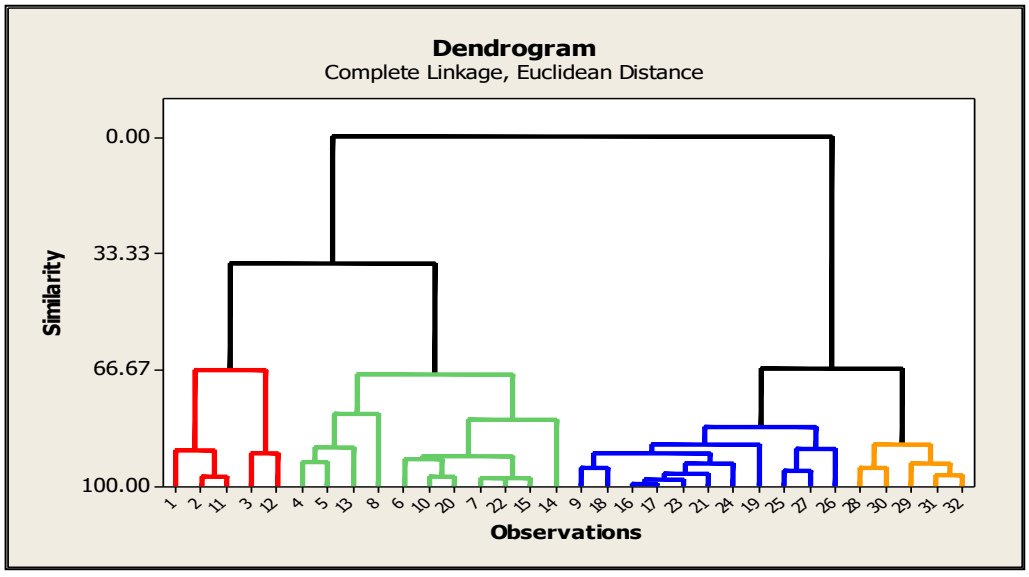


Figure 3.7: Dendrogram based on phytochemical markers reveals four major clusters having two subclusters in each cluster. Cluster 1 (Red)contains two sub clusters are from Punjab, and Himachal Pradesh, Cluster 2(Green) contains from Uttarakhand, Delhi,

Cluster 3(Blue) contains from Uttar Pradesh, Cluster 4 (Orange) contains from Uttar Pradesh and West Bengal

3.4.2. validation of method

Linerarity range of 500-2500ng.spot is showing for correlation coffecient.the sensitivity of method is shown by LOD and LOQ.

3.4.2.1.Precision

The precision of the new approach was tested using plant extracts containing 50, 100, and 150 percent stevioside and rebaudioside A.. presision by Interday and intraday , the percent RSD (coefficient variation) was examined (n=35) in order to assess the repeatability and reproducibility of the data. The values derived from these analytical parameters (average recoveries, intra-day and inter-day precision) are all listed in Table3.2.

3.4.2.2.Specificity

The method's specificity was tested using a densitometric chromatogram and peak purity.

All the chemicals investigated were separated properly using densitometric chromatograms (Fig. 3.4 and 3.5). Peak purities of examined substances (r2) were determined using regression data. The Rf values of the reference constituents were compared to all plant samples using superimposable spectra and found to be equal (Fig. 3.4 and 3.5).

Table 3.2:Method validation for stevioside and rebaudioside A quantification

S.No	Parameters	Stevioside	Rebaudioside A
1	Range of linerarity (ng/spot;*n*= 12[a])	200-1200	200-1200
2	Correlation coefficient (r^2)	0.998	0.987
3	Regression equation	Y = 1.68X + 115	Y=1.94X+204
4	Calculated SD value	0.42	0.34
5	(ng) [3×SD/S][b]Limit of detection (LOD)	80	60
6	[b]Limit of quantification (LOQ) (ng) [10×SD/S]	200	180
7	R_f	0.5	0.4

Accuracy and Precision

8	Intra-day RSD (%), n = 5	1.34	1.26
9	Inter-day RSD (%), n = 5	1.56	1.38
	Recovery		
10	standard in plant samples (μg mg^{-1}) containing bioactive compounds	59	19.5
11	standards added in plant samples (μg mg^{-1})	30, 60, 90	10, 20, 30
12	standard found (μg mg^{-1})	90.10, 118.70, 150.19	29.24, 39.53, 49.53
13	Recovery (%)	101.12, 99.75, 99.80	99.11, 100.07, 100.06
14	Mean Recovery (%)	100.22	99.74

Table3.3: Content of stevioside and Rebauidoside A from *Stevia rebaudiana*.

Accession No.	Location	Stevioside (% dry wt)	Rebaudioside A (% dry wt)
SrP1	Jalandhar	5.88± 0.11	1.95± 0.05
SrP2	Phagwara	5.41± 0.12	1.87± 0.04
SrP3	Kapurthala	5.23± 0.15	1.50± 0.07
SrP 4	Nawanshahr	5.08± 0.16	1.23± 0.02
SrP 5	Banga	4.70± 0.25	1.18± 0.15
SrP6	Hoshiarpur	3.53± 0.21	1.27± 0.13
SrP7	Ludhiana	3.11± 0.17	1.23± 0.17
SrP8	Amritsar	4.00± 0.19	1.04± 0.13
SrP9	Patiala	3.32± 0.21	1.02± 0.13
SrJ10	Jammu	3.11± 0.17	1.33± 0.19
SrH11	Palampur	5.28± 027	1.89± 0.14
SrH12	Kangra	4.66± 0.27	1.50± 0.18

SrH13	Solan	4.41± 0.25	1.27± 0.16
SrU14	Dehradun	3.81± 0.24	1.44± 0.22
SrP15	Chandigarh	3.20± 0.22	1.20± 0.04
SrH16	Hisar	3.13± 0.23	1.05± 0.08
SrH17	Kurukshetra	3.12± 0.27	1.06± 0.06
SrH18	Karnal	3.04± 0.15	0.99± 0.07
SrH19	Sonipat	2.96± 0.17	0.90± 0.07
SrD20	Delhi	3.15± 0.22	1.29± 0.06
SrU21	Moradabad	2.99± 0.16	1.09± 0.02
SrU22	Allahabad	3.07± 0.27	1.20± 0.06
SrU23	Lucknow	3.03± 0.19	1.05± 0.09
SrU24	Alamnagar	2.74± 0.13	1.04± 0.07
SrU25	Raibareli	2.25± 0.14	1.03± 0.11
SrU26	Varanasi	2.65± 0.15	1.15± 0.04
SrU27	Siwan	2.45± 0.27	0.99± 0.5
SrU28	Patna	2.10± 0.17	0.86± 0.07
SrWb29	Asansol	1.83± 0.19	0.77± 0.09
SrWb30	Kolkata	1.81± 0.12	0.89± 0.04
SrWb31	Kestopur	1.63± 0.16	0.78± 0.06
SrWb32	Bardhaman	1.43± 0.15	0.77± 0.04

3.5. Discussion

Many researchers has done studies on variation of content of ST and Reb A with geographical location in Stevia as measured by HPLC and HPTLC (Khiraoui et al., 2021; Jaitak et al., 2008). Similarly, HPTLC has been used to perform simultaneous quantification of stevioside and

rebaudioside, revealing variation in their content (Chester et al., 2012). These studies show a link between the content of steviol glycosides and climatic conditions. The content of steviol glycosides is strongly influenced by different environmental conditions (Yang et al., 2015). Ceunen and Geuns (2013) reported the variation in the content of steviol glycosides influenced by different photoperiod and genotype. Furthermore, there is high content of steviol glycosides found in high altitude regions and semi-arid regions of India (Ghaheri et al., 2018; Jaitak et al., 2008, Saifi et al., 2014).

In other plants, the geographical location plays an important role in content of secondary metabolite (Hennicke et al., 2016). There are genetic and photochemical variations in the *Swertia* in which there are variations at genetic level and phytochemical level based on variations in altitude (Kaur et al., 2019 a, b, c). Many studies were conducted on different medicinal plants which strengthen the correlation. There are many reports which supports that there is correlation between the geographical locations based on climatic conditions and production of secondary metabolites in plants (Kaur et al., 2021; Ray et al., 2019; Qaderi et al., 2019). In *Hedychium coronarium*, a study was conducted in which the morphological, molecular and phytochemical and diversity was determined in 50 *H. coronarium* accessions from various Eastern Indian states. Cluster analysis was done on the basis of molecular as well as phytochemical characterization which clearly showed the relationship between diversity pattern and geographical origin. The findings exhibited the high degree of phytochemical and molecular variations among *H. coronarium* accessions based on geographic locations (Ray et al., 2019). Similarly, a study on Iranian poppy (*Papaver bracteatum* Lindl.) was conducted to access the phytochemical variability and molecular diversity in Iran. The results showed the geographical parameters showed a significant and positive correlation with secondary metabolite extracted from the Plant (Qaderi et al., 2019). In Caucasian whortleberry, the phytochemical diversity has been accessed in Iran. They found that there are variations in the secondary metabolite of the plant which was correlated with the different province (Fathi et al., 2019). Similarly, another study has been carried out in which the influence of various climatic conditions was studied on the diversity phytochemicals and antioxidant activity of aqueous leaf extracts of *Aloe vera* obtained from a number of climatic zones of India. The results showed that there is enhanced antioxidant activity and variation in phytochemical content with different environmental conditions.

These studies clearly demonstrated the relationship between the influence of climatic conditions and geographical locations on plant secondary metabolites. India is well-known for its varied

geographical variations and climatic conditions. So, there can be variations in the secondary metabolites. In the present study, we have correlated the influence of different climatic conditions based on different geographical locations on the content of steviol glycosides in *S. rebaudiana*. The highest content (5.2 - 5.8 %) of stevioside and rebaudioside A was found in Northern Himalayan range having high lands and semi-arid region of northern India. The climatic conditions are favorable for the production of steviol glycosides in *S. rebaudiana* in these regions. Other regions showed the intermediate level of steviol glycosides which are having humid subtropical regions like Uttar Pradesh. These regions have different climatic conditions then Highlands which affects the production of metabolites. The contents was lowest in tropical wet and dry regions of India like West Bengal.

Since, stevioside and rebauidoside A are industrially important compounds which are mainly used by industry as an anti-diabetic and natural sweeteners (Mirzaei and Shakoory-Moghadam, 2022) so, it is important to find the accessions which are high yielding steviol glycosides. The phytochemical variations are also related to the genetic variations which are correlated. But there is very less work which has been done till yet to explore these correlations. In future, we can explore the genetic aspect and phytochemical aspect to explore and understand the dynamics of elite variety of *Stevia rebaudiana.*

3.5.1. Conclusion

The current rapid, validated, and reproducible HPTLC method presents compounds *viz.* ST and Reb A in 32 *Stevia rebaudiana* chemotypes collected from 32 districts of 9 geographically separated states of India. To select and screen the elite variety of *S. rebaudiana*, the assessment of phytochemical diversity associated with variation in the climatic factors and geographical location play a very significant role on difference in ST and Reb A content. In the present study, phytochemical variations in *S. rebaudiana* were observed which were related to the geographical location variation. The study reported significant intra-specific variation in stevioside and rebaudioside A content across *S. rebaudiana* chemotypes and screened the elite chemotype(s) for commercial propagation as well as for the production of stevioside and rebaudioside A. The elite *S. rebaudiana* chemotypes has been observed in Highlands and Semi-arid regions of India. These can be explored for the conservation and cultivation. Outcome of the study revealed that there is positive correlation between the chemical composition of plant accessions and different

geographical locations. Further, the elite accessions of *Stevia rebaudiana* can be conserved, mass propagated and can be used by the industries.

3.6. (RSM) for optimization of heat reflux extraction and hptlc analysis of Antioxidant compounds(gallic acid, quercitin and chlorogenic acid) from *Stevia rebaudiana* (Bertoni) leaves

Extraction process is an initial step in medicinal plant study. It is an important part for obtaining antioxidants from plants. Many researchers have reported both conventional and contemporary extraction method for antioxidant. It is very challenging for the researchers for effective and safe extraction of natural products from herbs. Conventional methods like maceration (Dacome et al 2005), hydro distillation (Marković et al 2008) extraction, Soxhlet method, reflux method for antioxidant compounds . Furthermore contemproray extraction techniques have been developed such as ultrasound assisted extraction (UAE)(Žlabur et al 2015) and microwave assisted extraction (MAE)(Jaitak et al 2009) method for antioxidant compounds. Quercetin is one of the flavonoids, used in human diet since a long time. Quercetin has shown various pharmacological and biological properties (Mukhopadhyay et al 2015, Wang et al 2016). Concequently, quercetin rich diet intake has shown the positive correlation in terms of good health (D'Andrea, 2015Quercetin can be used as an antioxidant to protect against free radical oxidative damage. Gallic acid (also known as 3,4,5-trihydroxybenzoic acid) has antibacterial, anticarcinogenic, antimutagenic, antioxidant, and anti-inflammatory .

Quercetin is also present in *Stevia rebaudiana* but it has not been extracted yet .So, Quercetin has been extracted by using reflux method by using ethanol as solvent as ethanol is green solvent. Similarly gallic acid has been extracted by Ultrasound assisted extraction(Zhang et al 2015) ,aqueous two-phase systems (Cláudio et al 2012) etc.

Beside extraction techniques various extraction parameters influence the yield of antioxidant compounds such as extraction time ,temperature, type of solvent used, number of extraction steps ,mean particle size and solid to liquid ratio influences the quality of herbal medicines (Gad et al 2013). So during extraction we have to consider the affects of these parameters. To obtain efficient and effective extraction, the process can be collaborate with the statistical method for optimization

and improvement of the process. **RSM, or response surface methodology**, has been extensively utilised to improve extraction conditions. It is the collection of mathematical and statistical technique which is useful for improving and optimizing the process (Myers et al 1995). The best advantage is it interactive effects on compound.

Rouhani et al. (2019) optimised the processes for stevioside extraction from *Stevia rebaudiana*. As a result, RSM has been regarded as a useful contemporary technique for examining the interactions between independent variables and simultaneously screening several significant factors. Antioxidant chemicals are more in demand globally as a result of health concerns, which has raised the industrial need for their manufacture.

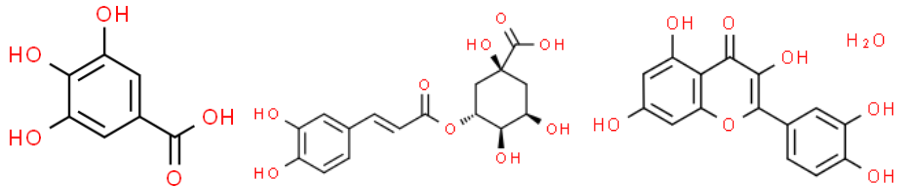
The optimization of the extraction parameters and HPTLC analysis of the antioxidants quercetin, gallic acid, and chlorogenic acid from Stevia rebaudiana are being done for the first time in this study. The goal of the current study was to enhance the recovery of antioxidant chemicals from *Stevia rebaudiana* (Bertoni) leaves by optimising a number of extraction parameters.

3.6.1.**Material and Method**

3.6.2. Chemical and Reagents

Sigma-Aldrich chemicals pvt Ltd,Banglore,India provided analytical reference compounds such as quercetin and gallic acid, as well as chlorogenic acid with a purity of 98%. All chemicals used in the experiment, including ethanol, formic acid, sulphuric acid, glacial acetic acid, ethyl acetate, and others, were of HPLC grade The HPTLC plates (20cm×10 cm) (E. Merck, Darmastadt, Germany) were used in the experiment.

Fig. 3.8. Chemical structure of Gallic acid (1), Chlorogenic acid, Quercetin



3.6.3Plant material

Stevia rebaudiana has been cultivated in the herbal medicinal garden, Lovely Professional University, Punjab .The leaves was obtained from cultivated plants of *Stevia rebaudiana*. The

leaves were ground into a fine powder using a laboratory-scale dry grinder before the extraction procedure.The stevia leaf powder was stored for the upcoming experiment in plastic bags.

