WEED MANAGEMENT STRATEGIES TO ENHANCE THE PRODUCTION OF *STEVIA REBAUDIANA* BERTONI

A Thesis

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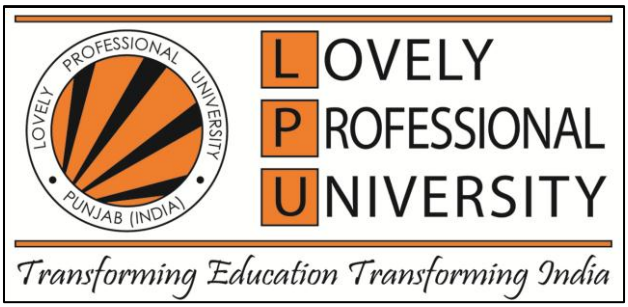

For the award of

DOCTOR OF PHILOSOPHY (Ph.D)

in

Botany

by

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SCHOOL OF BIOENGINEERING AND BIOSCIENCES

LOVELY PROFESSIONAL UNIVERSITY

PUNJAB

2021

Dedicated to my

Beloved Mother and Father

Declaration

I hereby declare that the thesis entitled, "**Weed Management Strategies to Enhance the Production of *Stevia rebaudiana* Bertoni**" submitted for Ph.D. Botany degree to School of Bioengineering and Biosciences, Lovely Professional University is entirely original work and all ideas and references have been duly acknowledged. The research work has not been formed the basis for the award of any other degree.

Date: 13 August 2021 **POOJA TAAK**

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Certificate

This is to certify that Ms. POOJA TAAK has completed the thesis entitled **"Weed Management Strategies to Enhance the Production of *Stevia rebaudiana* Bertoni"** under my guidance and supervision. To the best of my knowledge the present work is the result of her original investigation and study. No part of the thesis has ever been submitted for any other degree or diploma at any university.

The thesis is fit for the submission and the partial fulfillment of the condition for the award of DOCTOR OF PHILOSOPHY IN BOTANY.

Dr. Bhupendra Koul

Date: 13 August 2021 Signature of Advisor:

ABSTRACT

The increasing demands for food are directly proportional to the increasing world population. It shall lead to over-exploitation and depletion of available resources and hence may culminate in food and health insecurity. It has been estimated that the demands for food have doubled in recent years and especially in developed countries. Moreover, these increasing demands put a burden on the existing agriculture production system. Undoubtedly, agriculture is not only a production system which can fulfill the food-related demands of the teaming millions but also, it provides employment to nearly 40% of the world population. According to the FAO 2018 report, almost ½ billion small-scale farmers of the world produce near about 80% of consumable food. In the approaching years, sustainable plant productivity and crop yield(s) will be a major concern for food and nutritional security in emerging countries, particularly in India, where arable land per capita is declining while the human and livestock population is continuously expanding. As a result, in addition to a plant's genetic potential, the phenotypic performance of crop plants in the field is much varied and is impacted by a variety of physical, abiotic, and biotic factors. In a nutshell, the overall production of agricultural crops is governed by biotic stresses (weeds, bacteria, fungi nematodes etc.) and abiotic stresses (temperature, radiations, herbicides and other chemicals, floods, drought, salinity, and chemical toxicity). For a sustainable agriculture production system, it is necessary to maintain the balance between the production, supply and usage among the existing population for food (grains, pulses, vegetables and fruits).

Among all the major challenges, weeds are the most important, unmanageable threat which severely affects the crop(s) yield. It has been estimated that 10-15% of the total crop production gets affected due to weed competition which costs near about 40 billion USD annually (Oerke, 2006). Hence, weed management is crucial to maintain the sustainable production of every commercial crop without yield penalty. Various strategies such as manual weeding, crop rotation, allelopathy, mulching, soil treatments, ground cover systems, herbicides, herbicide-resistant crops, etc. have been adopted from time to time for weed management.

Since ancient times, ‘diabetes’ or ‘Madhumeha’ has been recognised as a debilitating disease, and it is still a serious worldwide health problem (especially India) today. Nowadays, people are more prone to this disease because of their changing life style

(eating junk food, stress, sedentary life style, alcoholism, heredity, carbohydrate rich diet, etc.). Although people have become health conscious, but they get tempted toward any artificial sweeteners, launched in the market, without being mindful of its long-term ill effects.

With the increasing awareness among people about the harmful effects of artificial sweeteners, the acceptance of natural sweeteners is expanding. Stevia (*Stevia rebaudiana* Bertoni) is a zero calorie natural sweetener commercially important plant (accepted by FAO) and its leaves are a source of steviosides which are responsible for its sweetness. Farmers in India and across the world are growing stevia to meet the growing demands of the diabetics. Unfortunately, stevia is a poor competitor of weeds, which hampers its growth and biomass upto 2-35%.

We took the initiative to find the most suitable solution to this problem of weeds in stevia cultivation (research gap). To accomplish the objectives of this study, weed samples from stevia fields from different location in Punjab were collected and identified. Two years stevia field-trial (in Lovely Professional University) was conducted to evaluate the effects of different herbicides and mulching treatments on weeds and crop yield. Several weed plants families commelinaceae, cyperaceae, primulaceae, caryophyllaceae, asteraceae, fabaceae, malvaceae, plantaginaceae, solanaceae, cucurbitaceae, amaranthaceae, caesalpinioideae, poaceae, etc. were witnessed in stevia field during the experiment. We suggested an amalgamation of herbicide and mulching treatments for weed control, which provided success to some extent. Moreover, to find a complete solution to the aforementioned problem of weeds in stevia field, we planned to raise herbicide-resistant transgenic stevia plants as an effective weed-management strategy in stevia cultivation. With the aim of raising transgenic stevia, in vitro regeneration of stevia was successfully optimized using various explants (shoot tip, seeds, nodal and inter-nodal section). We also optimized various others parameters (explant type, pre-incubation duration, Acetosyrigone concentration, *Agrobacterium* cell density, Agro-inoculation duration and co-cultivation duration) for *Agrobacterium tumefaciens*-mediated transformation (nuclear transformation) of stevia.

The best responding explant (nodal section) in terms of regeneration efficiency was used for *Agrobacterium tumefaciens*-mediated transformation to introduce the bialaphos

resistance (*bar*) gene into the stevia genome. Molecular characterizations (PCR, Reverse transcriptase PCR, real time PCR, Southern Hybridization) of putative transformants have confirmed the integration and expression of *bar* gene in stevia. The T_0 transgenic stevia plants were subjected to herbicide treatment in a green house. Interestingly, the transgenic stevia plants could tolerate 8mg/l of glufosinate ammonium (herbicide). The optimized protocol takes about 100 to 105 days from the start of co-cultivation to the planting of transformants into pots and does not alter the stevioside and rebaudioside contents. This is the first study on the integration of the *bar* gene into the stevia genome using the *Agrobacterium tumefaciens*-mediated transformation method.

PREFACE

Stevia rebaudiana Bertoni belongs to asteraceae family and commonly known as 'sweet leaf' or 'candy leaf'. It is an important zero calorie, natural sweetener plant. Its leaves are sweet due to the presence of 'steviosides'. It was first time introduced at the commercial level in markets of Japan as a natural sweetener in the early 1970s. Stevia sugar is almost a hundred times sweeter than sucrose. Stevia cultivation has been reported in the early 1970s. Steviosides and rebaudioside A are the two most important glycosides of stevia, followed by rebaudioside C and dulcoside A. Among them, steviosides and rebaudioside A are actually responsible for their sweet taste. Traditionally, dried leaves of stevia has been used in to sweeten tea and other drinks but, these days stevia sugar (purified form) is extensively being used as a natural sweetener in products like biscuits, ice creams, yogurts, desserts, chocolates, soft-drinks, smoothies, fruit drinks, and various beverages. The aforementioned products can be consumed by diabetics for the satisfaction of their cravings for sweets. That is why, stevia sugar is known as a 'boon to the diabetics'. Besides its sweet taste, stevia leaves also exhibits several pharmacological activities like anti-tumor, anti-hypertensive, immune-modulatory, anti-diarrheal, anti-viral, anti-microbial, hepato-protective, anti-fungal, anti-inflammatory, anti-oxidant, anti-diabetic, and diuretic, etc.

Nowadays, stevia is commercially cultivated in South-East Asia, Australia, Argentina, United States, China, Vietnam, Brazil, Malaysia, Israel, Columbia, Kenya, Paraguay, South Korea, USA, and Japan including India. At present, stevia is commercially grown in various Indian states, such as West Bengal, Rajasthan, Chhattisgarh, Punjab, Karnataka, Gujarat, Madhya Pradesh, and Uttar Pradesh. The stevia plants are poor competitor of weeds and they face heavy weed infestation during the initial growth period and rainy season. Weed infestation leads to a reduction in stevia branching which ultimately hampers the biomass yield and raises the production cost.

Therefore, the application of efficient weed management strategies is crucial for the successful cultivation of stevia without yield penalty. Till date, there are very few registered herbicides reported for weed control in stevia therefore, hand-picking (mechanical method) and mulching are the only options left with the stevia-farmers for controlling the weed population. Therefore, insertion of a desirable herbicide-resistant gene(s) into the stevia genome is required as a weed management strategy in stevia cultivation.

The *'bar'* gene encodes for phosphinothricin acetyltransferase (PAT) which provides resistance against broad-spectrum herbicide phosphinothricin or glufosinate-ammonium. There are several reports on the successful introduction of bialaphos resistance *bar* gene into many commercially important crop such as *Zea mays, Triticum aestivum, Avena sativa, Beta vulgaris, Sorghum bicolor, Brassica napus, B. oleracea, Solanum tuberosum, Daucus carota, Secale cereale, Festuca arundinacea, Lycopersicon esculentum, Medicago sativa, Nicotiana tabacum, Hordeum vulgare, Oryza sativa, Populus* spp etc.

The present thesis entitled **"Weed Management Strategies to Enhance the Production of *Stevia rebaudiana* Bertoni"** encompasses the details of the studies undertaken and analyses of results obtained under 9 major chapters as described below:

Chapter 1 - Introduction & Objectives: This chapter includes a brief introduction and objectives designed to address the problem of weeds in stevia cultivation.

Chapter 2 - Review of Literature: This chapter summarizes the distribution, cultivation, botanical description, pharmacology, phytochemistry, *in vitro* regeneration and extant weed management strategies in stevia cultivation. It also includes the reports on genetic transformation in stevia and the scope of developing herbicide resistant commercial crop plants.

Chapter 3 - Hypothesis: This chapter describes the hypothesis of the present work.

Chapter 4 - Aims and Objectives: This chapter describes the aims and objectives of the present study.

Chapter 5 - Materials and Methods: It includes the details of various experimental materials, procedures and protocols that were employed in order to accomplish the objectives.

Chapter 6 - Identification and collection of weeds found in stevia cultivation and comparative evaluation of different weed management approaches: This chapter summarizes the identification of most common weed species which were found in stevia fields of different locations in Punjab and evaluate the most efficient weed management strategy including the use of different mulches and herbicides.

Chapter 7 - Optimization of *in vitro* regeneration and establishment of *Agrobacterium*-mediated transformation of stevia and molecular characterization: This chapter describes the optimization of *in vitro* regeneration of stevia from different explants and includes the detailed procedure of *Agrobacterium*-mediated transformation of stevia. It also includes the molecular characterization, herbicide resistance assay and stevioside and rebaudioside profiling (through HPLC) of T_0 transgenic plants.