3.6.4. Extraction of plant sample

Using a heat reflux extraction, extraction studies were conducted under various extraction parameters. Plackett-Burman design (PBD) was used to determine the conditions of the extraction process. 1 g of dried plant material, or leaves, were extracted using a heat-reflux technique using an ethanol and water solvent .To determine the effect of extraction on the yield of antioxidant, trials were conducted using a variety of extraction variables, including temperature (X1 35-70 °C), extraction time (X2 30-60 min), solvent composition (X3 35-70%), solvent-to-solid ratio (X4 10-20 ml g1), particle size (X5 0.6-1.2 mm), and extraction steps (1-3). The reflux method was used to extract the dry leaf powder using a temperature-controlled hot water bath. In a 250 ml flask, dry leaf powder (1.0 g) was added, and various extraction settings (temperature, time, solvent composition, solvent-to-solid ratio,, particle size, and extraction steps) were used. All samples were concentrated, vacuum-dried, and stored in a refrigerator at 4 °C for further analysis.

3.6.5. Experimental design

The two phases of the experiments were the central composite design and Plackett-Burman design (PBD) was employed in the first stage in order to screen the significant independent parameters. Subsequently, BBD was employed in the subsequent phase to investigate the optimal level and any possible interplay among significant parameters. The statistical program Minitab 15 was utilized for the experimental setup.

3.6.6.Plackett–Burman model (PBM)

PBM was for the optimization of antioxidant extraction from *Stevia rebaudiana* for evaluation of the substantial parameters. PBD is based on the first-order model:

$Y = \beta_0 + \sum \beta i Xi$

where βi is the regression coefficients, β0 is the scaling constant, and Y is the anticipated target function. On antioxidant extraction, the effects of several factors (such as time, mean particle size, temperature, solvent composition, solid-solvent ratio, and extraction steps) were investigated. The Plackett-Burman model was tested using an experiment at two levels, where (+) denotes the maximum value and (-) denotes the minimum value (shown in Table 3.4). (depicted in Table 3.5). By carrying out 12 experiments, copies of each variable indicated were investigated (depicted in

Table 3.5). The relevant parameters from the regression analysis were assessed at the 5% level (P 0.05).

Table 3.4: *Different variables with coded levels employed in PBD for screening of extraction parameters influencing stevioside extraction*

Code	Variables	High level (+)	Low level (-)
X_1	Temperature (ºC)	70	35
X_2	Time (min)	60	30
X_3	Solid: solvent ratio(g/ml)	1:30	1:15
X_4	Solvent Composition (Ethanol and water)	70	30
X_5	Particle size (mm)	1.0	0.5
X_6	Cycles/steps	3	1

Table 3.5: *S. rebaudiana leaves using different levels of extraction variables of Plackett-Burman design criteria*

Run	Block	Temperature	Time	Sold: Solvent	Solvent Composition	Particle size	Extraction steps	Radical scavenging (%)	FITS1
1	1	70	30	30	30	0.5	1	15.50	15.14
2	1	70	60	15	70	0.5	1	10.34	8.18
3	1	35	60	30	30	1.0	1	19.67	19.42
4	1	70	30	30	70	0.5	3	8.98	9.03
5	1	70	60	15	70	1.0	1	10.56	12.71
6	1	70	60	30	30	1.0	3	20.12	18.48
7	1	35	60	30	70	0.5	3	6.61	8.77
8	1	35	30	30	70	1.0	1	14.50	14.51
9	1	35	30	15	70	1.0	3	10.23	7.99
10	1	70	30	15	30	1.0	3	11.23	13.16
11	1	35	60	15	30	0.5	3	8.67	8.37
12	1	35	30	15	30	0.5	1	8.98	9.56

3.6.7. Box-Behken design

Box-Behken design has been employed from the screened significant extraction parameter from Placket–Burman design On antioxidant extraction, the optimal levels of three significant variables, namely mean particle size, solid solvent ratio, and solvent composition, were investigated (Figure 3.5). Total antioxidant activity was calculated using the second-order polynomial equation, as shown below:

$Y=\beta_0 +\beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X^2_1 + \beta_{22}X^2_2 + \beta_{33}X^2_3 + \beta_{12}X_1X_2 + \mathbf{\beta_{13}}X_1X_3 + \beta_{21}X_2X_1 + \mathbf{\beta_{23}}X_2X_3 + \beta_{31}X_3X_1 + \mathbf{\beta_{32}}X_3X_2$

where Y is the predicted response, β0 is a scaling constant, X1, X3, and X5 are the extraction factor levels, β1, β2, and β3 are linear coefficients, β11, β22, and β33 are quadratic coefficients, and β12, β13, and β23, are interactive coefficients. The optimal conditions for a specific plant's reflux extraction were also determined using response surface methodology (RSM). Using the central composite design (Table 3), a total of twenty experiments were carried out with various combinations of significant variables. Minitab statistical software was used to create contour plots of antioxidant activity for each interactive coefficient.

3.6.8..Quantification of Gallic acid, Quercetin and Chlorogenic acid by HPTLC

3.6.8.1.Chromatographic Conditions

The stationary phase was made up of 20 cm by 10 cm pre-coated silica gel 60 F254 HPTLC plates. Samples were applied to the plates as 2.6 mm broad bands .The optimal mobile phases for GA, Q, and CA were n-hexane: ethyl acetate: acetone (16.4: 3.6: 0.2 v/v), toluene: ethyl acetate: formic acid (13.5: 9: 0.6 v/v), and glacial acetic acid: formic acid: water (20: 2.2: 2.2: 5.2 v/v). and 60% relative humidity. Plates were dried for 10 minutes at 100°C before being derivatized with a 100 ml solution of 10% (v/v) ethanolic sulphuric acid (H2SO4). The scanning speed was set to 20 mms-1, and the slit size was fixed at 4 mm by 0.33 mm. Densitometric scanning was performed at 375 nm. To examine the plant samples, one millilitre of extract from each sample was placed on

the plate. The amount of GA, Q, and CA was calculated using the assumption that the marker was 100% pure after development, derivitization, scanning, and peak area measurement.

3.6.8.2.Method Validation

By evaluating linearity, peak purity, limit of detection (LOD), repeatability, percentage recovery, intraday, and intermediate precision, the aforementioned method was shown to be reliable (Table 4). Three duplicate applications of the GA, Q, and CA standard solutions were made. By graphing peak area against the quantity of GA, Q, and CA, and determining the linearity range, the calibration plot was created. Six scans of the same GA, Q, and CA band were performed to verify the instrument's accuracy.

Peak area and retention factor (Rf) averages, standard deviations, and coefficients of variation (CV) (%) were calculated. The GA, Q, and CA bands were examined after administering the standard solution to the plate (n=10), and the% CV (n=10) was calculated. The accuracy of the approach was tested by analyzing the recovery of four levels after the addition of 0, 50, 100, and 150% quercetin to the sample. The amount of GA, Q, and CA in S. rebaudiana leaf samples was determined, and 100 mg of plant material was mixed with a known quantity of standard GA, Q, and CA. Each of the three stages included recovery documentation. (Table 5). Three bands of sample solution per plate were analysed on three different plates to observed precision (intraday precision) and on two different plates 3 bands were placed to determine precision (interday and intermediate precision) (Table 6) and to compute % CV. The absorbance spectra of the GA, Q, and CA standards, as well as the matching peak in the 200-800 nm region of the test sample, were used to calculate the procedure's specificity. The limits of detection (LOD) and quantitation (LOQ) of the standard solutions were determined using methanol as a blank and various dilutions of the standard solutions.

3.6.8.3.Determination of GA, Q and CA in Samples

The sample extract and standard solution mixture were applied to 60 HPTLC plates measuring 20 cm x 10 cm. Peak areas were measured and a calibration plot by comparing the peak area to the amount of Standard GA, Q, and CA used.

3.6.9 Measurement of DPPH scavenging potential

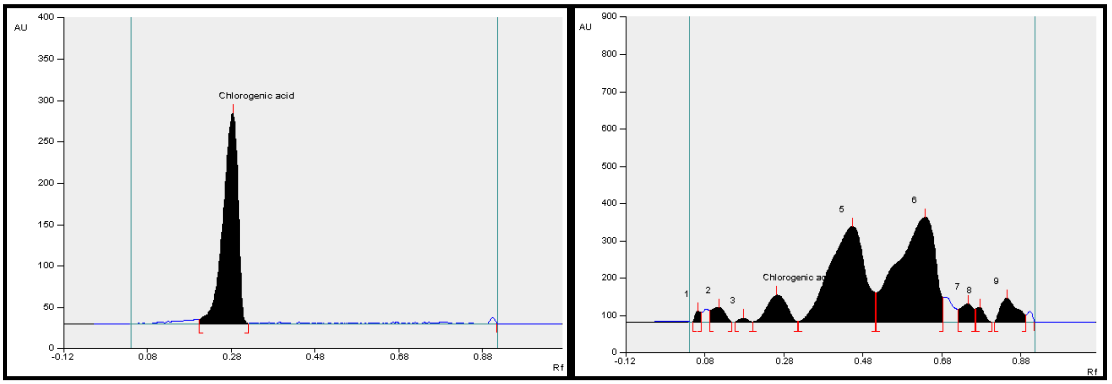
The ability of extracts to scavenge DPPH's free radical was used to evaluate their antioxidant capability (1,1-diphenyl-1-picrylhydrazyl). The test solution was created by combining 50 litres of aqueous ethanol leaf extract with 2 litres of methanol to dilute the 2 mL of DPPH solution (0.5 mM in methanol). After 30 minutes of incubation, the test solution's absorbance was determined using a spectrophotometric technique at 517 nm. Ascorbic acid served as the benchmark substance. The following formula has been used to compute the percentage of extract inhibition:

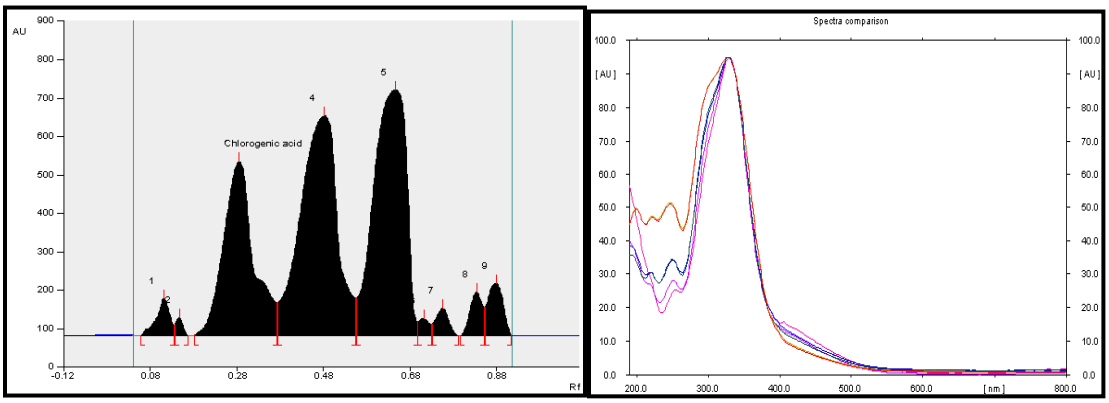
% inhibition = $(A^o\text{-}At)/A^o \times 100$

where At was the absorbance in the presence of the extract sample and A0 was the absorbance of the control (blank, without extract). The graph was plotted by using the mean values after each test was performed in triplicate.

3.6.10..Results and discussion

In this study, Optimization of Antioxidant activity of leaves of *Stevia rebaudiana* through RSM was estimated as well as quantitative estimation of antioxidant compound GA, Q and CA in different extracts was quantified by HPTLC method and HPTLC densitometric-peak of GA, Q and CA were shown in HPTLC fingerprinting on different extracts of *Stevia rebaudiana* showed that presence of antioxidant compound GA, Q and CA varies with different conditions(Figure3.9) .For antioxidant activity ,it is highest 9043% followed by other 54.73 % and so on. It is directly correlated with the highest GA, Q and CA quantity present in the sample .It shows that there is increase in the antioxidant activity which is due to high antioxidant compound GA, Q and CA and vice versa. The antioxidant activity also varies with different extraction conditions.


AU
Chlorogenic acid
Rf


AU
Chlorogenic acid
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Spectra comparison
[AU]
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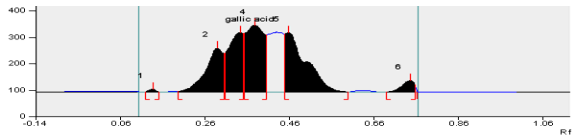
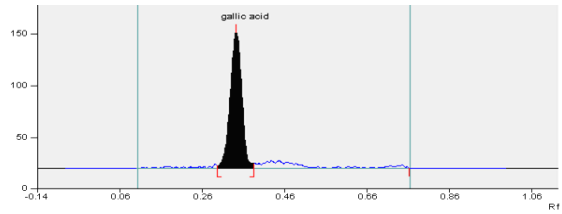
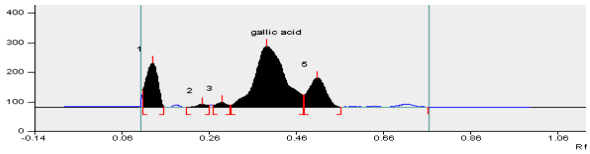
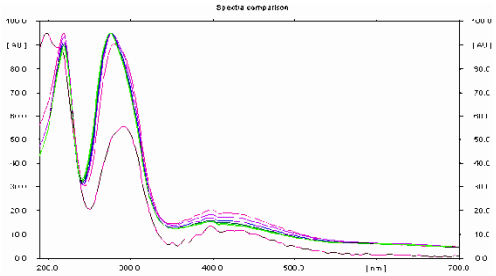

gallic acid5
Rf


gallic acid
Rf


gallic acid
Rf


Spectra comparison
[nm]

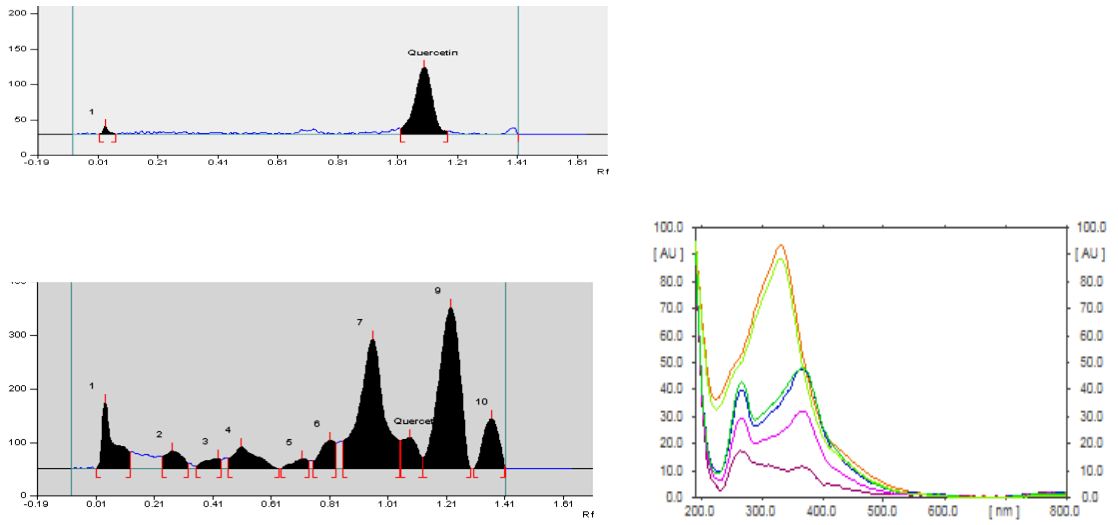
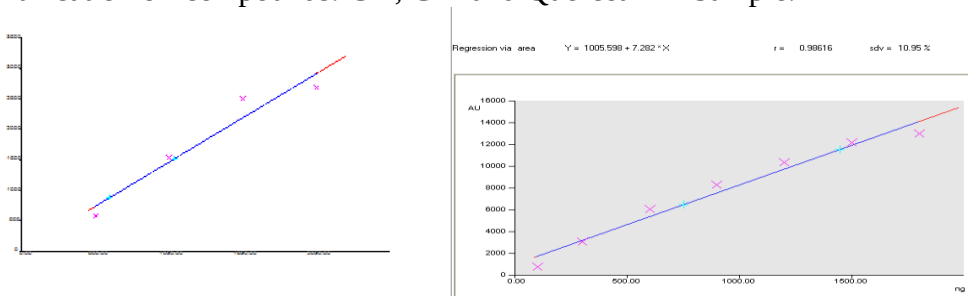
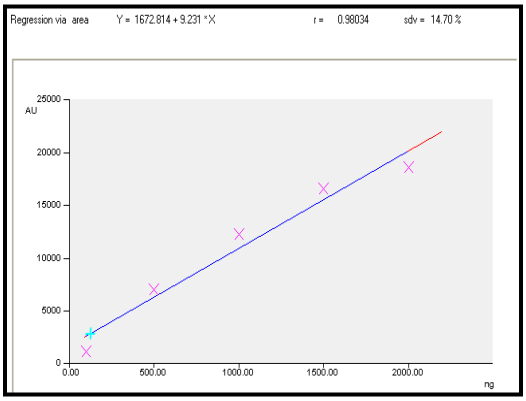


Fig.3.9. -HPTLC Densitometric (I-V) quantification of compounds: GA, CA and Quercetin; Standard compound: : GA, CA and Quercetin.(VI-VII) Densitometric graph showing quantification of compounds: GA, CA and Quercetin in Sample.



Standard curve of Gallic acid **Standard curve of Querceitin**



3.6.10.1.Standard curve of gallic, chlorogenic acid, quercitin

(a)Screening out significant extraction variables

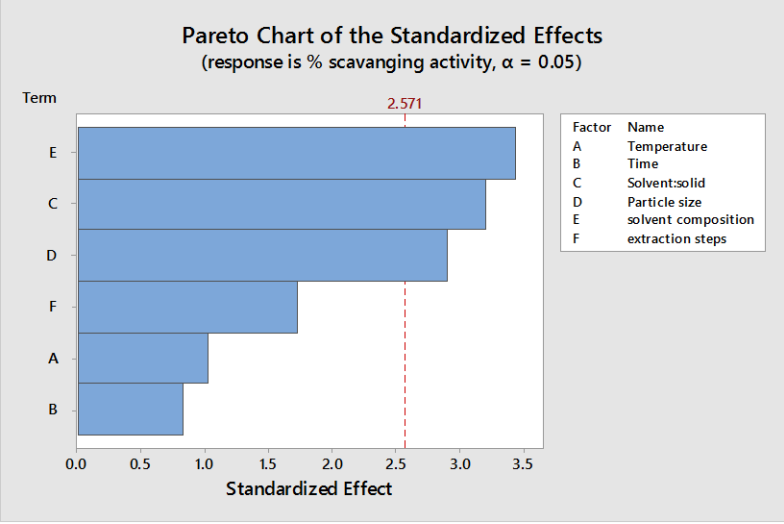
On antioxidant activity, the effects of six different independent variables have been assessed using the Plackett-Burman design criterion. In this technique, a particular constructed matrix was used, and Table 2 provides the resulting antioxidant activity content derived from Stevia

rebauidana leaf. By using regression analysis, the impact of extraction factors on antioxidant activity was examined . According to their P values at the 5% level (P 0.05 values), only three parameters—solvent:solid ratio, solvent composition, and mean particle size—had a significant impact on antioxidant activity.

Table 3.6: *Regression analysis for parameter of extractions by PBD criterion*

Term	Effect	Coef	SE Coef	T	P
Constant		12.116	0.6590	18.39	0.000
Temperature	1.345	0.672	0.6590	1.02	0.354
Time	1.092	0.546	0.6590	0.83	0.445
Sold: Solvent	4.228	2.114	0.6590	3.21	0.024
Solvent Composition	-3.825	-1.912	0.6590	-2.90	0.034
Particle size	4.538	2.269	0.6590	3.44	0.018
Extraction steps	-2.285	-1.142	0.6590	-1.73	0.143

Fig. 3.10: Comparative effect of, time, solvent composition solid–solvent ratio, , temperature ,extraction steps and particle size on antioxidant activity .



3.6.11. Variable effects on yield

In this method, 15 sets experiments were carried out using the Box-Behken design, which clearly displays experimental results alongside those that the model equation predicted. The quadratic model's contribution was substantial (0.0001), and the R^2 and R^2 adj values were,

respectively, 0.96 and 0.93, which supported the suitability of the model.

Following the application of multiple regression analysis to the experimental data, the resulting second-order polynomial equation is as follows:

$Y=20.260 +1.881X_1 -1.303X_2 + 1.825X_3 -3.465X_2^2 -3.875X_3^3 -1.643X1X2+ 0.920X1X3$ {2}

Table 3–7 lists the effects of the solvent:solid ratio, the solvent's makeup, and mean particle size on antioxidant activity, GA, Q, and CA. The solvent:solid ratio, solvent composition, and particle size all had substantial positive linear effects on antioxidant activity, according to the regression coefficient calculated from the study's data (Table 7). Due to their maximum linear coefficient value (1.881), GA, Q, and CA have demonstrated the greatest impact of the three parameters on antioxidant activity, followed by solvent composition (1.825) and particle size (-1.303). According to Table 3.7, the interaction between the solvent, the solid, and the particle size (X12) has a big impact on the antioxidant activity, GA, Q, and CA. While particle size and solvent composition (X23) were not shown to have impact on antioxidant activity, the interaction between the solid and the solvent composition (X13) had a substantial impact on antioxidant activity, GA, Q, and CA. Thus, in the aforementioned model regression [Eq. (2)], only interactions between solvent:solid and particle size (X12) and solvent:solid and solvent composition (X13) were displayed. Figure 3.14(a–c) shows contour plot maps showing the effects of various solvent:solid ratios, solvent compositions, and particle sizes on antioxidant activity, GA, Q, and CA. The particle size of 0.80 mm and the solvent solid ratio of 20:1 were maintained to achieve the highest antioxidant activity, as shown in Fig. 5a. This shows that the antioxidant activity is influenced by both particle size and the solvent to solid ratio. According to solvent solid ratio and solvent composition (% ethanol in ethanol-water mixture), Figure 5b depicts the evolution of antioxidant activity. Here, the solvent:solid ratio of 20:1 and 60% ethanol-water mixture result in an increase in antioxidant activity. The antioxidant activity can be raised still more by increasing the solvent:solid ratio.

Table 3.7. BBD with three extraction factors: solvent-solid ratio (A), Solvent composition (B) and Particle size(C).

Run Variables			Antioxidant activity, Phenolics and flavanoid (%)							
Solid:Solvent	Solvent composition	particle size	DPPH		Gallic acid		Quercitin		Chlorogenic acid	
			Exp	Pred	Exp	Pred	Exp	Pred	Exp	Pred
10	35	0.9	63.56	64.31	2.34	2.32	2.49	2.47	1.65	1.64
20	35	0.9	71.89	72.81	2.43	2.44	2.67	2.66	1.76	1.76
10	70	0.9	84.82	83.89	2.56	2.54	2.76	2.76	1.89	1.88
20	70	0.9	92.67	91.92	2.65	2.66	2.86	2.87	1.94	1.94
10	52.5	0.6	78.78	79.78	2.51	2.49	2.67	2.63	1.76	1.78
20	52.5	0.6	81.77	82.59	2.67	2.62	2.77	2.725	1.82	1.83
10	52.5	1.2	57.98	57.15	1.98	2.02	2.12	2.165	1.59	1.57
20	52.5	1.2	71.87	70.86	2.12	2.13	2.32	2.36	1.75	1.72
15	35	0.6	73.87	72.12	1.94	1.96	1.98	2.03	1.78	1.75
15	70	0.6	89.34	89.26	2.23	2.25	2.43	2.46	1.95	1.93
15	35	1.2	52.65	52.73	1.57	1.54	1.83	1.79	1.54	1.56
15	70	1.2	72.54	74.29	1.73	1.70	1.92	1.86	1.78	1.80
15	52.5	0.9	98.56	97.41	2.76	2.74	3.21	3.23	2.11	2.17
15	52.5	0.9	97.35	97.41	2.67	2.74	3.31	3.23	2.18	2.14
15	52.5	0.9	96.34	97.41	2.81	2.74	3.18	3.233	2.15	2.14

Table 3.7.2B. ANOVA for Response Surface Regression for DPPH,GA, Q, CA

Term	DPPH activity			Gallic acid			Quercitin			Chlorogenic acid		
	Coeff. ± S.E	**t**	**P>(t)**	**Coeff. ± S.E**	**t**	**P>(t)**	**Coeff. ± S.E**	**t**	**P>(t)**	**Coeff. ± S.E**	**t**	**P>(t)**
Con	97.41 ± 0.99	98.047	0.000	2.746 ± 0.034	80.664	0.000	3.233± 0.040	79.896	0.000	2.146 ± 0.020	102.794	0.000
A	4.132 ± 0.608	6.792	0.001	0.060 ± 0.020	2.877	0.035	0.072± 0.024	2.925	0.033	0.047± 0.012	3.714	0.014
B	9.675 ±0.608	15.901	0.000	0.111 ± 0.020	5.335	0.003	0.125± 0.024	5.044	0.004	0.103± 0.012	8.113	0.000
C	-8.5900 ± 0.608	-14.118	0.000	-0.243 ± 0.020	-11.690	0.000	-0.207 ± 0.024	-8.373	0.000	-0.081± 0.012	-6.353	0.001
A*A	-9.3408 ± 0.8956	-10.430	0.000	0.100 ± 0.030	3.272	0.022	-0.054 ±0.036	-1.485	0.198	-0.184 ± 0.018	-9.806	0.000
B*B	-9.8408 ± 0.8956	-10.988	0.000	-0.352 ± 0.030	-11.471	0.000	-0.484 ± 0.036	-13.273	0.000	-0.152± 0.018	-8.079	0.000
C*C	-15.4758 ± 0.8956	-17.280	0.000	-0.527 ± 0.030	-17.173	0.000	-0.709 ±0.036	-19.441	0.000	-0.232 ± 0.018	-12.329	0.000
A*B	-0.1200 ± 0.8605	-0.139	0.895	-0.000 ± 0.029	-0.000	1.000	-0.020 ±0.035	-0.571	0.593	-0.015± 0.018	-0.829	0.445
A*C	2.7250 ± 0.8605	3.167	0.025	-0.005 ± 0.029	-0.170	0.872	0.025 ±0.035	0.713	0.508	0.025 ± 0.018	1.382	0.225
B*C	1.1050 ± 0.8605	1.284	0.255	-0.032 ±0.029	-1.102	0.321	-0.090 ±0.035	-2.568	0.050	0.017 ± 0.018	0.968	0.378

WhereCoeff= Regression Coefficient

Solvent-solid (A); solvent comp (B); particle size (C); ** Very significant, where $p < 0.01$

According to the solvent composition and mean particle size, Figure 5c depicts the evolution of antioxidant activity. The association between them and antioxidant activity is very strong.
When using 55% ethanol and a 0.80 mm mean particle size in an ethanol-water solvent, the maximum antioxidant activity of GA, Q, and CA occurs.

3.6.11.1.Validation of the model

The best mean particle size (0.80 mm), and solvent:solid ratio (20:1),solvent composition, ((55% ethanol in ethanol-water mixture)) were determined by fitting the experimental data into the model equation. Antioxidant activity, GA, Q, and CA from *Stevia rebaudiana* leaf were 20.43% at these ideal levels of extraction parameters, which is extremely near to the expected value of 20.26% and is directly connected with the presence of the maximum GA, Q, and CA content(0.27%). The experimental extraction data were developed to the mathematical model with accuracy.

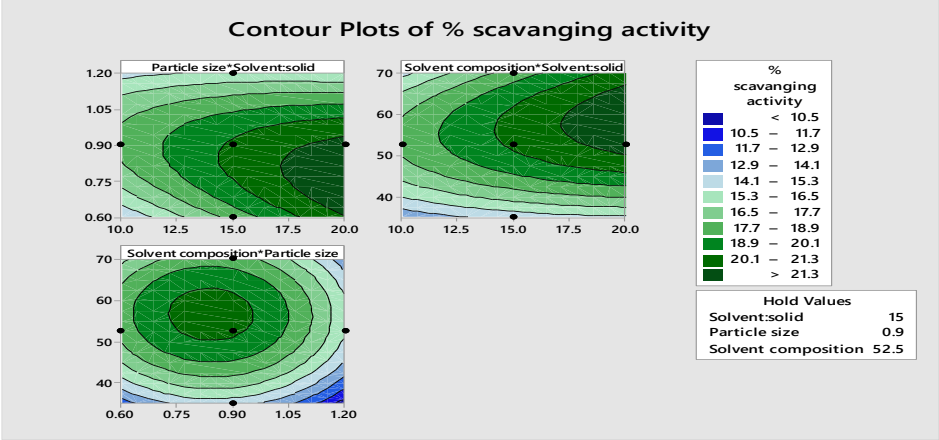


Figure 3.11. a.Contour plot for scavenging activity at various levels of particle size and the ratio of the solvent to the solid b. A contour figure showing the scavenging activity at different solvent composition levels (% ethanol in the ethanol-water combination) and solvent solid ratios c. A contour plot showing the scavenging activity at different solvent compositions (% ethanol in the ethanol-water mixture) and particle sizes.

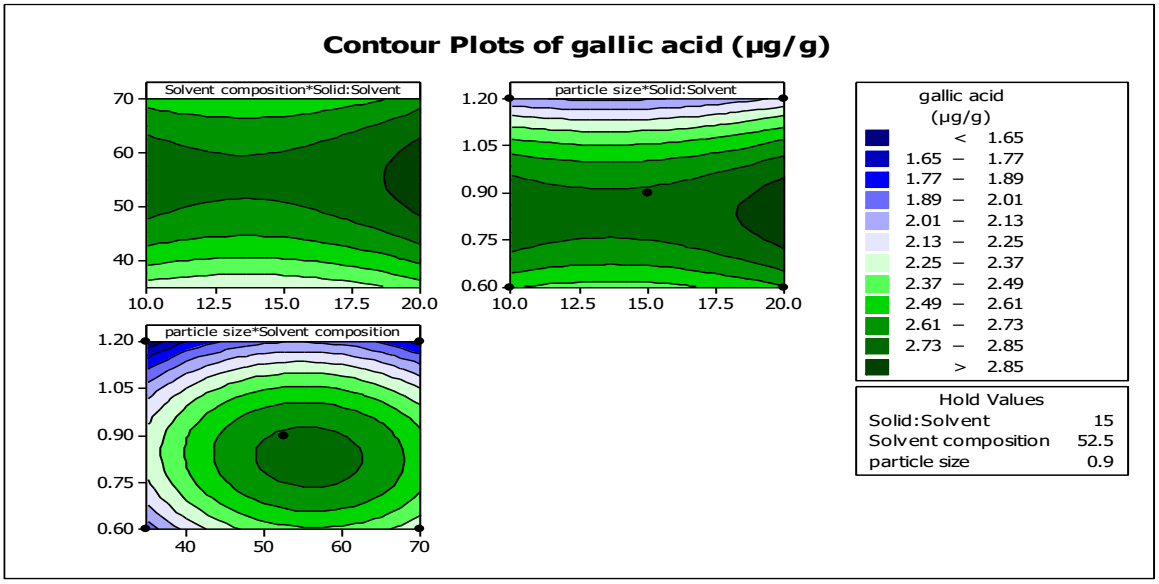


Figure 3.12. aGallic acid contour plot at different levels of particle size and solvent to solid ratio b. Gallic acid contour plot for different solvent composition levels (% ethanol in the ethanol-water mixture) and solvent solid ratios. c. A contour plot showing the scavenging

activity at different solvent composition levels (% ethanol in the ethanol-water mixture) and particle sizes.