Chapter 8 - Summary and Conclusions: This chapter briefly summarizes the work that has been presented in this thesis and conclusions drawn from this.

Chapter 9 - Bibiliography: This chapter contains citations of references used in the present investigation.

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POOJA TAAK

ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
ai/h	Active Ingredients Per Hectare
As	Acetosyringone
AMF	Arbuscularmycorrhizal Fungus
ANOVA	Analysis of Variance
BAP	6-Benzylaminopurine
bp	Base Pair
CaMV35S	Cauliflower Mosaic Virus 35S Promoter
cDNA	Complementary DNA
Cef	Cefotaxime
cm	Centimetre(s)
CPE	Cumulative Pan Evaporation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTAB	Cetrimonium Bromide
DAT	Days after Transplanting
DECaMV35S	Cauliflower Mosaic Virus 35S Promoter with Double Enhancer
DEPC	Diethyl Pyrocarbonate
DMSO	Dimethyl Sulfoxide
DNA	Deoxy Ribonucleic Acid
dNTP's	2′-deoxynucleoside-5′-triphosphate
EDTA	Ethylenediamine Tetra-acetic Acid
ERK	Extracellular-Signal-Regulated Kinase
FAO	Food and Agriculture Organization
Fe-EDTA	Ferric Ethylenediamine Tetra Acetic Acid
FYM	Farm Yard Manure
GA_3	Gibberellic Acid
GLUT1 and GLUT4	Glucose Transporter 1 and 4
GM	Genetically Modified
h	Hour
HCl	Hydrochloric Acid
HEK293T	Human Embryonic Kidney 293 Cells
HPLC	High Performance Liquid Chromatography
HT29	Human Colon Cancer Cell Line
IAA	Indole-3-Acetic acid
IBA	Indole-3-Butyric acid
IARC	International Agency for Research on Cancer

IL-beta	Interleukin-1β
ISAAA	International Service for the Acquisition of Agri-biotech Applications
IW	Irrigation Water
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JNK	c-Jun N-terminal kinases
PGR	Plant Growth Regulator
KIN	Kinetin, 6-furfuryl aminopurine
L	Litre
LAI	Leaf Area Index
LB	Luria Bertani medium
mg	Miligram
µ	Micro\Micron
µl	Microliter
min	Minute(s)
ml	Mililitre
mm	Millimetres (s)
MCF-7	Breast Cancer Cell Line
MS	Murashige and Skoog Medium
MAPK	Mitogen-Activated Protein Kinase
NAA	α-Naphthalene Acetic Acid
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NPK	Nitrogen Potassium Phosphate
NRCWS	National Research Centre for Weed Science
O.D./OD	Optical Density
PAT	Phosphinothricin Acetyltransferase
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PGPRs	Plant Growth-Promoting Rhizobacteria
PPB	Purple Phototrophic Bacteria
PVP	Polyvinylpyrrolidone
%	Percent
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
rpm	Rotations Per Minute
RNA	Ribonucleic Acid
sec	Seconds
SYBR	Synergy Brands
TALENs	Transcription Activator-like Effector Nucleases
THP-1	Human Monocytic Cell Line
T-DNA	Transfer-DNA
TDZ	Thidiazuron, N-phenyl-N′-(1,2,3-thiadiazol-5-yl) urea
TNF-alpha	Tumour Necrosis Factor Alpha

UV	Ultra Violet
USFDA	US Food and Drug Administration
V	Volt
v/v	Volume by Volume

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CHAPTER 1
INTRODUCTION

With an increase in the population, certain health-related problems such as cardiovascular disease, cancer, diabetes, etc. have also been increased among the people. Diabetes is a major threat to humanity on a global scale which affect near about ½ billion population of the World. The initial occurrence of diabetes was reported in the Eastern Mediterranean region during the 1980s. After that, the rate of diabetes occurrence was highly elevated with a 0.23% of yearly increase and reached about 2-3 folds greater than reported ever in the year 2014 (Zimdahl, 2013; Duarte et al., 2018). Approximately one and a half million deaths occurred in 2012 because of diabetes and about 43 % of these deaths occur under the age of 70 years. In the year 2014, 422 million people were reported with diabetes globally (Zimdahl, 2013; Roglic, 2016). Prevalence of diabetes during the early stages of life led to further complications like neurological, oncological, and cardiovascular diseases. There are various factors responsible for diabetes occurrence which acts in a collective manner are sedentary life cycle, high fat and salt-rich diets, smoking, stress, alcohol consumption, irregular physical examination, etc. (Alexandratos and Bruinsma, 2012; Duarte et al., 2018; Luo et al., 2018).

Continuously increasing burden of the human population at an alarming rate is a major constrain to fulfill the elementary need of every single human being in terms of food, water, and energy. At present, the crop production systems are not satisfactory to manage with the increasing demand. It has been estimated that around the year 2050, the human population will elevate to its peak value i.e. 9 billion (Alexandratos and Bruinsma, 2012). According to Global Health Observatory data of the World Health Organization, the 2020 population of urban areas will reach 60 % in 2030 and 66 % in 2050 from 54 % in 2015. An increase in diabetic rate among people has led to an increased in consumption and production of artificial sweeteners which are also known as high-intensity, non-nutritive, and low caloric sweeteners. There are six artificial sweeteners that are permitted by USFDA (US Food and Drug Administration) to be consumed are advantame, neotame, aspartame, sucralose, saccharin and acesulfame- potassium (acesulfame-K). Besides the extensive use of artificial sweeteners, various controversies regarding their safety and numerous health hazards effects have also been reported such as metabolic syndrome, obesity, cardiovascular disease, cancer, hepatotoxic and nephrotoxic effects. The rising concern of health-related issues of artificial sweeteners has led people towards the use

of natural sweeteners. The sweet herb stevia is a rapidly emerging natural sweetener which is the best alternative to synthetic or artificial sweeteners. It contains zero calories steviol glycosides (400 times sweeter than cane sugar) in its leaves which do not increase blood glucose levels. Steviol glycosides are now approved or adopted as safe natural commercial sweeteners by various authorities for their use in food products. 68th meeting of JECFA concluded that steviosides are hydrolytically and thermally stable and are acceptable to use as food additives (sweeteners) (FAO, 2007).

Stevia is now an important commercial crop in various countries such as Australia, Argentina, United States, China, Vietnam, Brazil, Malaysia, Israel, Columbia, Kenya, India, Paraguay, South Korea, and Japan (Alexandratos and Bruinsma, 2012; Samuel et al., 2018). Stevia plants face weed invasion in the rainy season. Various weed plant species belonging to different families retards the branching in stevia plants. Therefore, such kinds of strategies should be adopted which can reduce the weed content in stevia fields. To date, manual weeding and mulching are the only weed control methods in stevia cultivation.

Crop production or yield depends upon the interaction between various biochemical, physiological and metabolic phenomenon. With an increase in climatic deviations, crop plants also encounter various biotic and abiotic stresses, which directly or indirectly hit the economic value of commercial crops. The agricultural production system faces heavy losses due to biotic (anthropogenic activities or pathogen/insect attack,) and abiotic agencies of stress (Ramegowda and Senthil-Kumar, 2015; Fahad et al., 2017; Taak and Koul, 2018). Hence agricultural sustainability depends upon the implementation of such type of approach which can reduce the opposing effects of these constraints. Weeds are considered as unwanted and most challenging guests who are detrimental to agricultural production. Weed problems directly hit the cost of the crop by interfering or competing with crop plants for water, nutrition, resources, light, etc. which ultimately reduces crop yield (Alexandratos and Bruinsma, 2012; Zimdahl, 2013). About 8000 plant species among the total plants are considered weeds in the World. Most of the weeds belong to the families asteraceae, fabaceae, malvaceae and poaceae (Naidu, 2012; Ramegowda and Senthil-Kumar, 2015; Roglic, 2016). In India, crop yield reduction due to weed is about 36.5% in the rainy and summer season while 22.7% in the winter season, which led to an economic loss of

1050 billion annually INR (NRCWS, 2007). Losses caused by weeds may be from 5-10 % in the developed countries, while 20-30 % in developing countries. Generally, it can be concluded that reduction in plant yield is done by a group of weed plants species rather than single weed species.

Mulching is one of the important agriculture practices which conserves soil nutrients moisture content, enhances soil productivity, suppresses weeds as well as maintains the physical environment of soil. For maximum weed control and higher yield, different types of mulches have been used in commercial crops. Undoubtedly, chemicals or herbicides sprays are cost-effective than mulching and manual methods but these chemicals also impose several threats on environmental sustainability and human health. As per the report of IARC (International Agency for Research on Cancer) two important herbicides 2,4-D and glyphosate are termed as possible human carcinogens (EFSA, 2015). Besides that, Glufosinate is more beneficial as compared to other herbicides like short half-life, low toxicity, and degrades easily in soil (Duke, 2005). It is commercially available in the market with certain brand names such as Liberty R, Buster R, Basta R and Finale R. Hence, the production of glufosinate resistant crop plants could be a promising method for successful weed control. Alternatively, agro-biotechnological strategies should be adopted for successful weed control. Production of herbicide-resistant crop plants is one of the various biotechnological strategies and *Agrobacterium*-mediated transformation is more promising and highly efficient. There are several reports on the successful performance of *bar* gene (bialaphos resistance) into many commercially important crop plants (Gordon-Kamm et al., 1990; Fromm et al., 1990; Vasil et al., 1992, 1993; Weeks et al., 1993; Roglic, 2016; Heap and Duke, 2018).

CHAPTER 2
REVIEW OF LITERATURE

2.1 Introduction

It has been estimated that the world population could swell to 9 billion by the year 2050 (Alexandratos and Bruinsma, 2012). Unfortunately, the present crop production regimes are not robust to cope with the demands of the teaming millions. Crop productivity and yield are the results of the interaction of several physiological, bio-chemical, and metabolic processes over a defined period of time reflected in a gain of total biomass or converted harvestable commodities like seeds, fruits, or edible plant parts under a set of environmental conditions (physical, geochemical and biological components). Therefore, besides the genetic potential of crop species, the agricultural economy/productivity strongly depends and is influenced by several physicals, abiotic (intense light, herbicides, ozone, heat, chilling, freezing, drought, floods, salinity, and heavy metals), and biotic (pathogen/insect attack and anthropogenic activity) stress and is highly variable (Ramegowda and Senthil-Kumar, 2015; Fahad et al., 2017). Hence, both agricultural sustainability and global food security are subject to the implementation of strategies that can mitigate the severity and adverse effects of the aforementioned stresses. Among all the biotic factors, weeds are the most uninvited and demanding guests that are more harmful than other crop pests and are detrimental for agricultural production (Abouziena and Haggag, 2016; Gharde et al., 2018). Weeds compete with crop plants for nutrition, space, water, light, exhibit allelopathic effect, and may serve as an obligate or alternate host for insects and pathogens, etc., which ultimately reduces crop yield and raises the production cost (Zimdahl, 2013; Abouziena and Haggag, 2016). In India, the weeds-mediated crop-yield reduction is about 36.5% during the summer and rainy seasons while 22.7% during the winter season (Bhan et al., 1999), which accounts for an annual economic loss of 1,050 billion (NRCWS, 2007; Varshney and Prasad Babu, 2008). However, the yield loss is not caused by single weed species, but by a group of weed species having varying competitive abilities (Weaver and Ivany, 1998; Milberg and Hallgren, 2004). Among the total plant species in the world, approximately 8000 species are considered as weeds (Holm, 1991; Parker, 2012). Most of these weed species belong to the families Asteraceae, Poaceae, Malvaceae, and Fabaceae (Naidu, 2012).