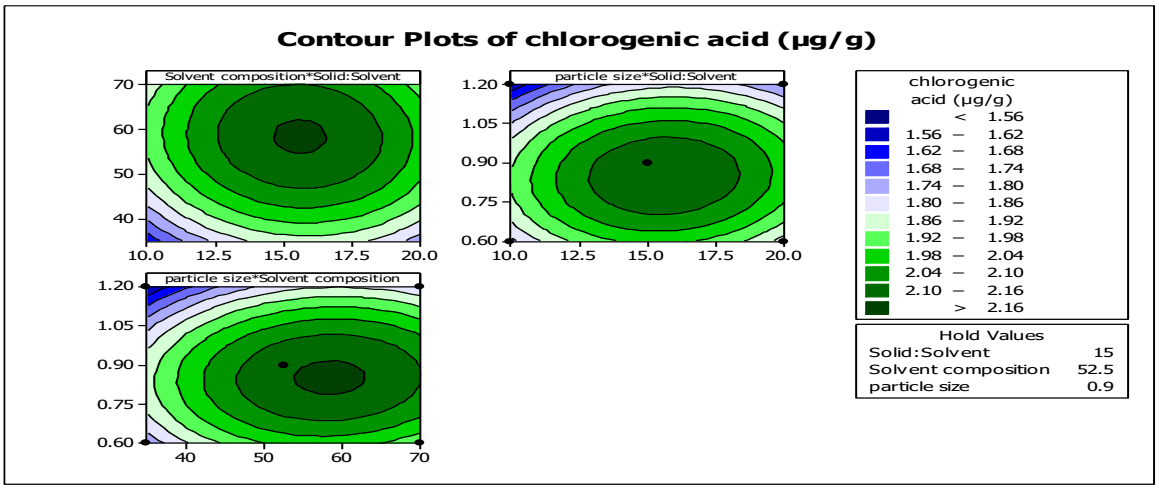


Figure 3.13. a. Contour plot for chlorogenic acid at different levels of particle size and solvent to solid ratio b. Contour plot for chlorogenic acid at different levels of solvent composition (% ethanol in ethanol-water combination) and solvent solid ratio c. A contour plot showing the scavenging activity at different solvent compositions (% ethanol in the ethanol-water mixture) and particle sizes.

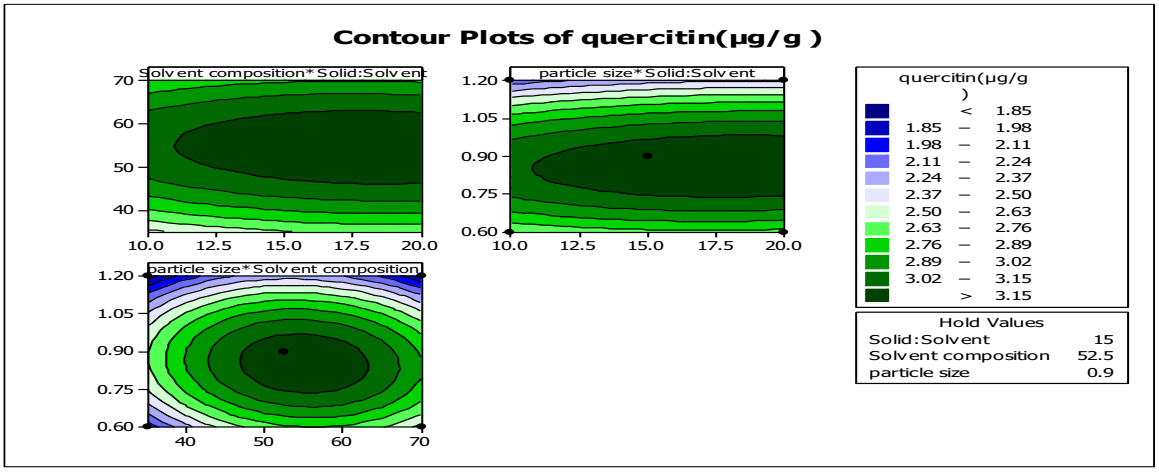


Figure 3.14. a.Contour plot for quercitin at varied levels of particle size and solvent to solid ratio b.Contour plot for quercitin at varying levels of solvent composition (% ethanol in ethanol-water mixture) and solvent solid ratio c. A contour plot showing the scavenging activity at different solvent compositions (% ethanol in the ethanol-water mixture) and particle sizes.

3.6.12.Conclusion

This study was done to optimise the extraction parameters for optimum antioxidant activity and antioxidant compound production due to the increased economic value of antioxidant compounds. In the *Stevia rebaudiana* leaf portion, the antioxidant activity as well as the antioxidant component Quercetin were determined. It demonstrated that the relationship between antioxidant activity and the antioxidant quercetin was direct and reciprocal. According to HPTLC fingerprinting, the leaf section was the most effective component for harbouring antioxidant chemicals. Three variables had effects on the antioxidant activity of the *Stevia rebaudiana* leaf when the six variables were examined using the Plackett-Burman design. Using the selected extraction variables, RSM has been successfully performed to optimise the antioxidant activity, GA, Q, and CA from Stevia rebaudiana leaf.

The optimal parameters were determined to be the solvent:solid ratio (20:1), size of particle (0.80 mm), and composition of solvent (55 percent ethanol in an ethanol-water mixture). At these optimal extraction parameter values, the maximum content of GA, Q, and CA was obtained, leading to the highest antioxidant activity of 0.27%. The antioxidant activity of stevia rebaudiana leaves was 20.43%, close to the predicted value of 20.26%.

CHAPTER-4

CHAPTER : 4

Cost effective tissue culture of *Stevia rebaudiana* by optimization of various cultural conditions

Stevia rebaudiana, a sweetener having variety of compounds in it which can be useful in various aspects The metabolites which are present in them like phenols, flavonoides and other present in them make them very useful. Due to huge demand of SGs there is need for large quantity of high yielding SGs containing *Stevia rebaudiana* for efficient agricultural production. The major challenges for large scale cultivation of Stevia plant were the low seed germination, vegetative propagation and low nodal plants for the propagation. Since the demand is very high in the current scenario ,there is need of alternatives of the production of the metabolite available from it.Plant tissue culture is widely used technique which has been used for the production of stevioside and others.There were many study conducted for the *In-vitro* production from *Stevia* by many researchers.The technique is being used for many different plants like Dioscorea for prodiction of metabolites .Due to excessive harvesting and major industrial use these plants are facing the danger of unavailability in sufficient amount of metabolites.The researchers have explored the horizon of tissue culture (Sivaram and Mukundan, 2003, Dwivedi et al 2014).

The Phytochemical analysis also plays an important role for the determination of content of available phytochemicals present in plants.There are many techniques for the analysis of these phytochemical and many researchers has also analyzed these by using different technique like ,HPLC, LC/MS, HPTLC and others.So to explore more about it,the study was carried out for the in-vitro cultures by using different plant growth hormones for sustainable production and the analysis of metabolites by HPTLC method.

There are various advantages of this technique like better stock,genetic variablility in genomes and increased metabolites can be achieved with different combinations of technique.Some researches have achieved the better growth in terms of shoot in plants .

Materials and method

4.1. Material

S. rebaudiana of same age were collected from Lovely Professional University, Punjab in the month of August 2017. Taxonomists authenticated and identified plants based on their morphology and submitted them to the Plant Biotechnology Laboratory Herbarium at Lovely Professional University., (Voucher Spicemen No. 2614-SRLPU) and the standard compounds like Reb A and Stevioside were precured and Chemicals of HPLC grade like ethanol, hydrochloric acid, chloroform, and analytical grade methanol were purchased.

4.2. culture media ,sterilization and Conditions

Young shoots of 1.5 cm were cut from healthy plants of Stevia ,it was rinsed for 30 minutes under the tap water .After this process,it was dipped in tween 20 for 10 mins and again reinsed with water for removing residue.The process of surface sterilization with 0.1 percent of mercuric chloride for four minutes followed by rinsed three to four times with sterile distilled water was done before inoculating in culture media.

MS medium having a pH of 5.8 and was supplemented with 3 percent sucrose and other plant hormones.The culture media was prepared and uniformally distributed 20ml of media in culture tube and these were transferred to autoclave for 20 minutes at 121°C and 1.06 kg cm2 pressure in an autoclave.The relative humidity of the culture room was kept at 60% and measured with a hygrometer on a regular basis.

4.3.Shoot and Root Growth

The explants from nodes were cultured on the medium containing different hormones i.e Kinetin(KIN), BA,meta-Topolin (mT), Indole-3-Acetic Acid (IAA), IBA, thidiazuron (TDZ).

MS media enriched with various concentrations,the explants were cultured on (BA), kinetin, and (2.5,1.5,2,1.5,1 and 0.5 mg /L) (KIN), (TDZ), meta-Topolin (mT) and (IBA) for direct shoot initiation (Table 1). After 4 weeks newly *in vitro* grown regenerants were sub-cultured into the rooting MS medium consists of various concentrations (2.5,1.5,2,1.5,1 and 0.5 mg /L) of mT and Indole-3-Acetic Acid (IAA) for four to five weeks (Table 2).

4.4.Acclimatization

When there is significant growth of roots in culture medium, these are transferred in the pots for the acclimatization. There were washed properly three times to remove any agar residue and then

they were transferred to cups having mixture of manure, sand and soil in 1:1:1 proportion and maintained at 28 degree Celsius. The plants which has been acclimatized were transferred to greenhouse after two weeks.

4.5. Phytochemical analysis

4.5.1. Sample preparation

Leaf samples of *S. rebaudiana* from plant and *in vitro*-raised plantlets were dried under shade. With the use of electric mixer grinder (Philips, India), the samples were ground to a fine powder. Each 1 g sample was placed in individual 50 ml culture tubes. To each sample, 30 mL of 70% ethanol was added, and the tubes were covered with perforated aluminum foil and agitated for three days. The resulting extract was passed using Whatman filter paper No. 1 and then dried. The dried extract was mixed with methanol, filtered using a syringe filter, and subsequently used for quantitative HPTLC analysis.

4.5.2. HPTLC analysis

The HPTLC analysis of *Steviol glycosides ST and REB A)* and antioxidant compounds (gallic acid (GA) and chlorogenic acid (CGA)) were done by method (Pandey et al 2015). stock solution of these standards were dissolved of 10mg in 10 ml of methanol. The HPTLC system was used and samples were place form of 6mm band on 20 X10 cm prepared on silica gel 60 F254 TLC plates were used as the stationary phase. A densitometric scan was conducted at 433 nm and it showed the presence of the reference compounds in samples.

4.6. Statistical analysis

After 28 days of culture ,the recording of data was done on parameter like response percentage with average length of shoot and numbers .After inoculation for 28 more days,the rooting was observed and recorded. The experiments were conducted in triplicates with twelve explants in each replication for the treatment.Stastical analysis was done by SPSS Software using DMRT(Dunca's Multiple Range Test) was used to determine the significance of variations between means at p = 0.05%.

4.7. Result and discussion

4.7.1.Shoot Multiplication

For the purpose of *S. rebaudiana* micropropagation, a reliable and efficient in vitro regeneration protocol was created. More successfully than nodal segments cultured on PGR-free medium (control), nodal segments cultured in vitro on MS medium supplemented with (BA), (Kin), thidiazuron (TDZ), meta-topolin (mT), and (IBA) developed. This implies that exogenous PGR supplementation promoted rapid shoot regeneration from nodal explants.For 28 days ,the culture was maintained without PGR and with different concentration of PGR .

The cytokinins (BAP and mT) and other hormones were treated in different concentrations ranging from 0.5-2.5 mg/l,showed significant difference in terms of shoot number ,shoot length according to the analysis of variance(Table 5.1).mT 1.5 and 2 mg/l showed good growth in terms of culture response, shoot formation, and multiple shoot induction, even though both mT and BAP promoted shoot initiation and multiplication independently.After 28 days of culture 2mg/l mT resulted in 11.77 ± 0.28 shoots and 4.96 ± 0.11 cm average length (with 100% culture response). However, with the medium containing TDZ (0.5 mg /L), there was a poor response in terms of shoot growth (46.68%), shoot number (1.80 ± 0.20), and length of shoots (2.20 ± 0.65 cm) .

In the production of micropropagation, mT has been considered as a possible substitute for BA.With 0.5 mg /l to 2.5 mg/l mT, the shoot regeneration rate of 6.23 to 11.77 shoots per explant with lengths ranging from 2.71 to 4.96 cm was obtained. A same kind of results were observed in Scaevola taccada (Gaertn.) Roxb., where 1.5 mg L-1 mT produced the greatest number of axillary shoots when compared to BAP (Shekhawat et al, 2021). Meta-Toplin induced maximum shoot numbers (23.36 shoots per explant) from 90.66% of *Sesamum indicum* explants.An aromatic cytokinin called meta-topolin was initially discovered in poplar leaves in the 1990s (Strnad, 1997). Different from benzylaminopurine's (BAP) metabolism.Meta-Topolin enhanced the multiplication rate and shoot quality of microshoots by enabling them to elongate, which increased the number of shoots accessible for roots (Wojtania, 2010; Gentile et al., 2014; Podwyszyska and Cieliska, 2018). It was observed that when TDZ concentration increased, multiple shooting were increased . According to Mithila et al. (2003), low concentrations of TDZ caused African violet explants to develop shoots, whereas higher concentrations (5–10 lM) caused the development of somatic

embryos. A relatively small dose of TDZ, compared to other agents with cytokinin action, encourages shoot expansion in a number of plants

In *Ribes grossularia* with the replacement of BAP, mT facilitated cyclical micropropagation and microshoots elongation, proficient rooting and increased shoot number from 1.8 to 2.8 compared to BAP (Kucharska et al., 2020). Meta-topolin and its derivatives, according to Bairu et al. (2007), can be used to replace BA and zeatin in terms of multiplication rate and rooting.

BA showed at 2mg/l significant shoot regeneration (7.93 shoots/explant) was seen, with an average shoot length of 3.20 cm. With 7.64 shoots per explant, BA at 1.5 mg L-1 likewise demonstrated excellent culture response (91.36%). Lower BA doses (0.5 and 1.0 mg L-1) had a reduced effect on shoot regeneration Thiyagarajan and Venkatachalam (2012) reported BAP 1.0 mg L^{-1} best for *in vitro* multiple shoot regeneration of *Stevia rebaudiana.* Shoot regeneration was observed in medium fortified with different doses of IBA (0.5 mg L^{-1} to 2.5 mg L^{-1}) and 83.32% response was observed at 2.0 mg L^{-1} IBA producing 5.18 ± 0.22 average shoot numbers. At low IBA doses low culture response, shoot numbers and length was observed. Beyond optimal concentration (2.0 mg L^{-1}) there was decrease in shoot regeneration. Less shoot regeneration and multiplication was found in medium supplemented with KIN and TDZ.

In this study, we discovered that mT is more effective in producing maximum number of shoots with good percentage of culture response and shoot length than BA, IBA, KIN, and TDZ. While Sivaram and Mukundan (2003) showed that nodal explants developed more shoots in BAP + IAA than kinetin + IAA. Shekhawat et al. (2021), found mT best for *in vitro* regeneration of *Dioscorea pentaphylla* and *Scaevola taccada*. In summary, it is clear that the optimal level and composition of PGRs are critical for shoot induction and multiplication in this plant, which can be influenced by explant source, cultivation duration, genotypes, and other factors.