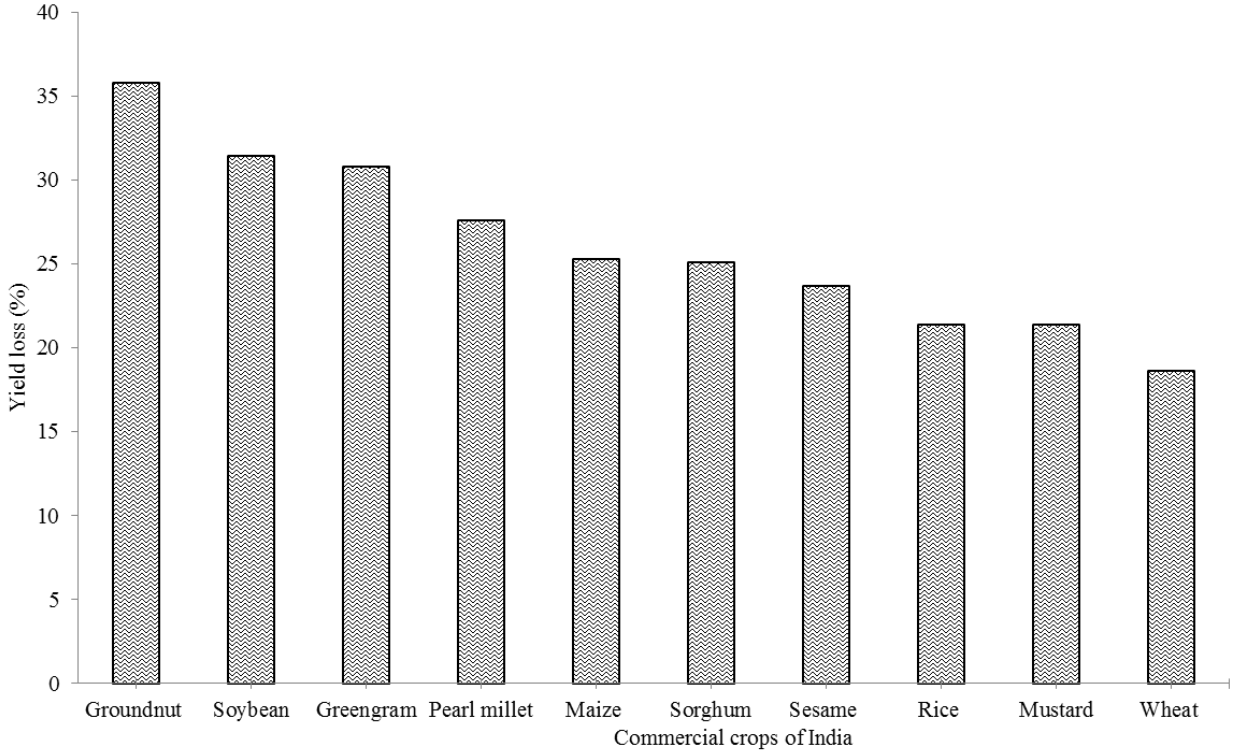


Fig. 2.1: Yield loss due to weeds in commercial crops of India (Rao et al., 2014).

The top ten notorious weeds of the world which affect commercial plantations are *Cynodon dactylon*, *Eleusine indica*, *Portulaca oleracea*, *Cyperus rotundus*, *Imperata cylindrical*, *Eichhornia crassipes*, *Echinochloa colonum*, *Chenopodium album* and *Sorghum haplepense* (Table 2.1). Rice, soybean, sugar-cane, cotton, coffee, potato, sugar-beet groundnut, maize, rubber, grape, sorghum, wheat and tea are the major crops which are affected by these weeds (Fig. 2.1) (Holm, 1991).

Table 2.1 Major weeds (monocot and dicot) of the world (Krahmer, 2016) (Image credit: http://www.theplantlist.org/)

Species	Image	Common name	Crop(s) affected	Countries
MONOCOT				
Avena fatua, *A. sterilis*		Common wild oat	Cotton sugar beet, Cereals, oilseed rape and canola	North America, Africa, Latin America, Asia Australia, Europa
Alopecurus myosuroides, *A. japonicas*, *A. aequalis*		Foxtail grass	Sugar beet, oilseed rape and cereals	Asia, Europa

Cyperus rotundus, *C. esculentus*, *C. difformis*		Khmer kravanh chruk, coco-grass, red nut sedge, Java grass, purple nutsedge and nut grass	Rice, maize, soybean, cotton	North America, Africa, Latin America, Asia Australia, Europa
Digitaria sanguinalis, *D. horizontalis*		Crabgrass, finger-grass and fonio	Maize, soybean, cotton	Africa, Asia Australia, Europa, Latin America, North America
Echinochloa crus-galli, *E. oryzicola*, *E. colona*		Barnyard grass or cockspur grass	Rice, maize, sugar beet	Africa, Asia Australia, Europa, Latin America, North America

Lolium multiflorum, L. rigidum		Ryegrass	Cereals	Africa, Asia Australia, Europa, Latin America, North America
Phalaris minor, P. paradoxa		Canarygrass, littleseed canarygrass, hood canarygrass	Cereals	Africa, Asia Australia, Europa, Latin America
Setaria viridis, S. glauca, S. faberi		Green foxtail, green bristlegrass, and wild foxtail millet	Sugar beet, cereals, soybean, canola, maize, oilseed rape	Africa, Asia Australia, Europa, Latin America, North America

Sorghum halepense		Great millet, durra, jowari, or milo	Maize, soybean, cotton	Africa, Asia Australia, Europa, Latin America, North America
Species		**Common name**	**Crops affected**	**Countries**
		DICOT		
Amaranthus A. viridis A. retroflexus, A. palmeri		Pigweed	Sugar beet, maize, cereals, cotton, soybean	Asia, Europe, North America, Latin America, Africa

Abutilon theophrasti		Indian mallow, velvetleaf, room maple, parlor maple, or flowering maple	Maize, soybean, cotton	Europe, North America
Ambrosia artemisiifolia, *A. trifida*		Tassel weed, American wormwood, bitterweed, stickweed, blackweed, stammerwort, carrot weed, short ragweed, Roman wormwood and hay fever weed	Cereals, maize, soybean	Asia, Europe
Chenopodium album		Fat-hen, lamb's quarters, manure weed, melde and goose foot	Sugar beet, maize, cereals, soybean and canola	Asia, Europe, North America, Latin America

Convolvulus arvensis		Field bindweed	Sugar beet, maize, cereals, soybean and canola	Asia, Europe, North America
Cirsium arvense		Creeping thistle	Sugar beet, maize, cereals, soybean and canola	Asia, Europe, North America
Galium aparine		Sticky willow, cleavers, sticky willy, clivers, robin-run-the-hedge, bedstraw, stickyback, goosegrass, stickybud, catchweed, sticky bob and stickyweed	Sugar beet, oilseed rape, cereals	Europe, Asia

Polygonum convolvulus, *P. aviculare*		Knotweed and knotgrass	Cereals, maize, soybean, sugar beet	Latin America, Asia, North America, Australia, Europe, Africa
Raphanus raphanistrum		Wild radish, charlock, white charlock, jointed radish, sea radish, jointed wild radish	Cereals	Africa, Australia, Europe, North America

2.2 Weed management

Weed management is a process that reduces weed infestation among the crop plants so that the crop can grow profitably without any kind of yield penalty. For successful weed management, knowledge of weed biology, habits, life cycle, dormancy period, susceptibilities, reproduction and seed dispersal of weed plants are the prerequisites. Weed management practices are broadly classified into four different classes i.e. cultural, mechanical, chemical, biological, and biotechnological. Various studies suggested that individual weed control practices are not very effective for weed suppression as integrated weed management strategies (Marshall et al., 2003; Koocheki et al., 2009; Chikowo et al., 2009).

2.2.1 Cultural methods

Cultural methods are most promising for weed control when other methods are not available or limited. Most commonly these methods include the maintenance of field conditions in such a way so as to reduce weed growth. These methods include crop competition, soil fertility, planting date, crop rotation and companion cropping.

2.2.2 Mechanical methods

These methods are among the most effective weed control methods but are laborious. These methods start with the preparation of seedbed and then plowing of field. Organic farming has stimulated the development of various new mechanical weed management methods over the last fifteen years (Bond and Grundy, 2001; Van der Weide et al., 2008; Pannacci and Tei, 2014). These methods include hand weeding, inter cultivation, hand hoeing, flooding, spudding, mulching, sickling, tillage, cutting, cheeling, mowing, soil solarization, digging, microwave radiations, and flaming (Fig. 2.2). There are various mechanical weed control methods that damage the weed plants by uprooting, cutting, and burial which causes withering and desiccation of weed plants (Chicouene, 2007). Hoes are one of the most ancient and commonly used hand tools for weed control and crop harvesting. Different types and shapes of hoes are available in the market and can be classified into two types scuffle hoes (for weed control) and draw hoes (for soil shaping). Scuffle hoe is further of two types Hoop hoe (USAID, 1984) and Dutch hoe (Loudon,

1871). Root talon and weed wrench are other important tools for mechanical weed eradication (Tu et al., 2001). Various mechanical weed control methods are used in various commercial crops such as Weed harrowing and inter-row weed hoeing in winter wheat crop (Rasmussen 2004); harrowing in barley crop (Rasmussen and Rasmussen, 2000); torsion weeding, manual weeding, weed harrowing and robovator in onion and cabbage crop (Melander et al., 2015); manual steering, camera hoe, camera hoe with finger, torsion weeders and rotary harrow in sugar beet crop (Kunz et al., 2016); harrow and hoe in soybean crop (Weber et al., 2016); intelligent intrarow weed hoeing in maize and sugarbeet crop (Gerhards et al., 2016); camera steering in sugarbeet and soybean crop (Kunz at al., 2016); flexible tine harrow in maize crop (Rueda-Ayala et al., 2015); rotor tine in sugarbeet crop (Rasmussen et al., 2012); hand weeding in soybean crop (Singh et al., 2016); mechanical digger in various vegetable crops (Hershenhorn et al., 2015); rotary hoeing in pepper crop (Campiglia et al., 2012); bar harrow and hand hoeing in wheat crop (Jabran et al., 2012); hand weeding and hand hoeing in rice crop (Akbar et al., 2011); flex tine harrow, finger weeder, torsion weeder in saffron crop (Cirujeda et al., 2014); weed harrowing in cereal crop (Armengot et al., 2013); torsion weeders in willow crop (Albertsson et al., 2016); steerage hoe in soybean crop (Tillett et al., 2002); rotating cultivar in Bok choy, radicchio, celery and lettuce (Fennimore et al., 2014); intrarow weeder in cotton crop (Saber et al., 2013); harrowing, and inter-row cultivation in spring-wheat (Kolb et al., 2012).