Table 4.1: *28 days of culture response by using PGR*

MS + growth regulators (mg/l)							
BA	**KIN**	**TDZ**	**mT**	**IBA**	% culture response	Shoots/explant	Length of shoots(cm)
0	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

0.5	-	-	-	-	56.43 ± 0.20^{m}	4.70 ± 0.25^{g}	2.30 ± 0.60^{n}
1.0	-	-	-	-	66.70 ± 0.20^{j}	5.40 ± 0.21^{e}	$2.51 \pm 0.65^{m,n}$
1.5	-	-	-	-	91.36 ± 0.15^{c}	$7.64 \pm 0.18^{c,d}$	$2.72 \pm 0.67^{k,l}$
2.0	-	-	-	-	94.53 ± 0.25^{b}	7.93 ± 0.27^{c}	3.20 ± 0.75^{g}
2.5	-	-	-	-	$81.23 \pm 0.15^{e,f}$	6.29 ± 0.22^{d}	3.13 ± 0.70^{h}
-	0.5	-	-	-	$56.03 \pm 1.02^{m,n}$	1.41 ± 0.20^{p}	$2.96 \pm 0.70^{i,j}$
-	1.0	-	-	-	63.01 ± 1.00^{k}	$2.55 \pm 0.13^{l,m}$	2.76 ± 0.70^{k}
-	1.5	-	-	-	69.35 ± 0.60^{i}	$3.76 \pm 0.28^{i,j}$	3.90 ± 0.75^{c}
-	2.0	-	-	-	73.01 ± 2.02^{h}	3.95 ± 0.28^{h}	3.76 ± 0.61^{d}
-	2.5	-	-	-	$67.60 \pm 0.20^{i,j}$	2.63 ± 0.50^{l}	2.23 ± 0.55^{o}
-	-	0.5	-	-	46.68 ± 1.14^{p}	1.80 ± 0.20^{o}	2.20 ± 0.65^{p}
-	-	1.0	-	-	$49.83 \pm 1.60^{o,p}$	1.83 ± 0.22^{n}	2.98 ± 0.55^{i}
-	-	1.5	-	-	$54.35 \pm 0.56^{n,o}$	$2.14 \pm 0.28^{m,n}$	$3.62 \pm 0.70^{e,f}$
-	-	2.0	-	-	57.07 ± 1.02^{l}	2.83 ± 0.14^{k}	$3.83 \pm 0.65^{c.d}$
-	-	2.5	-	-	50.46 ± 0.25^{o}	1.90 ± 0.18^{n}	3.51 ± 0.55^{f}
-	-	-	0.5	-	80.01 ± 2.00^{f}	$6.23 \pm 0.17^{d.e}$	2.71 ± 0.02^{l}
-	-	-	1.0	-	86.35 ± 2.29^{d}	$8.16 \pm 0.28^{b,c}$	3.63 ± 0.06^{e}
-	-	-	1.5	-	$93.01 \pm 0.97^{b,c}$	9.83 ± 0.20^{b}	4.73 ± 0.01^{b}
-	-	-	2.0	-	99.02 ± 2.49^{a}	11.77 ± 0.28^{a}	4.96 ± 0.11^{a}
-	-	-	2.5	-	$89.56 \pm 0.20^{c,d}$	8.53 ± 0.01^{b}	$4.61 \pm 0.13^{b,c}$
-	-	-	-	0.5	$62.01 \pm 1.02^{k,l}$	2.16 ± 0.21^{m}	2.67 ± 0.02^{l}

-	-	-	-	1.0	74.01 ± 1.08^{f}	3.87 ± 0.18^{i}	2.56 ± 0.01^{m}
-	-	-	-	1.5	77.01 ± 1.02^{g}	4.92 ± 0.13^{f}	2.62 ± 0.01^{l,m}
-	-	-	-	2.0	83.32 ± 1.05^{e}	5.18 ± 0.22^{e,f}	2.84 ± 0.01^{j}
-	-	-	-	2.5	79.01 ± 1.02^{f,g}	4.51 ± 0.28^{g,h}	2.83 ± 0.01^{j,k}

4.7.2. *Rooting of shoots*

The shoots grown on cultured tubes were cultivated on rooting medium supplemented with different amounts of IAA and mT. As indicated in Table 2. Roots grown within two weeks of subculturing, with the exception of PGR free media. Root initiation was directly correlated with mT and IAA concentration, as Table 2 illustrates. Maximum rooting (97.53%) was noted in MS media that had 1.5 mg/l mT added to it. (Figure 1 C).Concentration 1.0 mg L^{-1} mT also showed good culture response (91.24%) with 6.48 ± 0.19 average number of shoots per explant. As the concentration increases to 2.0 mg L^{-1} mT slight decrease in the root regeneration was observed. Out of the various IAA dosages, the cultures responding to 1.0 mg L-1 and 1.5 mg L-1 showed 73% and 79%, respectively, with 3.16 ± 0.05 and 3.70 ± 0.10 shoots per explant. The root number and culture response differ significantly when mT and IAA are compared for root induction. As a result, when mT was used at an appropriate concentration, the root growth parameters increased significantly in comparison to when IAA was used for rooting. (Table 2). Hence, mT (1.5 mg/l) was selected to be fit for root growth. Various researchers have used different PGRs for *in vitro* rooting of *Stevia rebaudiana* (Ahmad et al., 2007).Similar to shooting,all data were recorded twelve explants per treatment which were represented Mean ± standard error.

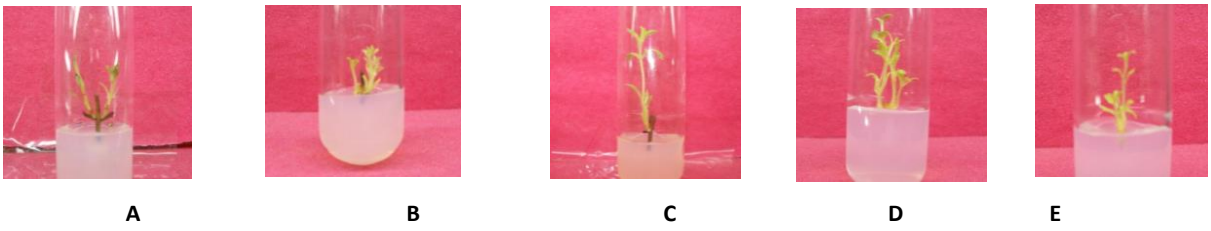
4.7.3. Acclimatization

The tissue cultured plants from all treatments of rooting were successfully acclimatised (96%) in polybags containing a 1:2 mixture of sand:soil. The plantlets were effectively developed in the greenhouse (25 2C) under natural photoperiod circumstances after 1 month (Fig. 1g, h). The plantlets were kept in a controlled setting for 1 month under natural photoperiod circumstances to allow the plantlets to acclimatized to greenhouse conditions. The survival rate was comparable to those earlier reported for this plant (80–95%) by various researchers .Plantlets were transferred to

the field after a 6-week acclimatisation period. The protocol utilised provided a high percentage of survival in the field of this commercially important species.

Table 4.2: Effects of IAA on *in vitro* root generation

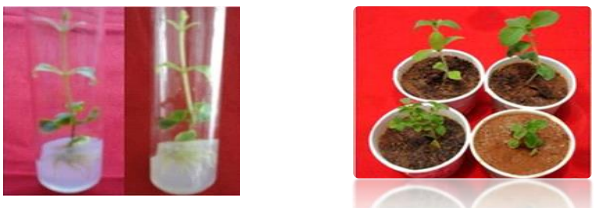
MS + PGR (mg/l)		Response (%)	Rooting/explant	length of roots (cm)
mT	**IAA**			
0.5	-	76.00 ± 0.19^{e}	4.96 ± 0.15^{d}	1.96 ± 0.11^{f}
1.0	-	91.24 ± 0.21^{b}	6.48 ± 0.19^{b}	3.48 ± 0.09^{b}
1.5	-	97.53 ± 0.18^{a}	7.91 ± 0.21^{a}	4.91 ± 0.22^{a}
2.0	-	81.12 ± 0.23^{b,c}	5.82 ± 0.25^{c}	3.12 ± 0.27^{c}
	0.5	64.00 ± 1.00^{g}	2.06 ± 0.05^{g}	1.06 ± 0.31^{h}
	1.0	73.33 ± 1.53^{f}	3.16 ± 0.05^{f}	2.48 ± 0.19^{e}
	1.5	79.00 ± 1.00^{d}	3.70 ± 0.10^{e}	2.91 ± 0.23^{d}
	2.0	57.00 ± 2.00^{g,h}	2.01 ± 0.11^{h}	1.82 ± 0.25^{g}



A B C D E

S.rebaudiana In vitro multiplication from nodal explant on MS media supplemented with Kin.A.After 7 day B.20 days C.45days of culture

D,E-Multiple shoot formation on MS media supplemented with TDz .



F ,G

F,G-Development of rooted plant on mT.
G-Two weks old hardened plants

Different Stages of Micropropogation of Stevia

Figure4.1 : *Different Stages of Micropropogation of Stevia: In vitro shoot regeneration*

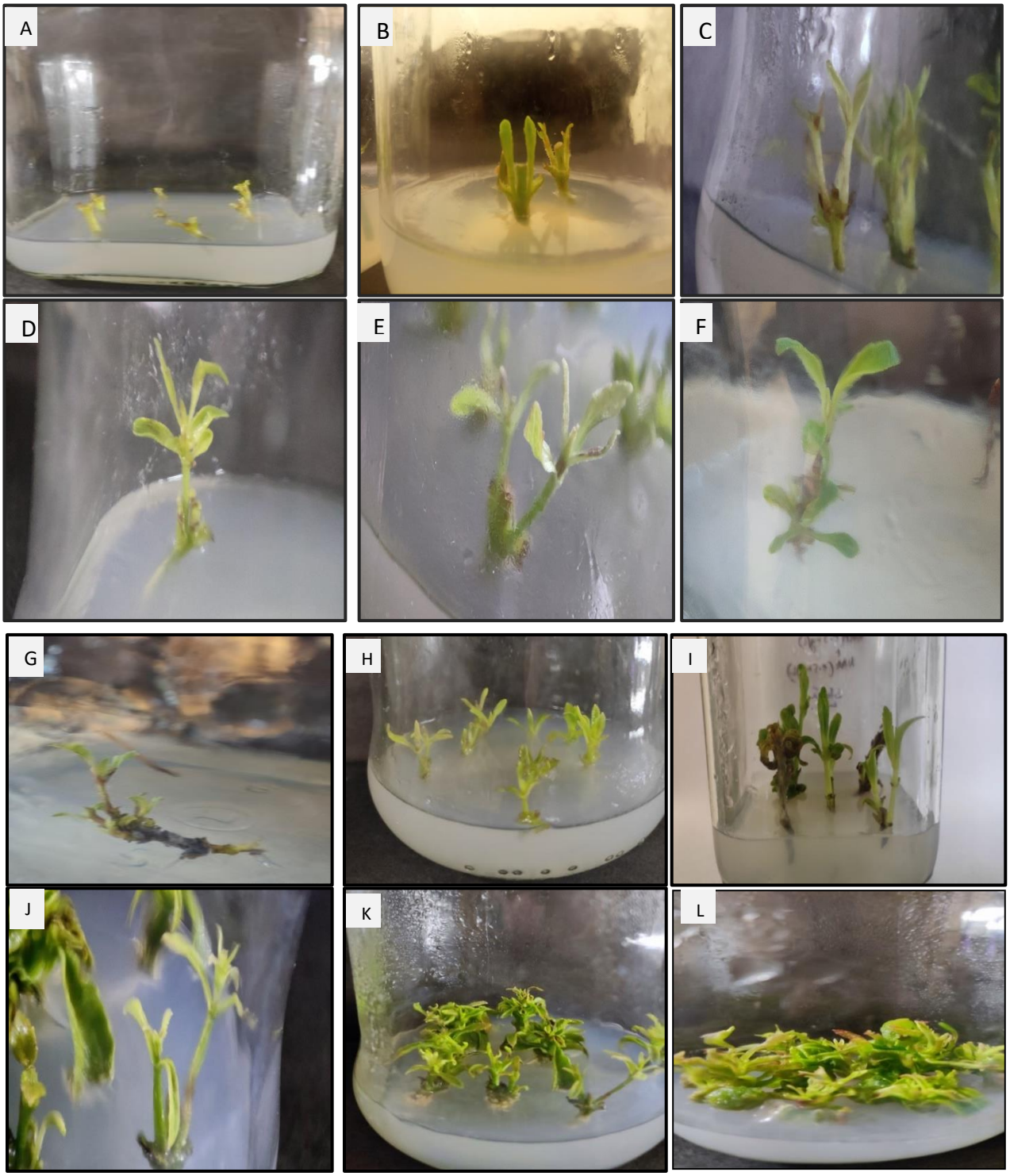


Figure 4.2 : ***MS media(A,B-Shoot initiation from nodat explant,K,L-shoot multiplication and elongation,(A).BA0.5mg/l (B,C)KIN1 mg/l (D,E)IBA 1.5mg/l (G.H)KIN 1.5 mg/l (I,J)TDZ 1.5mg/l (K,L)2.0 mg/l***

4.7.5. Phytochemical fidelity

An proposed tissue culture process's efficiency is determined by how uniformly the plants and tissue culture regenerants exhibit the same level of phytochemistry. The metabolites that are available in plants give them their therapeutic qualities. In the current study, antioxidant compounds (gallic acid and chlorogenic acid) and SGs (ST and Reb A) were quantified using HPTLC. Stevioside was detected in the stevia plant at 4.79% and in the in vitro plantlets at 5.85%. In vitro plantlets, about 2.13% Rebaudioside A was found, while in the mother plant, 1.70%. Additionally, CGA was detected in 0.033% of the mother plant and 0.049%) of the in vitro regenerants, indicating a slight increase in this compound in the in vitro plantlets. GA was measured in both the mother plant and the in vitro plants, and the results showed that the mother plant had 0.026% GA and the in vitro plants had 0.031% GA. in Table 4.3.

The secondary metabolite which is produced during in-vivo culture in terms of Stevioside is 4.7% and Reb A is 1.7% while in case of In-vitro cultue it terms of Stevioside is 5.8% as well as Reb A is 2.1% .The content of Stevioside and Rebauioside A has been increased in *In-vitro* cultures in comparision to In vivo Culture .Similar results were found in callus culture exposed to stevioside.

Table 4.3: Stevioside, rebaudioside A, Chlorogenic acid and Gallic acid content

Compound	*In vivo* (%)	*In vitro* (%)
Stevioside	4.79 ± 0.008	5.85 ± 0.006
Rebaudioside A	1.70± 0.009	2.13± 0.005
Chlorogenic acid	0.033± 0.006	0.049 ± 0.006
Gallic acid	0.026± 0.006	0.031± 0.006

4.8. Conclusion

Tissue culture technology was used to produce a practical, easy, effective, or adaptable method to produce mass multiplication of *Stevia rebaudiana*, a sweetener plant. In shoots and root regeneration, our research found that mT resulted in improved shoot proliferation and root induction. The protocol will aid in the long-term utilisation of the species without causing harm to

its native habitat. The in vitro regenerants survived well.As a result, in vitro produced plants appear to be superior for secondary metabolite extraction, as they displayed a wider diversity of phytochemicals. The findings could aid in the development of a more effective micropropagation method for large scale plant production with a greater diversity of bioactive compounds while reducing unpredictability.

CHAPTER : 5

Biotechnological Strategies for production of secondary metabolite by using biotic and abiotic elicitors and precursors

Stevia rebaudiana belongs to Asteraceae family which is an important medicinal plant..The secondary metabolite produced by stevia have commercial use.There are other secondary metabolite which are of importance like ajmalicin, paclitaxel and shikonin (Mehravaran et al., 2021).Stevia is generally propagated through seeds but there is limitations through it,as the rate of germination is low.So,to overcome this problem,Plant tissue culture is widely used.There are many plants where secondary metabolites are obtained through this technique. To enhance tissue culture for the production, elicitors are widely used (Kaur et al., 2020)

Elicitors can increase the secondary metabolites production by doing some physiological changes in the organism and changes in the metabolite biosynthesis of plants.In order to accumulate the secondary metabolite . The secondary biosynthetic pathway are activated by them, so they can enhance the production (Zehra et al., 2021; Ahmad et al., 2020, Pradhan et al., 2020; Dey et al., 2013,)The only parts of *S. rebaudiana* leaves that can produce SGs are the mesophyll cells. Because chloroplasts are necessary for precursor synthesis, glycoside levels in tissue lacking chlorophyll, such as roots and lower stems, are extremely low (Singh and Rao 2005). There are many researchers worked to enhance the SG in *stevia(*Jain et al., 2020; Blinstrubienė et al., 2020; Ramezani et al., 2020)

In medium growth of plants was induced in presence of elicitors to know the effects and the way of interactions among them for the production of important compounds in plant..

Tissue culture of *Stevia* in presence of elicitors may produce the high shoot growth as well as steviol glycosides production. So it may help to achieve the better commercial production with the increase in demand in the market. After many years of use, *in vitro* culture techniques are now able to produce quality plantlets, transform the plant genome, produce secondary metabolites with significant commercial value, and multiply threatened plant genotypes rapidly to achieve our goal of sustainable growth.

5.2. Material and Method

5.2.1. Shoot cultures *In-vitro*

The shoots of *Stevia rebaudiana* were produced in tissue culture by the nodal explants collected from herbal garden LPU, Phagwara, Punjab, India. Shoot cultures grown from nodal explants in a statistically optimised MS (Murashige and Skoog) medium having 4mg/l BA(Benzyladenine) and 8mg/l IBA(Indole butyric acid).