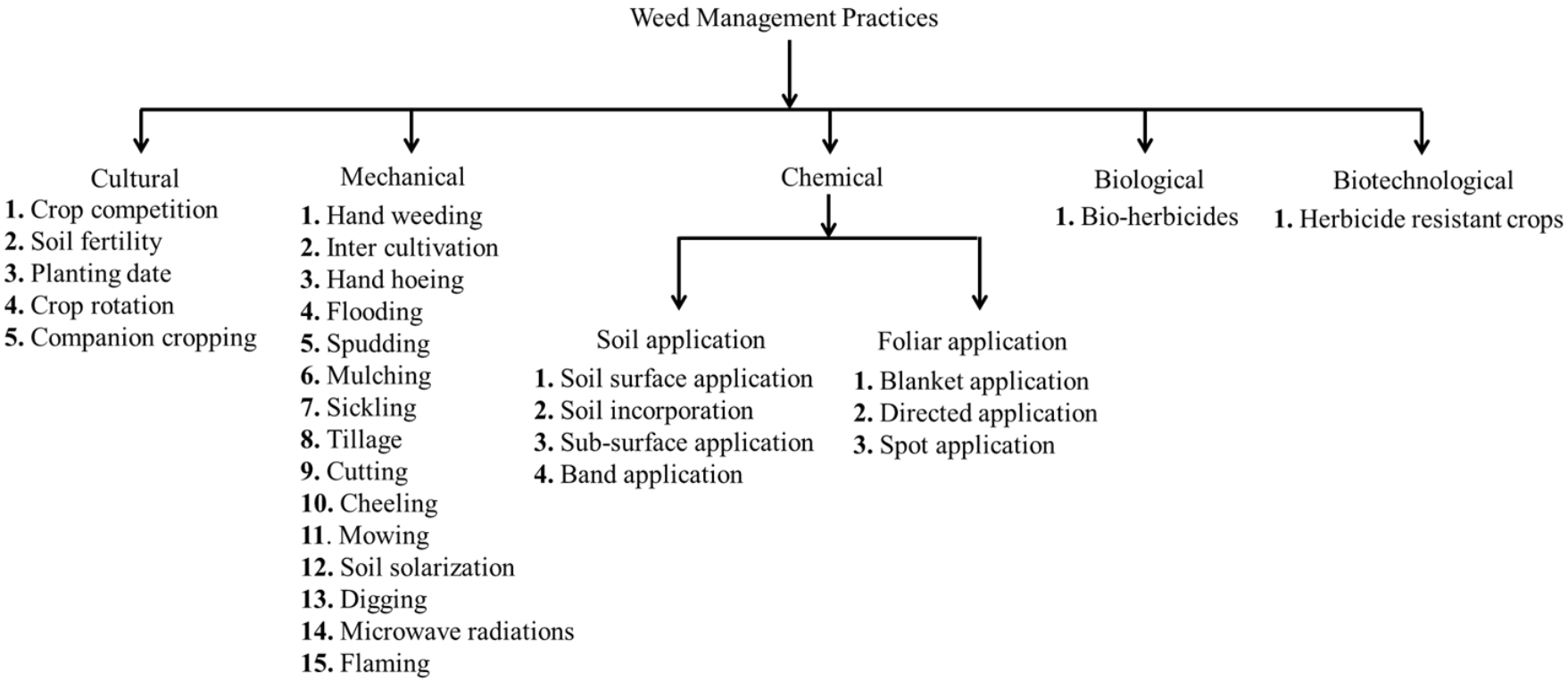
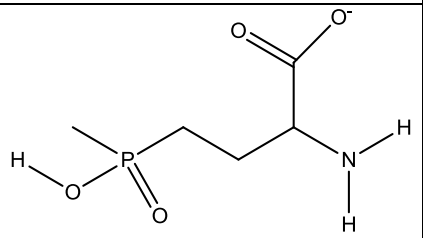
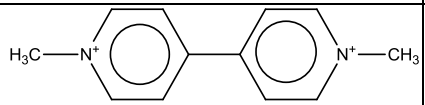
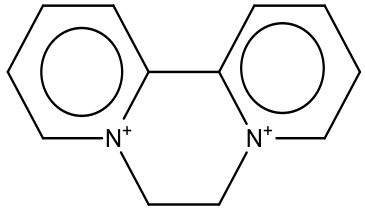
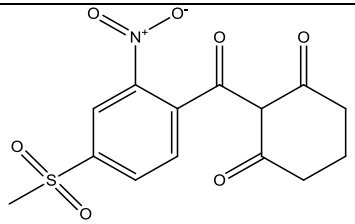


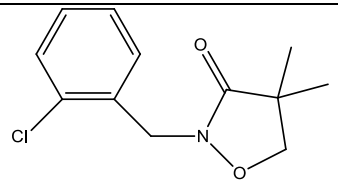
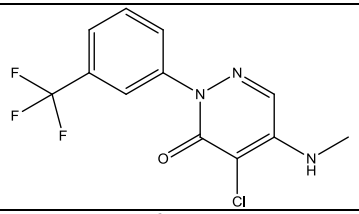
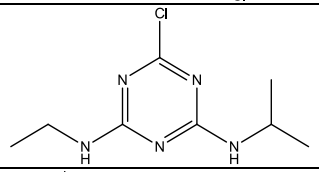
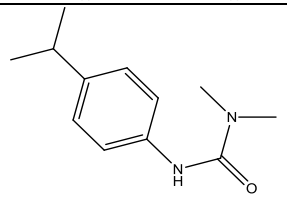
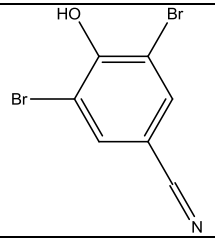
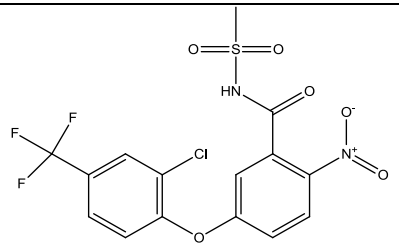
Fig. 2.2 List of various weed management strategies

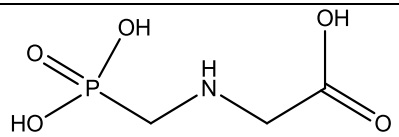
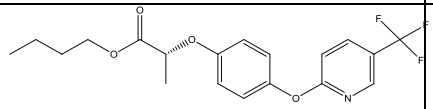
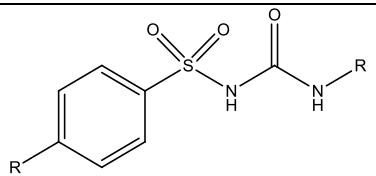
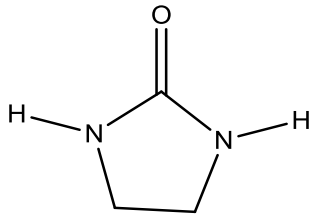
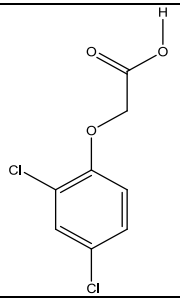
2.2.3 Chemical methods

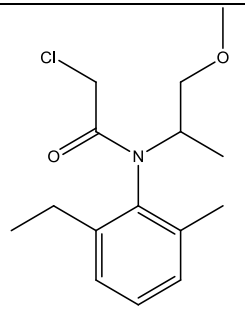
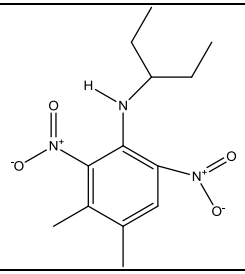
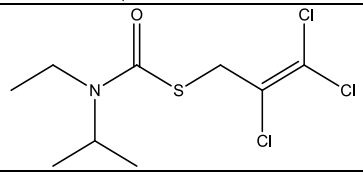
These methods include various types of chemicals which are used to kill weed plants. Although these methods are highly efficient and cost-effective besides that these chemicals also impose harmful effects on soil and crop plants. Extensive use of these chemicals caused various problems such as acidification of soil (Koopman et al., 1995; Bennett et al., 2004), herbicide-resistant weeds (Cirujeda and Taberner 2010; Marshall et al., 2010), environmental pollution (Fernandez-Perez, 2007; Knee et al., 2010; Koesu kwiwat et al., 2010; Zhang et al. 2010), loss of biological diversity (Ros et al., 2006; Schooler et al., 2010; Potts et al., 2010). These chemicals can be applied to the field in two ways i.e. soil application and foliar application. Soil applications include soil surface application, soil incorporation, sub-surface application, and band application. Foliar applications include blanket application, directed application, and spot application. The mode of action of various herbicides is enlisted in Table 2.2.

Table 2.2 Mechanism of action of different herbicides (Rana and Rana 2016)

Mechanism of action	Example	Structure	Effects on weed plants
Glutamine synthetase	Glufosinate		>Ammonia accumulation in cells >Destroy cell membrane >Stops photorespiration and photosynthesis in cells >Shoots yellowing and desiccation >Control broad spectrum of young weeds
Photosystem I (PSI) inhibition	Paraquat		>Affects photosynthesis >Destructs cell membrane >Leaves yellowing and desiccation >Broad spectrum herbicide
	Diquat		
Carotenoid biosynthesis inhibition, eg 4-hydroxyphenyl-pyruvatedioxygenase (HPPD)	Mesotrione		>Degrades leaf pigments >Shoots whitening >Acts as pre or post-emergence herbicide

	Clomazone		
	Norflurazon		
Photosystem II (PSII) inhibition	Atrazine		>Affect photosynthesis >Leaves yellowing and desiccate from tips, edges, and veins >Soil residual effects
	Isoproturon		
	Bromoxynil		
Protoporphyrinogen oxygenase (PPO) inhibition	Fomesafen		>Rapid desiccation of all green tissues >Systematic in action, when enters through roots >Controls broadleaf weeds

EPSP synthase	Glyphosate		>Inhibits the formation of phenylalanine, tryptophan and tyrosine amino acids >Degrades chlorophyll, root and shoot death, stunted growth >Leaf yellowing and purple pigmentation >Inactive in soil
Acetyl CoA carboxylase (ACC-ase) inhibition	Fluazifop-pbutyl		>Inhibit the formation of cell membrane fatty acids > yellow, purple, brown leaves then death >Death of rhizomes and roots >Post emergence control of grass weeds
Acetolactate synthase (ALS) inhibition	Sulfonylureas		>Inhibits the formation of leucine, isoleucine and valine amino acids > yellow, purple, brown leaves then death
	Imidazolinones		
Synthetic auxins	2,4-D		>Hormonal effect on plant growth >Leaf curling, stem twisting and leaf browning and yellowing >Post emergence control of broadleaf weeds

Cell division disruption	Metolachlor		>Inhibits cell division >Stunted seedling growth >Pre emergence control of grassy weeds
	Pendimethalin		
Seedling growth inhibitors	Triallate		>Inhibits the formation of waxy cuticle >Stunted seedling and root growth >Pre emergence control of grassy weeds

These herbicides are classified according to their time of application and mode of action i.e. contact and systematic, selective and nonselective, post and pre-emergent. Systematic herbicides are absorbed quickly by the plant and move to other parts also to kill the weed plant effectively. While contact herbicides kill only particular plant parts by acting only at the site of contact. Sulfonylurea and glyphosate are examples of systematic herbicides and glufosinate is an example of contact herbicide (Qasem, 2011). Postemergence herbicides are applied after the weed emergence and pre-emergence herbicides are applied before the seed germination. These can act either in contact or in a systematic way. Approved dosage of registered herbicides for various crops is enlisted in Table 2.3.