5.2.2.Preperation of Elicitors

SA and MJ (Salicylic acid and Methyl jasmonic acid) stock solutions were made. The acids were dissolved in distilled water to bring the pH down to 5.8 in order to create stock solutions. Gibberlic acid stock solution was prepared. The weighed amounts of each chemical were separately dissolved in a small amount of absolute alcohol to make the stock solution, to which the necessary amount of distilled water was added. Different concentrations of GA3 were prepared as a solution.

Glacial acetic acid 2ml was added in dropwise manner to 1 g chitosan for 15 min at 60 °C.the final volume was made 100ml and 1 M NaOH was used to adjust the pH to 5.8. Before being used as an elicitor, these solutions were autoclaved at 120 °C for 20 minutes.

Table 5.1:Type of Elicitor and Precursor

S.No	Elicitor	Category
1	Chitosan	Biotic
2	Methyl jasmonic acid (MJ)	Abiotic
3	Salicylic acid (SA)	Abiotic
Precursor		
4	Gibberellic Acid($GA_{3)}$	Abiotic

5.2.3. Screening of elicitors

The Nodal explants were cultured for 4 weeks .These were further subcultured on proliferative media for 4 weeks for growth. Elicitation was achieved by transferring 8 week-old shoot cultures

to 20 ml of optimised media and supplementing with the required elicitor concentration.These were cultured in triplicate in culture bottles of MS media in 20 ml enriched with with salicylic acid (SA: 100–200 M), chitosan (CH: 100–200 M), and methyl jasmonate (MJ: 100–200 M) with ten explants in each replication. In growing environment with a cool white fluorescent light (20 mol/m2 s), all of the cultures were kept at 25 °C. On the 14th day of growth, the biomass of shoot culture and the content of SGs in control and elicitor-treated flasks were measured. Each replication's fresh biomass weights were determined by accumulating all of the plantlets. All plantlets were harvested and dived leaves and other parts to identify fresh biomass weight of each replication.After freeze drying ,dry weight(DW) for each application were determained. HPTLC method the contents of stevioside and rebaudioside A were examined.

5.2.4 HPTLC sample preparation

For qualitative and quantitative estimation of (SGs) in tissue cultured grown plantlets, shoots were washed under tap water to washout all the traces of culture media. For extraction, Microwave assisted extraction (MAE) with 10 mL of ethanol was used by following Nawaz et al., 2022 method three times for 20 minutes on the leaf samples (about 100 mg). Prior to HPTLC examination, the samples were processed through 0.45 m PTFE filter to eliminate any remaining particles. Next, 10 mL of the methanol solvent were used to dilute 1 mL of the stock solution.

5.2.4.1.HPTLC detection analysis

Using HPTLC System analysis was done the plates were developed with the mobile phase (ethyl acetate-methanol: water; 8.5-1.0-0.5 (v/v/v) after drying. Anisaldehyde-sulphuric acid reagent (1 mL anisaldehyde mixed with 20 mL glacial acetic acid, 175 mL methanol, and 7 mL concentrated sulphuric acid) was used to post-derivatize the plate for 10 seconds in a CAMAG immersion chamber. The plate was then heated for 5 minutes at 110 °C (dry plate). The plates were scanned at 530 nm reflectance wavelength after band formation. Slit size was 4.00 x 0.30 mm, scanning speed was 20 mm/s, and data resolution was 100 m/step for densitometric scanning. ST and Reb A were quantified using calibration curves made by an SGs mixture that also contained standards.

5.2.5. Response Surface Methodology

20 Sets were carried out in duplication using MINITAB .Salicylic acid, chitosan, and methyl jasmonate were evaluated for their effects on the total synthesis of Steviol glycosides and fresh

biomass using the Response Surface Methodology. A Central Composite Design (CCD) with 6 axial points, 6 duplicates and 8 quadrant points at the center points was created using the software Minitab 15 (Table 5.2).

Table 5.2. CCD for experimental and predicted values of fresh biomass (g) and steviol glycosides (SGs)

Run	Elicitor variables			Fresh biomass (g)		Stevioside Yield (%)		Rebaudioside Yield (%)	
	MJ (µM) (A)	SA ((µM) (B)	CS (µM) (C)	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1.	100.00	100.00	100.00	2.51	2.51	5.77	5.76	1.31	1.31
2.	200.00	100.00	100.00	2.45	2.44	8.03	8.00	1.77	1.77
3.	100.00	200.00	100.00	2.3	2.29	6.78	6.89	1.43	1.42
4.	200.00	200.00	100.00	2.79	2.78	8.45	8.57	1.87	1.87
5.	100.00	100.00	200.00	2.22	2.21	7.02	6.97	1.57	1.57
6.	200.00	100.00	200.00	2.37	2.36	7.78	7.73	1.75	1.76
7.	100.00	200.00	200.00	2.35	2.35	8.12	8.21	1.58	1.58
8.	200.00	200.00	200.00	3.08	3.07	8.35	8.42	1.77	1.77
9.	65.91	150.00	150.00	2.18	2.18	6.48	6.42	1.55	1.55
10.	234.08	150.00	150.00	2.71	2.72	8.51	8.47	2.1	2.10
11.	150.00	65.91	150.00	2.36	2.36	7.09	7.19	1.59	1.57

12.	150.00	234.08	150.00	2.77	2.77	8.94	8.72	1.66	1.66
13.	150.00	150.00	65.91	2.61	2.61	7.34	7.25	1.5	1.50
14.	150.00	150.00	234.08	2.6	2.60	8.15	8.13	1.64	1.63
15.	150.00	150.00	150.00	3.14	3.14	9.76	9.81	2.23	2.28
16.	150.00	150.00	150.00	3.18	3.14	9.98	9.80	2.34	2.28
17.	150.00	150.00	150.00	3.16	3.14	9.67	9.80	2.21	2.28
18.	150.00	150.00	150.00	3.11	3.14	9.76	9.80	2.27	2.28
19.	150.00	150.00	150.00	3.15	3.14	9.88	9.80	2.29	2.28
20.	150.00	150.00	150.00	3.12	3.14	9.78	9.80	2.35	2.28

5.3. Results and discussion

5.3.1. Screening of elicitors

As a result of plant defence response activation, the overproduction of secondary metabolites has been well documented for the elicitor addition. On the content of DW and SGs, the impacts of various elicitors (individually, 20 sets of experiment, Table 5.2) are discussed. The culture tubes of *Stevia rebaudiana* showed elicited shoot cultures and control plants.

5.3.2. Response Surface Methodology

Salicylic acid(SA), and chitosan(CH), methyl jasmonate(MJ) were evaluated for their effects on the total synthesis of Steviol glycosides and fresh biomass using the Response Surface Methodology.20 sets of experimrnt was conducted using Minitab software 15,having eight quadrant points,six axial points, six duplicate at centre with CCD(centralcomposit design).To

calculate the SGs production as a function of the test variables, the regression equation below was used (A, B, C)

Y1 (Stevioside) = 9.8078 + 0.61025 {A} + 0.45481 {B} + 0.26377 {C} - 0.83562 $\{A\}^2$ - 0.65177 $\{B\}^2$ - 0.74723 $\{C\}^2$ - 0.14000 {AB} - 0.36750 {AC} + 0.03000 {BC} **(1)**

Y2 (Rebaudioside) = 2.28183 + 0.16072 (A) + 0.02693 (B) + 0.03848 (C) - 0.16255 $(A)^2$ - 0.23326$(B)^2$ - 0.25271 $(C)^2$ - 0.00125 (AB) - 0.06625 (AC) - 0.02375 (BC) **(2)**

Y3 (Fresh Biomass) = 3.14310 + 0.16119 (A) + 0.12152 (B) - 0.00343 (C) - 0.24540 $(A)^2$ - 0.20297 $(B)^2$ - 0.18883 $(C)^2$ + 0.14125 (AB) + 0.05625 (AC) + 0.08875 (BC) **(3)**

where A is the methyl jasmonic acid , B is the salicylic acid , and C is the chitosan . In order to create contour plots to investigate the response to different elicitor interactions for various conc of elicitors the model equation describing the accumulation of SGs was used.

Table 5.3. Estimated Regression Coefficients for Stevioside, Rebaudioside and Fresh biomass (coded units)

Term	Stevioside			Rebaudioside			Fresh biomass		
	Coef ± S.E. Coef	**T**	**P**	**Coef ± S.E. Coef**	**T**	**P**	**Coef ± S.E. Coef**	**T**	**P**
Constant	9.807 ± 0.054	180.104	0.000**	2.281 ± 0.016	136.444	0.000**	3.143 ± 0.007	398.630	0.000**
A	0.610 ± 0.036	16.890	0.000**	0.160 ± 0.011	14.485	0.000**	0.161 ± 0.005	30.812	0.000**
B	0.454 ± 0.036	12.588	0.000**	0.026 ± 0.011	2.427	0.036	0.121 ± 0.005	23.228	0.000**
C	0.263 ± 0.036	7.300	0.000**	0.038 ± 0.011	3.468	0.006**	-0.003 ± 0.005	-0.655	0.527
A × A	-0.835 ± 0.035	-23.758	0.000**	-0.162 ± 0.011	-15.049	0.000**	-0.245 ± 0.005	-48.187	0.000**
B × B	-0.651 ± 0.035	-18.531	0.000**	-0.233 ± 0.011	-21.595	0.000**	-0.202 ± 0.005	-39.856	0.000**

C × C	-0.747 ± 0.035	-21.245	0.000**	-0.252 ± 0.010	-23.396	0.000**	-0.188 ± 0.005	-37.079	0.000**
A × B	-0.140 ± 0.047	-2.966	0.014	-0.001 ± 0.014	-0.086	0.933	0.141 ± 0.006	20.665	0.000**
A × C	-0.367 ± 0.047	-7.785	0.000**	-0.066 ± 0.014	-4.570	0.001**	0.056 ± 0.006	8.230	0.000**
B × C	0.030 ± 0.047	0.635	0.539	-0.023 ± 0.014	-1.638	0.132	0.088 ± 0.006	12.984	0.000**

Where A_= Methyl Jasmonate; B = Salicylic Acid; C = Chitosan; Coef = Regression Coefficients;

Table 5.4. ANOVA for Stevioside, Rebaudioside and Fresh biomass.

		Stevioside			**Rebaudioside**			**Fresh biomass**		
Source	**DF**	**Seq SS**	**F**	**P**	**Seq SS**	**F**	**P**	**Seq SS**	**F**	**P**
Regression	9	30.3892	189.40	0.000**	2.1801	144.07	0.000**	2.4595	731.20	0.000**
Linear	3	8.8610	165.67	0.000**	0.3829	75.91	0.000**	0.5566	496.46	0.000**
Square	3	20.2838	379.25	0.000**	1.7576	348.45	0.000**	1.6549	1476.02	0.000**
Interaction	3	1.2444	23.27	0.000**	0.0396	7.86	0.000**	0.2479	221.13	0.000**
Residual Error	10	0.1783			0.0168			0.0037		
Lack-of-Fit	5	0.1191			0.0007	0.05	0.998	0.0004	0.12	0.981
Pure Error	5	0.0591	2.01	0.230	0.0160			0.0033		
Total	19	30.5675			2.1969			2.4633		

** Very significant, where $p < 0.01$

One elicitor was kept constant ,response surface was made and examined all the possible scenarios for the combination of elicitors to establish the best concentration and te examination of the combination of elicitor for the SG production. . Some of these Contour plots are depicted in Figures 2-4, with one variable held constant and 2 variables have been changed in experiment range (MJ-SA,CH-SA,MJ-CH) .The comparative countor plots for fresh biomass (g), % o have been shown

of rebaudioside and stevioside at different concentrations (Fig 5.2) of methyl jasmonates, salicylic acid and chitosan.

Based on the several response curves obtained, the predicted concentrations of all elicitors (MJ, (MJ, SA and CH) at 172 µM, 158 µM and 152µM respectively for maximum fresh biomass production is 3.14 g and % yield of stevioside, and rebaudioside A is 9.80%, and 2.28%, respectively. Experiments employing the optimum doses of the elicitors were undertaken to confirm the expected findings of the model, and maximum values for fresh biomass, stevioside and rebaudioside A are 3.18 g, 9.98 % and 2.35 %, respectively representing 98-99.9% validity of the projected model. When the elicitor was added indivisually the result was less but in combination it was more like its 1.8 time larger than the highest increase achieved in adding single elicitor (8.9).The reasons for the different effects of these combination elicitors on SG production are still unknown A. The significance of each of the coefficients is determined by the P value, which reflects the size of the effect of distinct factors and their interactive actions. Table 2 shows that MJ (A), SA (B) and CH (C) had substantial individual effects, quadratic effects of A^2, B^2, C^2, and interactive effects of MJ and CH on stevioside and rebaudioside A production. Each elicitor in this study has a unique mechanism of action, and determining it for each combined treatment may be difficult . SA play an important role for SG synthesis at higher doses, except when combined with the highest concentrations of MJ and CH (Table 1). Vazquez-Hernandez et al., 2019 report similar significant effects of SA on the production of steviol glycoside compounds that are related to the gene expression of biosynthetic pathways. When all three elicitors were combined at their highest concentration, SG synthesis was reduced, indicating that they inhibit the biosynthetic machinery of S. rebaudiana microshoots. Different elicitors have different effects on cell physiology and defense chemical activation, resulting in either negative or additive interactions (Ramezani et al., 2020). The One Variable at a Time (OVAT) optimization technique, on the other hand, is time consuming and does not allow for the simultaneous investigation of multiple elicitors. A successful strategy is to use statistical experimental design to investigate the numerous interactions between all process components.

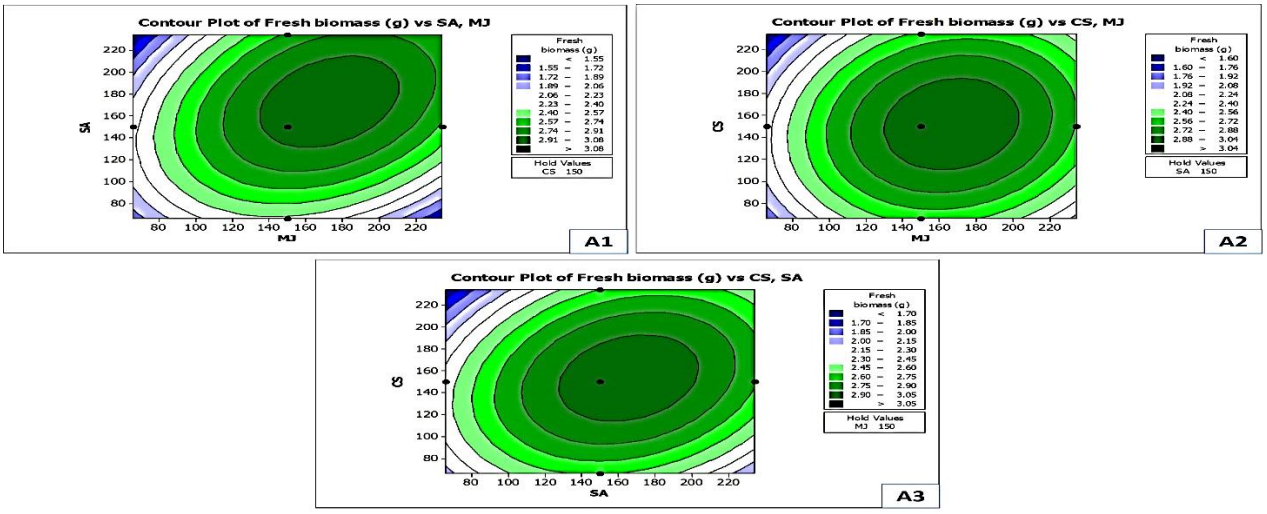


Figure 5.1: Contour plots for Fresh Biomass (g) of *Stevia rebaudiana* at different concentrations of (MJ), (SA) and (CS).

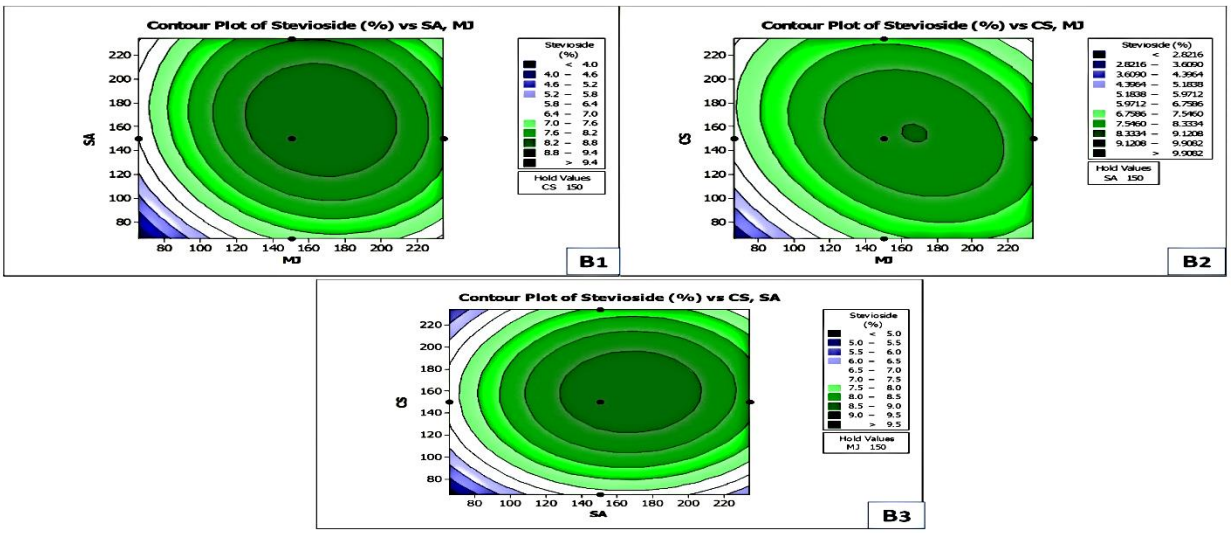


Figure 5.2: Contour plots for Stevioside (%) in *Stevia rebaudiana* at different concentrations of (MJ), (SA) and (CS).

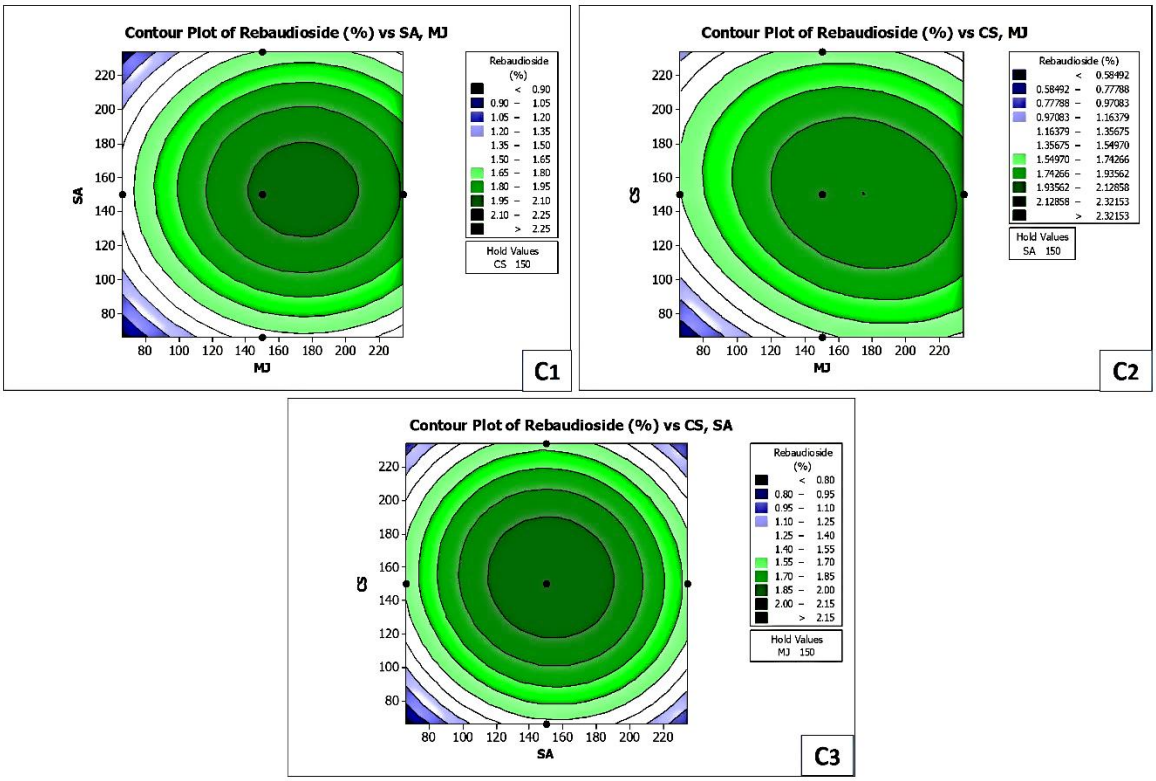


Figure *5.3*: **Contour plots for Rebaudioside (%) in *Stevia rebaudiana* at different concentrations of (MJ), (SA) and (CS).**

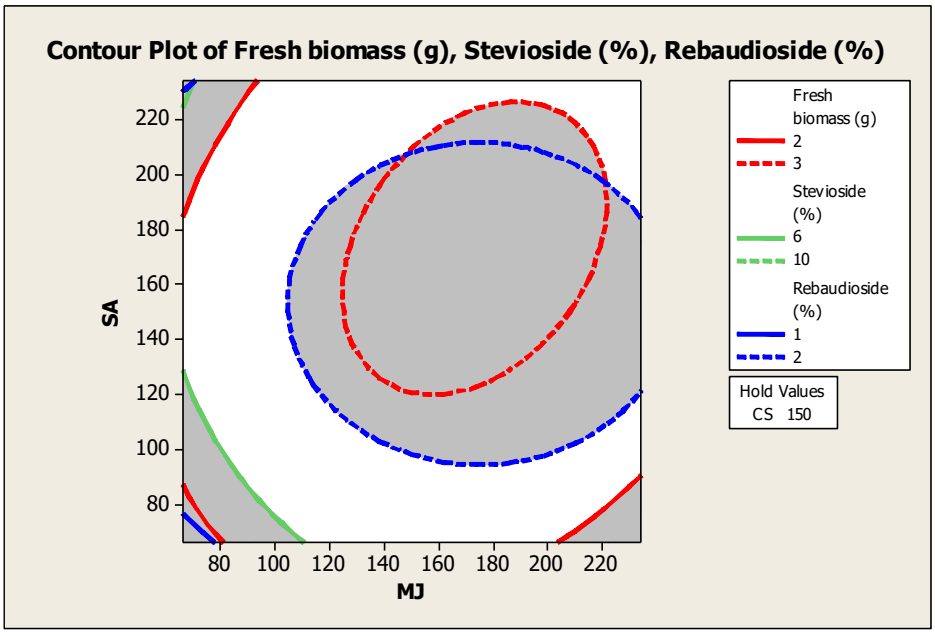


Figure *5.4*: **Comparative contour plots for Fresh Biomass (g), % of Stevioside, and Rebaudioside in *Stevia rebaudiana* at different concentrations of (MJ), (SA) and Chitosan (CS).**

Fig 5.5: Preliminary experiment to set the level of CS, MeJA and SA.

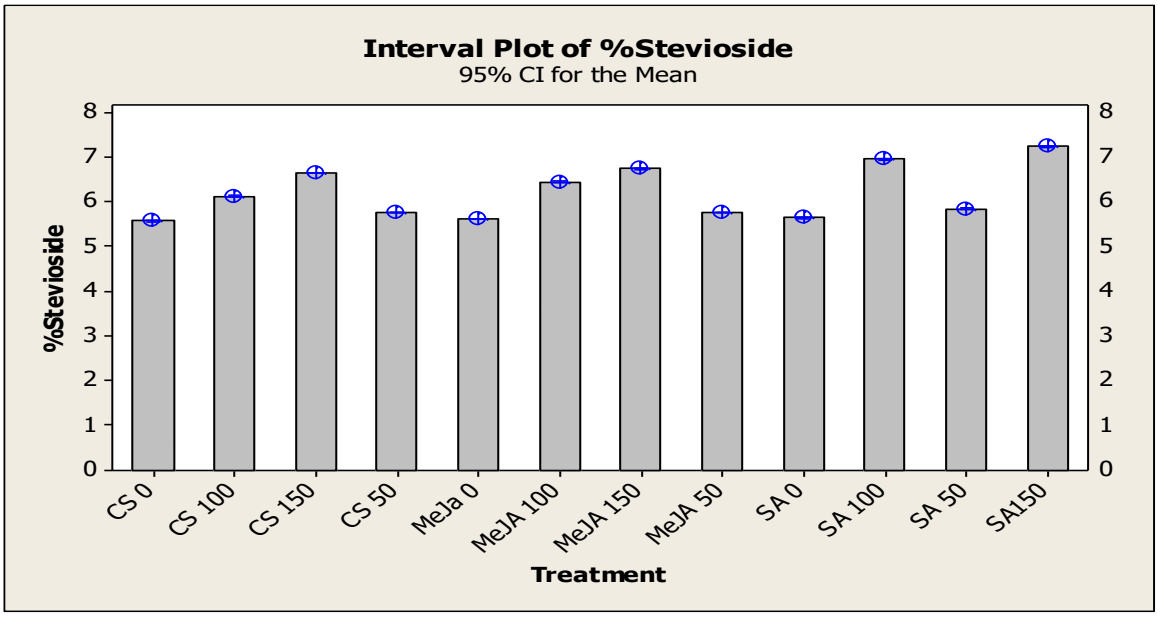
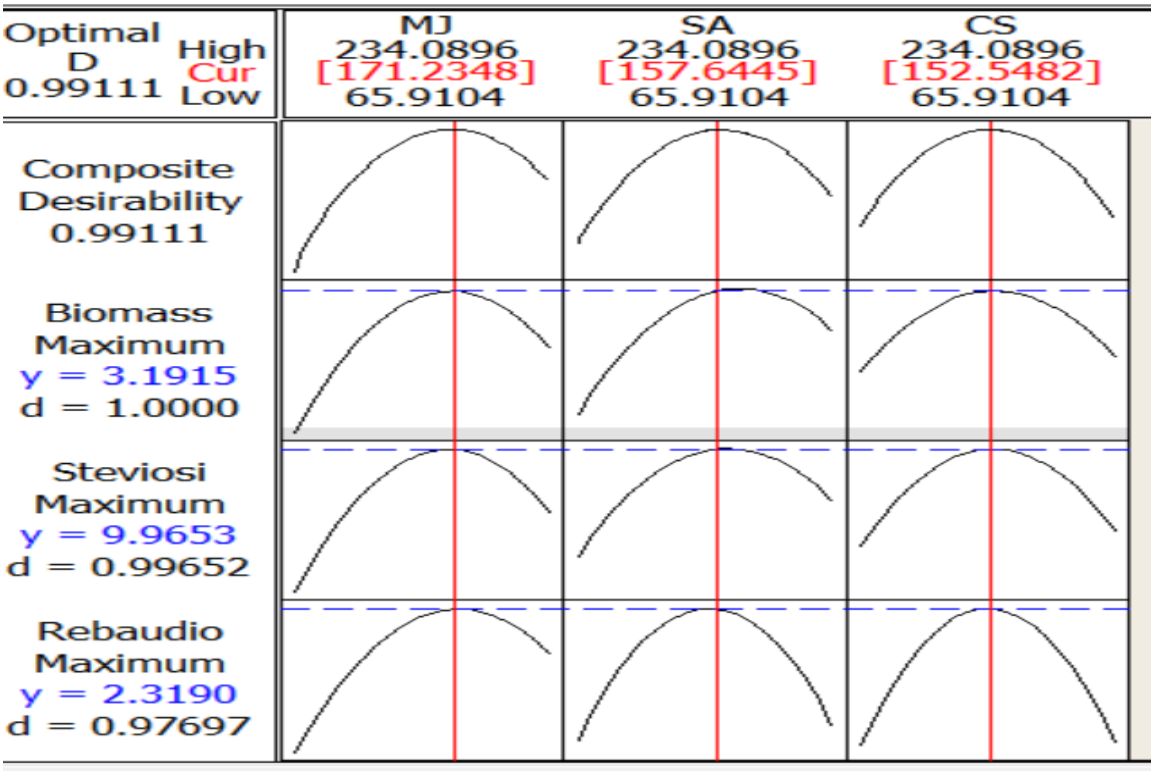


Fig.5.6: Response optimization.



5.5.1.Effect of Precursor Gibberellic Acid(GA_3)

S.No	MS + Gibberellic Acid (mg L^{-1})	Stevioside Yield (%)	Rebaudioside Yield (%)
1	MS	2.5± 0.50	1.2± 0.10
2	MS 1/2	2.7± 0.60	1.3± 0.10
3	0.5	3.05± 0.20	1.9 ± 0.10
4	1.0	5.05± 0.50	1.6± 0.50
5	1.5	4.70± 0.40	1.50± 0.30
6	2.0	4.50± 0.40	1.70± 0.30

Table 5.5: *GA3 on tissue cultured Stevia rebaudiana content*

PGRs are one of the major variables influencing plant growth and the important compounds production. According to Medina & Saavedra (1999), Lang, and other sources, the main class of phytohormone known as gibberellin (GA) is known to regulate plant growth and flowering. GAs are known to induce cell elongation and cell division, hence enhancing plant growth as seen in changes to the size, shape, and length of shoots, internodes, leaves, etc.

Gibberellins have significant effects on the leaf production of various medicinal plants, according to scientific investigations (Hassanpouraghdam et al., 2011). The medicinal plant Coleus amboinicus Lour's leaf fresh and dry weights were apparently impacted by GA3 treatment (Payan et al 2005). The largest quantity of stevioside content in stevia plants treated with foliar GA spraying on a dry weight basis was also observed (Modi et al., 2011).

By increasing the quantity of GA that cells absorb by foliar spray, the GA process may be shut off and the pathway may be redirected to the formation of steviol glycosides. However, in vitro stevia research found that GA3 given to S. rebaudiana cell suspension cultures quickly converted to stevioside (Striedner et al., 1991). The finding that SGs and gibberellins may have a similar biosynthetic pathway may be related to this.

Nodal segments cultured in vitro on MS medium fortified with GA3 concentrations developed more successfully than those cultured on PGR-free medium (control). This suggests that adding exogenous PGRs aided in the rapid growth of shoots from nodal explants. The rate of shoot regeneration after 15 days of precursor treatment in culture differed significantly between control and PGR-containing medium. (Table 5.5). The analysis of variance showed that the culture response, shoot number, and length were significantly impacted by the GA3 concentrations (0.5–2.5 mg L-1).Though GA3 promoted shoot initiation and multiplication independently, (1.0 mg L-1 GA3) was found to be more effective in terms of culture response, shoot formation content of higher Stevioside (5.05± 0.50) and Reb A (1.6± 0.50) . After 15 days of incubation, a maximum of 9.77 ± 0.28 shoots and 5.86 ± 0.11 cm average length (with 100% culture response) were reported at 1.0 mg L^{-1} concentration of GA_3 (Table 1). However, with the medium containing GA_3 (0.5 mg L^{-1}), there was a moderate response in terms of), shoot number (2.80 ± 0.20), and shoot length (3.20 ± 0.65 cm) .

When chitosan was added *in vitro*, after four weeks the fresh weight was increased by three times ,in an other study ,GA3 increases the dry weight hen sprayed at conc of 1 mg/l under in vitro cultures.

The elicitors have different set of action of mechanism and the combination between interaction of physiological effects make it difficult to determine the mechanism of action.When at the same time exogenous and endogenous elicitors have been used ,the mutually beneficial effects of them was observed on production of secondary metabolite.

The method of One variable at a time is very time taking and it doesn't determine the effects of different elicitors at same time.One of the best method known is Response surface methodology which helps to determine the various factor interaction at same time.