2.2.4 Biological methods

These methods include the use of biological agents (microbes, pathogens, phytophagous animals, fungus, natural products, or extracts) to control the weeds, without affecting the environment and soil fertility (Goeden, 1988). These are also known as bio-herbicides. However, the uses of bio-herbicides need further investigation in order to improve their efficiencies (Cai and Gu, 2016). No doubt, biological weed control methods are of low cost, besides that, precautions should be taken while choosing biological agents because they can also affect the non-target microbes in soil (Van Lenteren, 2012; Van Wilgen et al., 2013; Weyl and Martin, 2016; Jones et al., 2017; Myers and Cory, 2017). In a recent study by Caser et al., 2020, a plant phytotoxin (ailanthone) extracted from the leaves, secondary roots, rachises, and samaras of *Ailanthus altissima* was used as a bioherbicide against two weed species i.e. *Raphanus sativus* L. and *Lepidium sativum* L. in the cultivation of three crops *Salvia rosmarinus*, *Salvia officinalis* and *Dianthus caryophyllus*. In their study, a reduction in weed growth was reported when the extract was sprayed post-emergence to the crop. No weed growth was reported in any of the pots of *S. rosmarinus* and *S. officinalis* when sprayed with 100 and 200 mg/l of leaf extract (Caser et al., 2020). In a very innovative study by Cavalcante and the team, extracts from agro-industrial residues (shrimp shell and orange peel) were prepared enzymatically and successfully used as bio-herbicides under controlled conditions (Cavalcante et al., 2021).

Table 2.3 Registered herbicides and their recommended dose (Rana and Rana, 2016)

Herbicide	Crop(s)	Dosage/ha	
		Active ingredient (g/kg)	Formulations (kg/l, g/ml)
Alachlor (50%)	Maize	2.5 kg	5 l
	Cotton	2.0-2.5 kg	4-5 l
	Groundnut	2.5 kg	5 l
	Soybean	2.5 kg	5 l
Anilofos (30%)	Transplanted rice	0.3-0.45 kg	1-1.5 l
Atrazine (50%)	Maize	0.5-1.0 kg	1-2 kg
Bensulfuron Methyl (60%)	Transplanted Rice	60 g	100 g
	Transplanted Rice	60 g	100 g
Butachlor (50%)	Transplanted Rice	1.25-2.0 kg	2.5-4 l
Carfentrazone ethyl (40%)	Wheat	20g	50 g
Clomazone (50%)	Soybean	0.75-1.00 kg	1.5-2.0 l
	Transplanted rice	0.4 - 0.5 kg	0.8-1.0 l
2,4-D Dimethyl Amine salt (58%)	Maize	0.5 kg	0.86 l
	Wheat	0.5-0.75 kg	0.86-1.29 l
	Sorghum	1.8 kg	3.1l
	Potato	2.0 kg	3.44 l
	Sugarcane	3.5g	6.3l
2,4-D Sodium salt	Citrus	1.00-2.5 kg	1.25-3.2 kg
	Grapes	2.0g	2.5l
	Maize	1.00 kg	1.25l
	Sugarcane	2.0-2.6	2.5-3.25l
	Wheat	0.5-0.84 kg	0.625-1.0l
Diuron (80%)	Rubber	1.6-3.2 kg	2-4 kg
	Citrus (sweet orange)	2-4.0 kg	2.5-5.0 kg
	Cotton	0.75-1.5 kg	1-2.2 kg
	Maize	0.80 kg	1.0 kg
	Banana	1.60 kg	2.0 kg

	Sugarcane	1.6-3.2 kg	2.0-4.0 kg
	Grapes	1.6 kg	2.0 kg
Fenoxaprop-p-ethyl 9.3% w/w EC (9% w/v)	Soybean	100g	1111 ml
	Rice (transplanted)	56.25 g	625 ml
	Blackgram	56.25-67.5 g	625-750 ml
	Cotton	67.5 g	750 ml
Fluchloralin (45%)	Soybean	1.0-1.5 kg	2.22-3.33l
	Cotton	0.9-1.2 kg	2.0-2.68 l
Glufosinate Ammonium 13.5% SL (15% w/v)	Tea	0.375-0.500 g	2.5-3.3l
	Cotton	375-450 g	2.5-3.0l
Glyphosate	Tea	0.820-1.230 kg	2.0-3.0l
Imazethapyr Technical	Soybean	100 g	1000l
	Ground nut	100-150 g	1000-1500l
Metolachlor (50%)	Soybean	1.0 kg	2.0 l
Metribuzin (70%)	Soybean	0.35-0.525 kg	0.5-0.75 kg
	Wheat	Medium soil - 0.175 kg Heavy soil - 0.21 kg	0.25 kg, 0.30 kg
Metsulfuron Methyl (20%)	Wheat	4 g	20 g
	Rice (transplanted)	4 g	20 g
	Sugarcane	6 g	30 l
Orthosulfamuron (50%)	Transplanted Rice	60-75 g	1503 l
Oxadiargy (l 6%)	Transplanted Rice	100 g	1.66 l
	Mustard	90 g	1500l
	Cumin	60-75 g	1.0-1.25 l
Oxyflourfen (23.5%)	Onion	100-200 g	425-850 l
	Tea	150-250 g	650-1000 l
	Rice	150-240 g	650-1000 l
	Groundnut	100-200 g	425-850 l
	Potato	100-200 g	425-850 l
Pendimethalin (30%)	Wheat	Light soil - 1.0 kg	3.3 l, 4.2, 5.0 l

		Medium soil - 1.25 kg Heavy soil - 1.5 kg	
	Rice (transplanted & direct sown upland)	Light to Heavy soil 1 - 1.5 kg	3.3-5 l
	Cotton	0.75-1.25 kg	2.5-4.165 l
	Soybean	0.75-1.0 kg	2.5-3.3 l
Pendimethalin (5%)	Rice (transplanted & direct sown puddled)	1.0-1.5 kg	20-30 kg
	Soybean	580.5- 677.25 g	1500-1750 l
	Cotton	677.27 g	1500-1750 l
	Chilly	677.27 g	1500-1750 l
	Onion	580.50- 677.25g	1500-1750 l
	Wheat	40-45 g	800-900 ml
Propaquizafop (10%)	Soybean	50-75 g	500-750 l
	Onion	62.5 g	625 l
	Blackgram	75-100 g	750-1000 l
Paraquat dichloride (24%)	Tea	0.2-1.0 kg	0.8-4.25 l
	Potato	0.5 kg	2.0 l
	Cotton	0.3-0.5 kg	1.25-2.0 l
	Rubber	0.3-0.6 kg	1.5-2.5 l
	Coffee	250 g	1.0 l
	Sugarcane	500 g	2.0 l
	Sunflower	400 g	1.6 l
	Rice	0.3-0.8 kg	1.25-3.5 l
	Wheat	1.0 kg	4.25 l
	Maize	0.2-0.5 kg	0.8-2.0 l
	Grapes	0.5 kg	2.0 l
	Apple	0.75 kg	3.25 l
Pyrithiobac Sodium (10%)	Cotton	62.5-75 g	625-750 l
Tembotrione (34.4%)	Maize	120 g	286 ml
Triallate (50%)	Wheat	1.25 kg	2.5 kg

2.2.5 Biotechnological methods

2.2.5.1 Herbicide resistant crops

Among genetically modified crops, herbicide-resistant crops are most commonly used. These crops have consistently been used in the agriculture production system from the year of their introduction. It has been estimated that these crops have occupied 53% of genetically modified crops (according to the ISAAA GM Approval Database report). Different types of strategies can be used to make the herbicide-tolerant. It includes cisgenic (introduce the resistant gene from plant origin), transgenic (introduce the resistant gene from diverse origin). Recently CRISPR/Cas9 and TALENs systems were also used for herbicide resistance through targeted genome editing (James, 2008; Endo and Toki, 2013). Commercially used and worldwide approved genes for herbicide tolerance are *bxn* (against oxynil), hppd (against isoxaflutole), *bar* and *pat* (against glufosinate), *epsps* (against glyphosate), *aad-1, aad-12* (against 2,4-D), and *als* (against sulfonylurea) (Heap and Duke, 2018).

Glufosinate herbicide is also known as glufosinate ammonium, Phosphinothricin, and basta is an important herbicide compound that hinders the action of glutamine (enzyme synthetase) and ultimately leads to ammonia assimilation and plant death. Glufosinate was first time found in two actinomycetes *Streptomyces viridochromogenes* and *S. hygroscopicus.* These actinomycetes produce bialaphos (a tripetide) under fermentation conditions. Bialaphos was used as a broad-spectrum and non-selective herbicide in the eastern Asia region (Bayer et al., 1972; Dayan et al., 2019). Glufosinate was adopted as a commercial broad-spectrum herbicide for weed suppression in Canada and the USA for the first time in the year 1993. According to a study in the year 2014, near about twelve million hectare area of the world was treated with glufosinate (Busi et al., 2018).

It has been confirmed that glufosinate had no harmful effect on soil organisms and microbes such as earthworms, actinobacteria, proteobacteria, gemmatimonadetes, acidobacteria and bacteroidetes (Dorn et al., 1992; Tang et al., 2019). Application of glufosinate inhibits the activity of glutamine synthetase enzyme, leading to reduction of glutamate and glutamine and accumulation of ammonia. In the absence of glutamate and

glutamine, glyoxylate is accumulated, which ultimately leads to the inhibition of photorespiration and the Calvin cycle (Wild and Wendler 1993; Lu et al., 2014; Takano et al., 2020). Oxidative stress is generated in the chloroplast due to the inhibition of photorespiration and the Calvin cycle. In full sunlight reactive oxygen species are produced in chloroplast which leads to peroxidation of cell membrane and ultimately cell death (Demidchik, 2015; Takano et al., 2019).

There are several reports on the successful performance of *bar* gene (bialaphos resistance) into many commercially important crop plants such as *Zea mays* (Gordon-Kamm et al., 1990; Fromm et al., 1990), *Triticum aestivum* (Vasil et al., 1992, 1993; Weeks et al., 1993), *Sorghum bicolor* (Casas et al., 1993), *Brassica napus*, B. oleracea (DeBlock et al., 1989), *Solanum tuberosum* (DeBlock et al., 1987), *Avena sativa* (Somers et al., 1992), *Daucus carota* (Droge et al., 1992), *Secale cereale* (Castillo et al., 1994), *Festuca arundinacea* (Wang et al., 1992), *Hordeum vulgare* (Wan and Lemaux, 1994), *Lycopersicon esculentum* (DeBlock et al., 1987, 1989), *Medicago sativa* (Eckes et al., 1989; D'Halluin et al., 1990), *Nicotiana tabacum* (DeBlock et al., 1987), *Oryza sativa* (Datta et al., 1992; Toki et al., 1992), *Populus spp* (DeBlock, 1990; Devillard, 1992; Chupeau et al., 1994). The introduction of *bar* gene into various important crops has been of great value to make them resistant to glufosinate herbicide. Field trials of these glufosinate-resistant crops have confirmed that there is no harmful impact of this gene on the quality or yield of crops. Earlier studies on the *Agrobacterium*-mediated transformation of the *bar* gene in different crops are enlisted in Table 2.4.