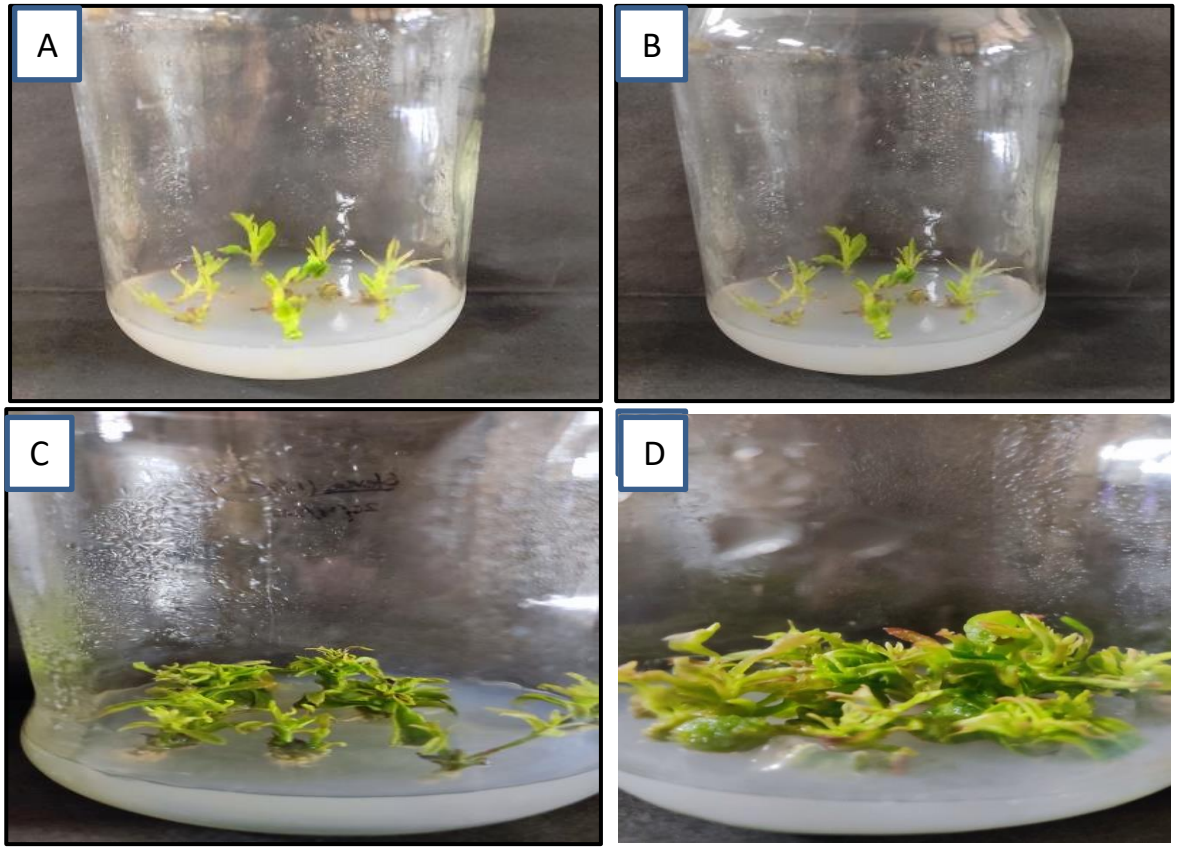


Figure 5.7: Shoot culture *In vitro* in MS media *of stevia rebaudiana* (A,B):4 week culture (C,D) 8 week of culture

3.3 Simultaneous quantification of Rebaudioside A and stevioside

High performance thin layer chromatography (HPTLC) is an effective analytical tool for qualitative and/or quantitative analysis of industrially important compounds (Kaur et al., 2021b). Nawaz et al., 2022 used an HPTLC protocol for simultaneous estimation of stevioside and rebaudioside A compounds in in-vitro Stevia plantlets.

In present study, the mobile phase (ethyl acetate-methanol: water; 8.5-1.0-0.5 (v/v/v)) provided good separation and symmetric peaks at an *Rf* value of 0.3 for Rebaudioside A and 0.4 for stevioside, respectively. Figure 5.7 shows HPTLC fingerprints produced from tissue cultured *Stevia rebaudiana* sample plants and standard compounds *viz*., stevioside and rebaudioside.

Reference solutions of 0.5–2.5 mg/mL for stevioside and 0.5–2.5 mg/mL for rebaudioside were used to produce the calibration curve for rebaudioside A. The calibration curve regression coefficient for Reb A and ST was 0.998. To verify and compare the separated bands of the standard compound with those of the plant samples, the corresponding Rf values in their scanning densitometric chromatograms were used.

Figure *5.8*: HPTLC Fingerprints of standard compounds viz. Stevioside (S) and Rebaudioside (R); whereas S1-S22 represent tissue cultured *Stevia rebaudiana* sample plants.

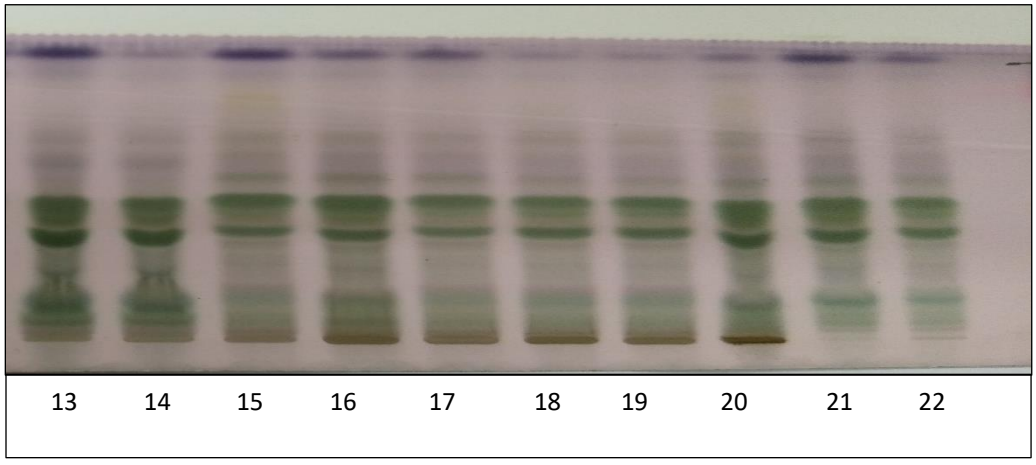


Figure *5.8.1*: HPTLC Fingerprints Stevioside (S) and Rebaudioside (R); whereas S13-S22 represent tissue cultured *Stevia rebaudiana* sample plants (1 and 2 are control plants; 3 – 22 elicited plant samples with different concentrations of (MJ), (SA)and (CH).

Conclusion

In this investigation, elicitors (MJ, SA, and CH) were applied to micropropagated S. rebaudiana plants to enhance the synthesis of steviol glycosides in comparison to untreated in vitro plantlets. *In vitro* plantlets fed with all the three elicitors i.e MJ, SA and CS at (MJ, SA and CH) at 172 µM, 158 µM and 152µM respectively respectively produced the maximum biomass (3.19 g), stevioside (9.96%) and rebaudioside A (2.20%). The method was validated and the predicted value were found to be close to the experimental value 3.14 g fresh biomass and % yield of stevioside and rebaudioside A is 9.80%, and 2.28%, respectively representing 98-99.9% validity of the projected model. This finding is 100% greater than the control plants, while the quantities of stevioside in the field-grown plants were practically identical. The findings of this study can serve as a guide for future research into increasing steviol glycosides production. *In* vitro grown S. rebaudiana plantlets can be treated with elicitors on a large scale in bioreactors, allowing the production of stevioside in desired quantities to be maximized under controlled conditions. Future research could focus on rebaudioside A's metabolic pathway.A well-established system could be used to investigate stevioside synthesis routes. In addition, elicitors can be used in the field.

CHAPTER – 6

Summary and Conclusion

Summary and Conclusion

Stevia rebaudiana is native to Paraguay, its leaves have been used as sweetners since many centuries. *S. rebaudiana* is a perennial plant that exhibits the habit of both herbs and shrubs. The species *S. rebaudiana*, popularly described as sugar-leaf sweet-leaf and meethipatti. To select and screen the elite variety ,the assessment of phytochemical diversity associated with variation in the climatic factors and geographical location play a very significant role on secondary metabolite. In the present study, phytochemical variations in *Stevia rebaudiana* were observed which were related to the geographical location variation There are variations in the content of stevioside and Rebaudioside A based on geographical locations. The highest contents (%dry wt) was found from climatic zones of semi arid region like Punjab(5.8) and Highland like Himachal Pradesh(5.2)..The populations of high amount of secondary has been observed in Highlands and Semi arid regions of India. These can be explored for the conservation and cultivation. *Stevia* leaves contains non caloric sweetening compounds as well as antioxidant compounds.

In order to optimise various extraction variables, including extraction time (X2 30-60 min), temperature (X1 30-70 °C), solvent-to-solid ratio (X4 10-20 ml g1), solvent composition (X3 35-70%), particle size (X5 0.6-1.2 mm), and extraction steps (X1–3) on Stevia rebaudiana, RSM (Response Surface Methodology) has been used. Mean particle size, solid solvent ratio, and solvent composition were tested on the antioxidant activity and yield of quercetin, chlorogenic acid (CA), and gallic acid using the Plackett-Burman design. For a higher yield, the three extraction parameters—solvent:solid ratio (20:1), mean particle size (0.80 mm), and solvent composition (55% ethanol in ethanol-water mixture—can be regarded as significant.Tissue culture of *Stevia rebaudiana* is very useful for Industries. In vitro culture of stevia from nodal explants with various combinations of growth regulators yields better results. The effects of (BA), kinetin (Kin),(IBA), (TDZ), (IAA), and mT at various concentrations (0.5-2.5 mg L-1) on shoot induction, multiplication, and root organogenesis were compared. MS medium supplemented with mT and BA resulted in superior shoot regeneration. However, mT at 2.0 mg/l showed maximum shoot growth (99.02%), mean shoot number (11.77 ± 0.28) and length (4.96 ± 0.11 cm) after 4 weeks of culture. Among mT and IAA employed for *in vitro* rooting, 97.53% shoots showed rooting on MS medium fortified with 1.5 mg L^{-1} mT with 7.91 ± 0.21 mean root number and 4.91 ± 0.22 cm root length.These can be used for large scale production and conservation of medicinal plants. To enhance the natural sweetener steviol glycosides, i.e. steviosides and rebaudiosides A, in vitro

shoot culture of Stevia rebaudiana on MS media containing the biotic and abiotic elicitors was used. In shoot culture of Stevia rebaudiana, different doses of elicitors (salicylic acid, chitosan, methyl jasmonic acid) were applied. The largest increases in SGs content were promoted by chitosan, salicylic acid, and methyl jasmonate, which were 2 to 3-fold more than the control. RSM (Response Surface Methodology) was used to investigate the combined effect of these elicitors on SG content.It showed increase in the content of natural sweetners.

The screening of Stevia rebaudiana for elite population from different geographical location showed the variations in ST and Reb A yield.The mass propagation Stevia rebaudiana has been attempted and elicitation enhanced Stevioside yield .The techniques developed in the present study can be utilized by cultivators and researchers associated with Stevia production.It is important to further study the various other factors which can be utilized for the enhancement of stevioside yield.

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Conference/ Publications

- Training on "Diagnostics of Medicinal Potential and DNA Fingerprinting in Plants" at Bose Institute ,Kolkata",India in 2016.
- Participated in International Symposium on "Sweet Revolution-Stevia"in 2016 .
- Participated in International Conference on "Innovative Strategies for Sustainable Water Management " in 2017.
- Attended Workshop on "Quantitative Data Analysis and Statistical Design of Scientific Experiments" in 2017.
- "Hands on Training in Plant Tissue Culture" at CSIR-Institute of Himalayan Bioresource Technology Palampur(H.P),India in 2018.
- Participated in"International Conference on Biosciences and Biotechnology"in 2019.
- Short term course on "Material Characterization Technique"at Dr.B.R.Ambedkar National Institute of Technology,Jalandhar,India in 2019.
- Workshop on" Liquid Chromatography and Mass Spectrometry Applications in Clinical and Medical Research at Banaras Hindu University ,Uttar Pradesh ,India in 2020.
- Shahnawaz, Pandey, D. K., & Dey, A. (2018). HPTLC Analysis of the Antioxidant and Possible Antidiabetic Chlorogenic Acid in the in situ and in vitro Populations of the Low-calorie Sweetener Stevia rebaudiana (Bert.) Bertoni. *Analytical Chemistry Letters*, *8*(6), 872-881.
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Publications

Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-021-11580-9

MINI-REVIEW

Biotechnological interventions of in vitro propagation and production of valuable secondary metabolites in *Stevia rebaudiana*

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Received: 30 May 2021 / Revised: 8 September 2021 / Accepted: 10 September 2021

Abstract
Plant cell and tissue culture makes provision of a sustainable and nature-friendly strategy for the production of secondary metabolites, and modern progress in gene editing and genome engineering provides novel possibilities to improve both the qualitative and quantitative aspects of such phytochemicals. The ever-expanding quest for plant-based medicine to treat diabetes facilitates large-scale cultivation of *Stevia rebaudiana* to enhance the yield of its much-coveted low-calorie sweetener glycosides. The potential to process stevia as a "natural" product should enhance the acceptance of steviosides as a natural calorie-free sweetener especially suitable for use in diabetic and weight control drinks and foods. Besides sweetener agents, *S. rebaudiana* is a potent source of many antioxidant compounds and is used to cure immunodeficiencies, neurologic disorders, inflammation, diabetes mellitus, Parkinson's disease, and Alzheimer's disease. This comprehensive review presents the research outcomes of the many biotechnological interventions implicated to upscale the yield of steviol glycosides and its derivatives in in vitro cell, callus, tissue, and organ cultures with notes on the use of bioreactor and genetic engineering in relation to the production of these valuable compounds in *S. rebaudiana*.

Key points
- *Critical and updated assessment on sustainable production of steviol glycosides from Stevia rebaudiana.*
- In vitro *propagation of S. rebaudiana and elicitation of steviol glycosides production.*
- *Genetic fidelity and diversity assessment of S. rebaudiana using molecular markers.*

Keywords Steviol glycosides · Phytochemistry · Micropropagation · Elicitation · Biotechnology

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Screening of elite germplasms for industrially valuable medicinal crop *Stevia rebaudiana* for stevioside and rebaudioside A production: An HPTLC-linked chemotaxonomic assessment

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ARTICLE INFO

Article History:
Received 18 May 2022
Revised 29 July 2022
Accepted 4 September 2022
Available online xxx

Edited by A. Ndhlala

Keywords:
Stevia rebaudiana
Stevioside
Rebaudioside A
High performance thin layer chromatography (HPTLC)
Validation

ABSTRACT

Stevia rebaudiana Bertoni (Asteraceae) is a South American perennial herb native to the Paraguay region. It is high-value commercial crop in the food industry because of an array of bioactive diterpenoid glycosides, the most important of which are the steviol glycosides, stevioside (Stev) and rebaudioside A (Reb A). Standardized extract from *Stevia* leaves, which is high in steviol glycosides, can be used as a high-potency sweetener. The current study proposes a high-performance thin layer chromatography (HPTLC) approach for estimating stevioside and rebaudioside that is rapid, verified, and repeatable. A collection of 32 chemotypes of *Stevia rebaudiana* were collected from 32 districts across nine Indian states. Stevioside and rebaudioside A content ranged between 1.43 - 5.88 % and 0.77 - 1.95 % respectively across 32 chemotypes of *S. rebaudiana*. Among all the studied samples, SrP1 chemotype was found to possess the highest quantity of stevioside (5.88 %) and rebaudioside A (1.95 %), followed by SrH11 (5.28 % Stev; 1.89 % Reb A) chemotypes obtained from Jalandhar, Punjab and Palampur, Himachal Pradesh respectively. Chemotype SrWb32 (1.43 % Stev; 0.77 % Reb A) collected from Bardhaman, West Bengal exhibited least amount of stevioside and rebaudioside A. The current fast, reproducible, and validated HPTLC method presents an efficacious toolkit to explore the quantitative variation in the stevioside and rebaudioside A contents in natural *S. rebaudiana* chemotypes. This methodology may be employed to screen high stevioside and rebaudioside A yielding elite genotypes for future propagation as well as commercialization of these highly potent sweeteners to deal with the ever-expanding demand for alternative yet natural sweeteners in food industries.

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Date of Issue: 14-09-2019
Place of Issue: Phagwara (India)

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at the "World Biotechnology Congress"
held during February 20-22, 2017 in New Delhi, India

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