Table 2.4 Earlier reports on *Agrobacterium* mediated transformation of crops with *bar* gene

Plant name	Explant used	Transformation efficiency	References
Medicago saliva L (alfalfa)	Stem and petiole	Not mentioned	D'Halluin et al., 1990
Beta vulgaris L. (sugar beet)	Embryogenic callus	Not mentioned	D'Halluin et al., 1992
Solanum tuberosum (potato)	Leaf discs	Not mentioned	Figueira Filho et al., 1994
Saccharum officinarum L (sugarcane)	Meristematic region	10-35 %	Enriquez-Obregon et al., 1998
Lactuca sativa (lettuce)	Seeds	Not mentioned	Mohapatra et al., 1999
Populus alba L (white poplar)	Internodal stem segments (5–10 mm)	7 %	Confalonieri et al., 2000
Phaseolus vulgaris L. (dry bean)	Embryonic axes	Not mentioned	Aragao et al., 2002
Allium cepa (onion)	Immature embryos	0.9 %	Eady et al., 2003
Saccharum species hybrids (sugarcane)	Axillary buds	50 %	Manickavasagam et al., 2003
Cynodon dactylon X *C. transvaalensis* (triplod bermuda grass)	Compact and globular calluses (5mm)	18 independent transgenic lines	Hu et al., 2005
Coffea canephora P (Coffee)	Leaves	Not mentioned	Ribas et al., 2006
Agrostis stolonifera L (bentgrass)	Callus	Not mentioned	Kim et al., 2007
Ipomoea batatas (L.) Lam. (Sweet potato)	Shoot apex	Not mentioned	Choi et al., 2007
Vaccinium spp (Blueberries)	Leaves	Not mentioned	Song et al., 2007
Ipomoea batatas L. (Sweet potato)	Embryogenic callus	Not mentioned	Yi et al., 2007
	Embryogenic suspension cultures	Not mentioned	Zang et al., 2009

Quercus suber L. (cork oak)	Embryogenic cell lines	42 %	Alvarez et al., 2009
Eustoma grandiflorum (Lisianthus)	Nodes	67–80%	Chen et al., 2010
Lolium perenne L (Ryegrass)	Callus	Not mentioned	Jin-Xia et al., 2012
Vigna unguiculata L (cowpea)	Whole immature cotyledons of 0.5–0.6 cm in length	62 %	Aasim et al., 2013
Lotus corniculatus L. (bird's foot trefoil)	Seeds	Not mentioned	Nikolic et al., 2013
Glycine max L. Merr (soybeans)	Cotyledonary node	1.06 %	Liu et al., 2014
Salvia miltiorrhiza (Dan Shen)	Leaves	Not mentioned	Liu et al., 2015
Saccharum officinarum L (sugarcane)	Embryogenic callus	Each gram of callus produced 10 transgenic lines approximately	Wang et al., 2017
Zea mays (maize)	Embryo	1.01 to 2.74%	Hong et al., 2019

2.3 Diabetes

Diabetes is a major threat to human beings on a global scale. The highest occurrence of diabetes was reported in the Eastern Mediterranean region in the year 1980, and still increasing continuously 0.23 % annually (Table 2.5). It has been estimated that there will be more than 600 million people affected with diabetes by the year 2045 (Duarte et al., 2018). Imbalanced nutrition, obesity, sedentary or unhealthy lifestyle, smoking, regular alcohol uptake, unhealthy diet, high caloric food, genetic predisposition, etc. (Parkkola et al., 2017; Mercader and Florez, 2017; Luo et al., 2018) are the major reasons of diabetes. Most commonly, diabetic people suffer from neurodegenerative disorders, different types of cancers, aggressive metastatic and cardiovascular diseases (Cebioglu et al., 2010; Yeghiazaryan et al., 2013), blindness (Golubnitschaja, 2013), chronic wounds, and impaired wound healing (Adler et al., 1997; Sheehan et al., 2003; Illigens and Gibbons, 2013).

2.3.1 Artificial sweeteners

These days overconsumption of sugar-rich food leads to various health-related problems in the people of developed as well as in developing countries. This drastic increase in health-related problems promoted the search for low calories sweeteners as an alternative to high calories sweeteners. Although a number of synthetic or artificial sweeteners have already been present in the market (O'Brien, 2012), nowadays people are aware of the consequences of artificial sweeteners and are more focused on the use of natural sweeteners as compared to synthetic ones (Cheron et al., 2018).

Table 2.5 Top ten countries of the world recorded with maximum numbers of diabetic patients in 2015 (Luo et al., 2018)

Position	Country	Adult diabetic patients (million)
10	Bangladesh	7,1
9	Japan	7,2
8	Egypt	7,8
7	Indonesia	10,0
6	Mexico	11,5
5	Russian Federation	12,1
4	Brazil	14,3
3	USA	29,3
2	India	69,2
1	China	109,6

Artificial sweeteners are food additives that provide a sweet taste without increasing the calorie count. They are also known as low caloric, non-nutritive and high-intensity sweeteners (Shankar et al., 2013; USFDA, 2015; Sylvetsky and Rother, 2016). Among the artificial sweeteners acesulfame-K, aspartame and sucralose are the most popular (Araújo et al., 2014; Nettleton et al., 2016). In the USA, the most commonly used artificial sweeteners are acesulfame, sucralose, and aspartame (Yang, 2010). Usage of aspartame is approved as an artificial sweetener in near about > ninty countries of the World (Magnuson et al., 2007; Chattopadhyay et al., 2014). While in Germany and Switzerland, there is more consumption of cyclamate (Scheurer et al., 2009; Buerge et al., 2009).

Various animal studies have revealed that long-term exposure to artificial sweeteners led to neurobehavioral effects, increased body weight, alteration in gut microbiota, and impairment of glucose and insulin homeostasis (Attari et al., 2018). Their more consumption causes several side effects such as weight gain, metabolic syndrome, type II diabetes, cardiovascular diseases, and hypertension (Swithers, 2013) (Fig. 2.3). Besides that, their long term consumption affects the diversity of microflora (lactobacilli and bifido) (Nettleton et al., 2016; Suez et al., 2014; Abou-Donia et al., 2008) of both humans and mice (Anderson and Kirkland, 1980; Abou-Donia et al., 2008, Schiffman and Rother, 2013; Shreiner et al., 2015; Frankenfeld et al., 2015).

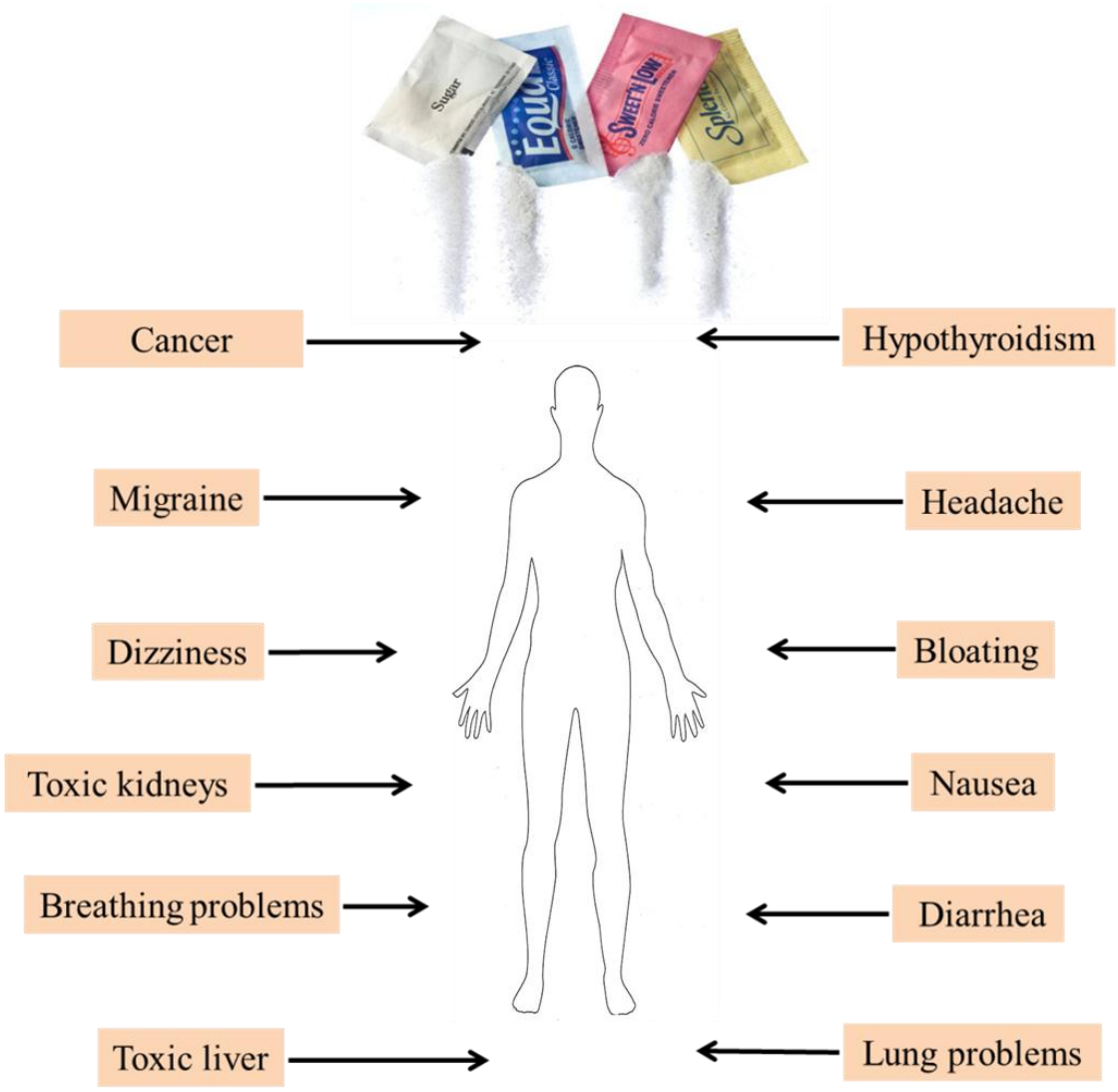


Fig. 2.3 Harmful effects associated with use of artificial sweeteners on human body

2.3.2 Natural sweeteners

Nowadays, people are more conscious in terms of their health and hence consumption of high-calorie sugar has been replaced by low-calorie sweeteners especially natural sweeteners. These sweeteners are of low calories and can be consumed by health-conscious people. Presently, there are ten most commonly used natural sweeteners having much higher sweetness as compared to sucrose. Among them, miraculin (400,000 times sweeter than sucrose), thaumatin (1,600 to 3,000 times sweeter), and pentadin (500 to

2,000 times sweeter) have maximum sweet content while, thaumatin and stevia sugar are used most commonly (Swiąder et al., 2019). It is strongly recommended that stevia sugar can be the best substitute for diabetic and health-conscious people to fulfill their desire for sweet taste (Kalpana et al., 2011).

Various types of natural sweeteners have already been used in beverages or food products (Beltram et al. 2018). Glycyrrhizin, erythritol, and thaumatin are types of natural sweeteners. Among them, glycyrrhizin and thaumatin have been used to enhance the flavor of chewing gum and soft drinks (Jain and Grover 2015). While, erythritol is blended with certain other sweeteners for cooking or baking. Sweeteners can be divided into two categories i.e. low potency (sweetening potency 1 or less than 1) and high potency sweeteners (sweetening potency more than 10). Saccharin, aspartame, and acesulfame potassium are high potency sweeteners while sucrose, glucose, xylitol, and mannitol are some examples of low potency sweeteners.

Gwak et al, measured the relative sweetness of twelve different types of sweeteners i.e. fructose, corn syrup, maltitol, fructooligosaccharides, sucralose, xylose, rebaudioside A, aspartame, tagatose, stevia, erythritol, and xylitol. The 2-alternative forced-choice method was adopted in their study and sucrose solution (5%) was taken as a control. In this study relative sweetness of rebaudioside A 97, tagatose, stevia, and erythritol were found to be 227, 0.85, 64.1, and 0.63 respectively (Gwak et al., 2012). In another study by Baek, low calorie ssanghwa beverage was prepared with different types of sweeteners such as acesulfame-K, aspartame, and glucosylated stevia. Sucrose solution (10%) was taken as control and a binary solution model was used in their study. The relative sweet content of stevia, aspartame and acesulfame-K was found to be 100, 140, and 170 respectively (Baek et al., 2008).

2.4 *Stevia rebaudiana* Bertoni

This plant is popular for its high sweet content due to the presence of steviol glycosides (Fig. 2.4). Stevia powder was introduced at the commercial level into the market of Japan as a natural sweetener (Ashok et al., 2011). There are total of thirteen species of the stevia genus having sweetening content are *S. lemmonii*, *S. dianthoidea*, *S. viscida*, *S.*

phlebophylla, *S. serrate*, *S. anisostemma*, *S. salicifolia*, *S. bertholdii*, *S. crenata*, *S. rebaudiana*, *S. enigmatica*, *S. plummerae*, *S. eupatoria* and *S. micrantha*, among these species S. rebaudiana is the only species having maximum sweetness (Singh et al., 2019). Several cultivars of stevia have already been developed, some of them are: Him stevia (stevioside =5.87% and rebaudioside-A = 7.34%), stevia UEM-13 (stevioside = 4.1%; and rebaudioside-A= 9.1%), CIM Mithi, RSIT 94-1306 (stevioside = 17.25% and rebaudioside-A = 0%), CIM Madhu (stevioside = 12.57 %, rebaudioside A = 5.8% and dulcoside A = 0.2%) (Lal et al., 2011), RSIT 94-75, SRB-128 (stevioside = 21 %), RSIT 95-166-1, SRB-123 (glycosides = 9-12%), Morita II (stevioside = 3.97% and rebaudioside A = 15.15%), AC Black Bird (glycosides = 14%), Yungri SM 4 and Madhuguna.

United States Department of Agriculture gives stevia classification (USDA 2019).

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Asterales

Family: Asteraceae/Compositae

Genus: Stevia Cav

Species: *Stevia rebaudiana* Bertoni

Stevia sugar is almost a hundred times sweeter than sucrose and contains zero calories (Prakash et al., 2008; Belloir et al., 2017). Steviosides (4-13%) and rebaudiosides A (2-4%) are the two most important glycosides of stevia, followed by rebaudioside C (1-2%) and dulcoside A (0.4-0.7%). Among them, steviosides and rebaudiosides A are actually responsible for sweet taste (Kurek and Krejpcio, 2019). Steviol glycosides do not absorb

in the human body and do not hydrolyze enzymatically; hence they are considered to be safe for use (Geuns et al., 2007). Steviol glycosides are also resistant to pH alterations, high temperature, and longtime sunlight exposure (Brandle et al., 1998). Traditionally, stevia was used in form of dried leaves to sweeten tea and other drinks but, these days stevia sugar is extensively used as a natural sweetener to sweeten the food products like biscuits, ice-cream, chocolates, yogurt, fruit, and soft drinks, and various other beverages (Table 2.6).

Jabeen and co-workers have found that 4% of stevia extract can be a possible substitute for other sugars in guava drinks. It has been recommended in this study that stevia extract enhance the nutritional properties of guava drinks without compromising their taste (Jabeen et al., 2019). An experiment was conducted by Šic Žlabur J and the team to find the difference between the nutritional composition of chokeberry juice sweetened with stevia sugar and sucrose. It has been reported that chokeberry juice having stevia sugar has higher vitamin C content (2 times), anti-oxidant activity (3%), and phenol content (6%) than sucrose sugar (Šic Žlabur J et al., 2018).

Fig. 2.4 *Stevia rebaudiana*: plant habit

Table 2.6 List of food products containing stevia sugar

Product name	Stevia form	Amount	References
Yoghurt cake	Leaf extract (liquid)	3.33 %	Abdel-Salam et al., 2009
Custard, kulfi and sandesh	Stevia extract	25 mg	Agarwal et al., 2010
Strawberry flavored yoghurt	Leaf powder	6 % / 100 g yoghurt	Lisak et al., 2011
Kulfi	Steviosides	0.05–0.07 %	Giri et al., 2012
Kuih Baulu (traditional cake in Malaysia)	Rebaudioside A	40% stevia + 60% isomalt	Hamzah et al., 2013
Mango nectar	Rebaudioside	0.052 %	Cadena et al., 2013
Ice cream	Leaf powder	0.02–0.11 %	Alizadeh et al., 2014
Ready-to-serve fruit beverages	Stevia aqueous extract	100 g	Balaswamy et al., 2014
Ice cream	Leaf powder	0.862 %	Ozdemir et al., 2015
Passion fruit juice	Leaf extract	0.09924 %	Rocha and Bolini 2015
Ice cream + Cocoa	Leaf powder	0.786 %	Ozdemir et al., 2015
Ice cream	Rebaudioside –A	0.6-1.7 %	Pon et al., 2015
Orange nectar	Stevioside	0.02–0.06 %	Hosseini et al., 2015
Muffin	Rebaudioside A	9.97 and 19.76 %	Gao et al., 2016
Muffin	Steviol glycosides	0.075–0.300 %	Karp et al., 2016
Muffin	Steviol glycosides	0.09 %	Karp et al., 2017
Bittersweet chocolate	Stevia extract	0.16 %	Azevedo et al., 2017

2.4.1 Biochemical constitution of stevia

The Biochemical constitution of stevia depends upon geographical area, cultivars as well as methods used for leaf drying and their processing also affects its constituents (Gasmalla et al., 2014; Khiraoui et al., 2017). It has been reported from previous researches that stevia is a nutrient-rich plant, which contains minerals (Fig. 2.6), calcium, protein, amino acids (Fig. 2.5), vitamin C, phosphorous, folic acid, etc. in its leaves (Viana and Metivier, 1980; Lemus-Mondaca et al., 2012). The composition of stevia plants grown in Venezuela, Colombia, and France was reported in a study. It has been found that it contains crude protein (9.9-11.3%), crude fat (1.2-1.8%), and ash contents (6.3-7.6 %) (González et al., 2014). Total 62 compounds including major compounds quercetin glucosyl, α-cadinol, protocatechuic acid, carvacrol caryophyllene oxide,

quercetin dihydrate, (−)-spathulenol, ibuprofen, β-guaiene, isopinocarveol, α-pinene and limonene, and has been reported in stevia leaves. 86.50% of palmitic acid was the abundant fatty acid found in leaves of stevia (Muanda et al., 2011; Siddique et al., 2012). Stevia leaves also contain carbohydrates (Braz de Oliveira et al., 2011), proteins (Abou-Arab et al., 2010; Mohammad et al., 2007), minerals (Calcium, phosphorus, sodium, potassium, iron, magnesium, zinc) (Tadhani and Subhash, 2006; Mishra et al., 2010; Abou-Arab et al., 2010; Serio, 2010; Goyal et al., 2010; Kaushik et al., 2010), lipids (Tadhani and Subhash, 2006) and vitamins C, B2, B6, folic acid, niacin, thiamine (Kim et al., 2011).

2.4.2 Phytochemical constituents

Tannins, alkaloids and polyphenols are the most important phytochemicals of plants (Edeoga et al., 2005). Stevia is rich in thiamine, austroinullin, stevioside, b-carotene, steviol, dulcoside, riboflavin, nilacin and rebaudi-oxides (Jayaraman et al., 2008).

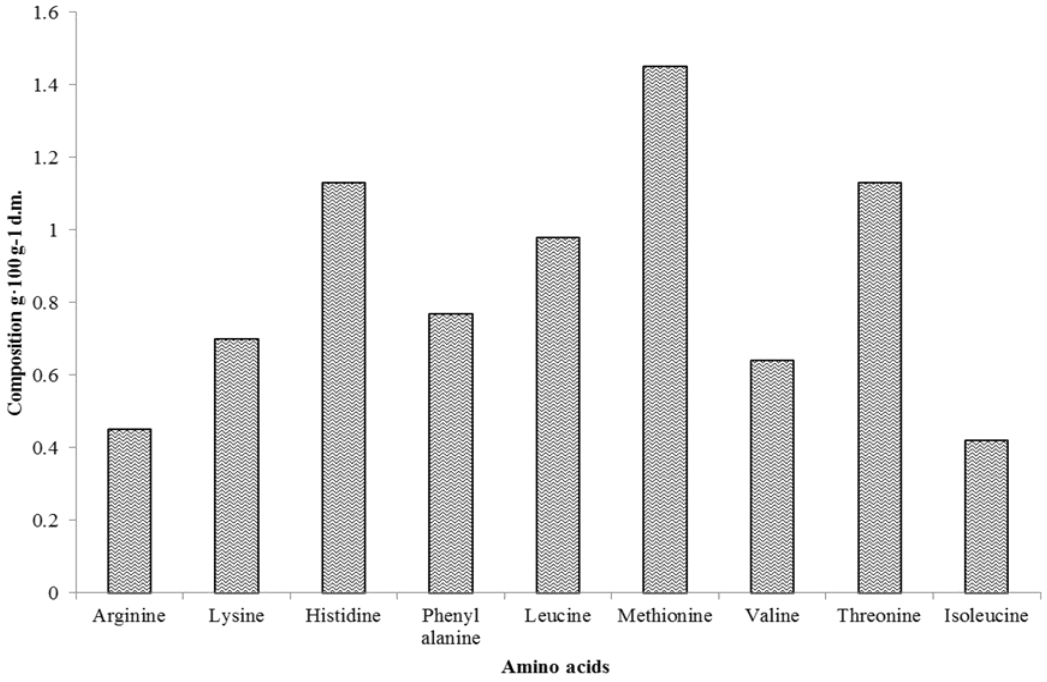
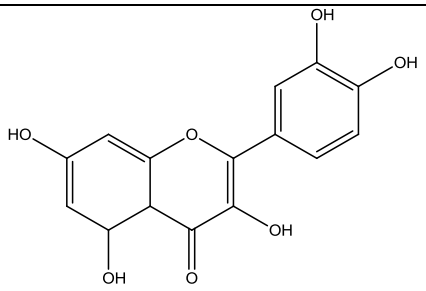
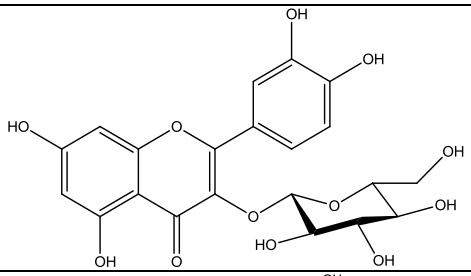
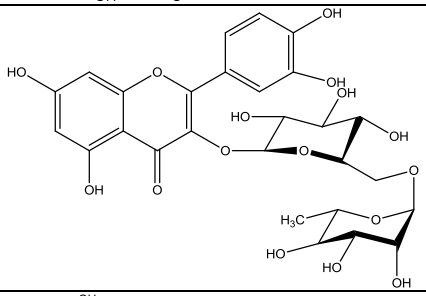
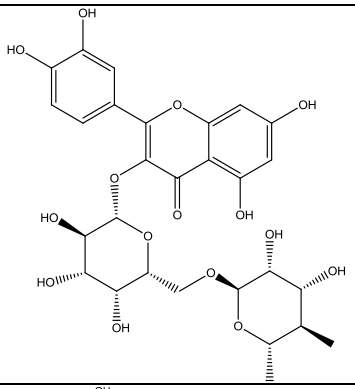
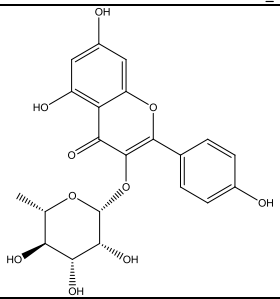
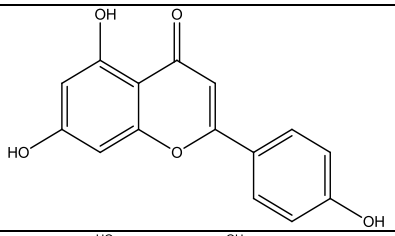
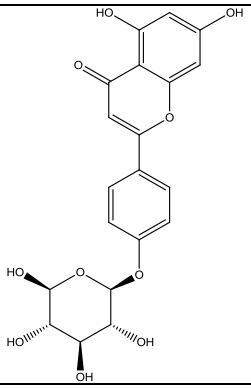
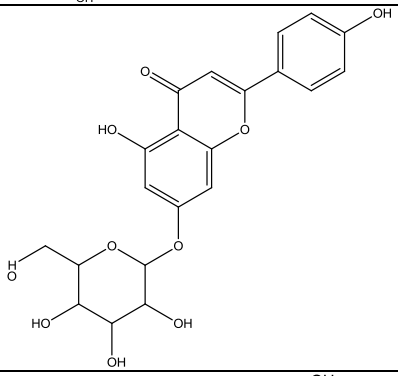
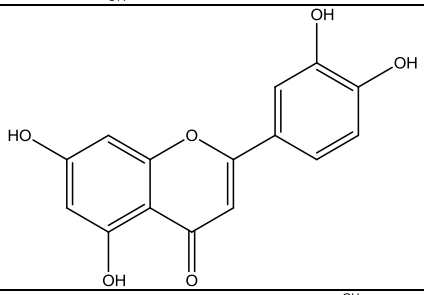
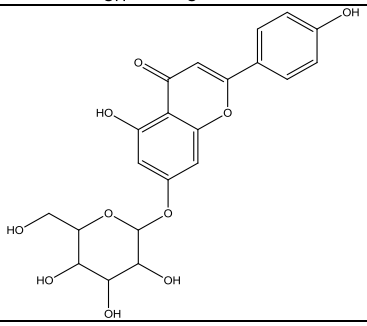


Fig. 2.5 Composition of amino acid in *Stevia* leaves (Abou-Arab et al., 2010)

Table 2.7 Flavones and flavonols found in stevia leaves

Compound	Structure	Reference (s)
Quercetin		Li et al., 2010
Quercetin-3-O-glucoside		Cacciola et al., 2011
Quercetin-3-O-rutinoside		Cacciola et al., 2011
Quercetin-3-O-(4-O-transkafeoil)-α-ramno-pyranosil(1-6)-β-D-galactopyranoside		Li et al., 2010
Kaempferol-3-O-rhamnoside		Ghanta et al., 2007

Apigenin		Ghanta et al., 2007
Apigenin-40-O-β-D-glicoside		Ghanta et al., 2007
Apigenin-70-O-β-D-glicoside		Li et al., 2010; Cacciola et al., 2011
Luteolin		Ghanta et al., 2007; Li et al., 2010
Luteolin-70-O-β-D-glicoside		Li et al., 2010; Cacciola et al., 2011

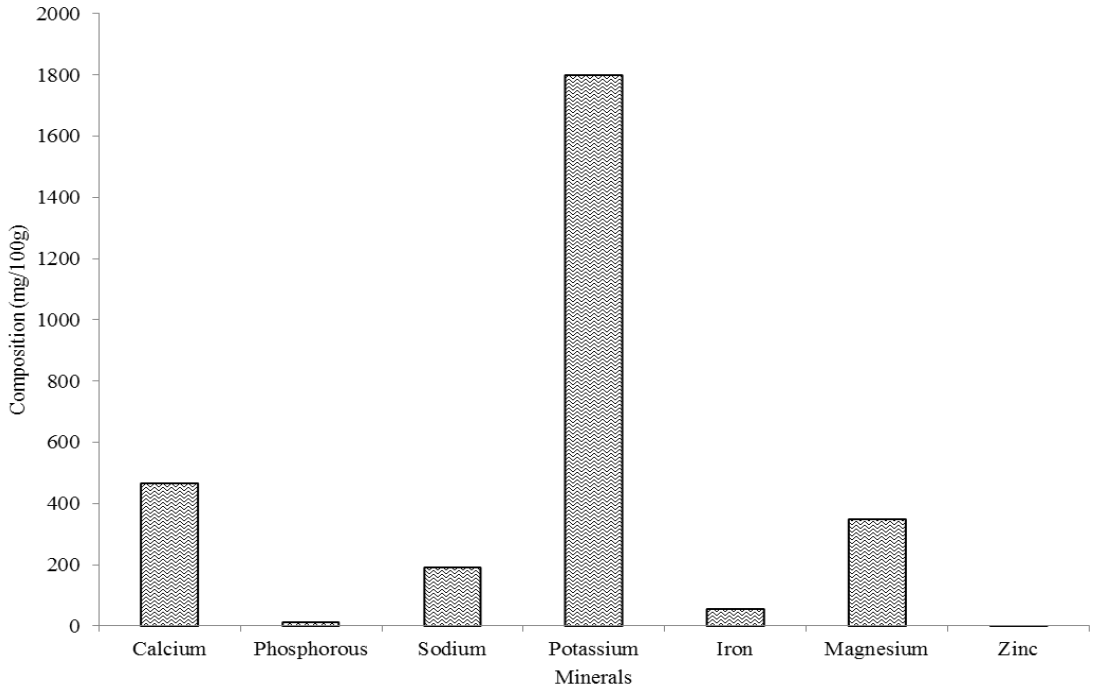


Fig. 2.6 Minerals content (mg/100g) of dried stevia leaves (Mishra et al., 2010)

Steviosides were found to be most abundant in stevia leaves as compared to rebaudioside B (Fig. 2.7). The concentration of steviosides highly depends on the agriculture conditions (Pol et al., 2007) and agronomical strategy (Geuns, 2003; Nepovim et al., 1998). All the diterpene glycosides obtained from stevia leaves have similar steviol backbone, the only difference is in the carbohydrate residue (Kochikyan et al., 2006) (Fig. 2.8). The trend of stevioside and rebaudioside content in various plant parts of stevia is leaves > flowers > stems > seeds > roots. The sweetness of the leaves is almost twice as compared to inflorescence (Dwivedi, 1999, Bondarev et al., 2003). According to Kumar et al., 2012 maximum stevioside content was reported in the leaves present at the third node of the plant.

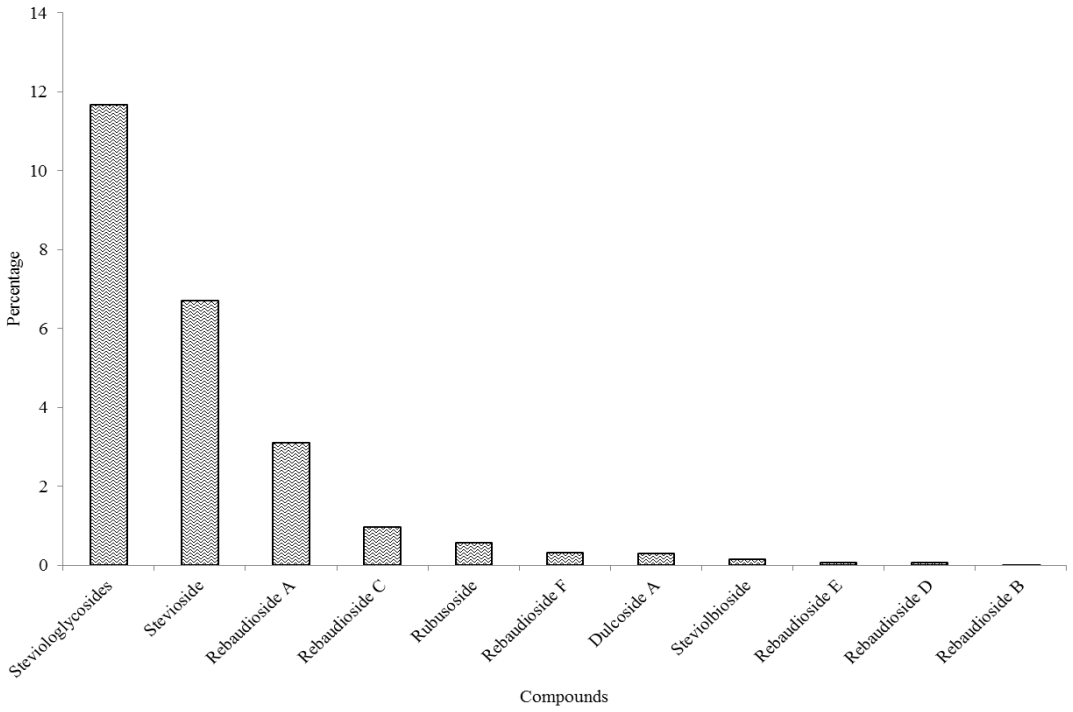


Fig. 2.7 Composistion of steviol glycosides in leaves of Stevia (% of dry weight) (Makapugay et al., 1984; Brandle et al., 1998; Bender et al., 2015)

2.4.3 Metabolism of steviol glycosides

Concerns regarding the steviol glycosides safety in food products have been reviewed in a number of studies (Carakostas et al., 2008; EFSA 2010, 2015; Momtazi-Borojeni et al., 2017). Various reports are available regarding the absorption, metabolism, and excretion of steviol glycosides in the human body. Some *in vitro* studies reported that brush borders enzymes of hamsters, rats, and mice, and human salivary and pancreatic enzymes are not able to degrade these steviol glycosides, while, microbes present in the gut of humans, hamsters, and rodents are able to do so (Hutapea et al., 1997). In a report by Magnuson et al., 2016 it has been reported that these glycosides remain undigested in the upper part of the gastrointestinal tract and degraded (cleave glycosidic bonds) by the microflora present in the colon. Degraded glycosides then glucuronidated in the liver and excreted in form of urine or feces.

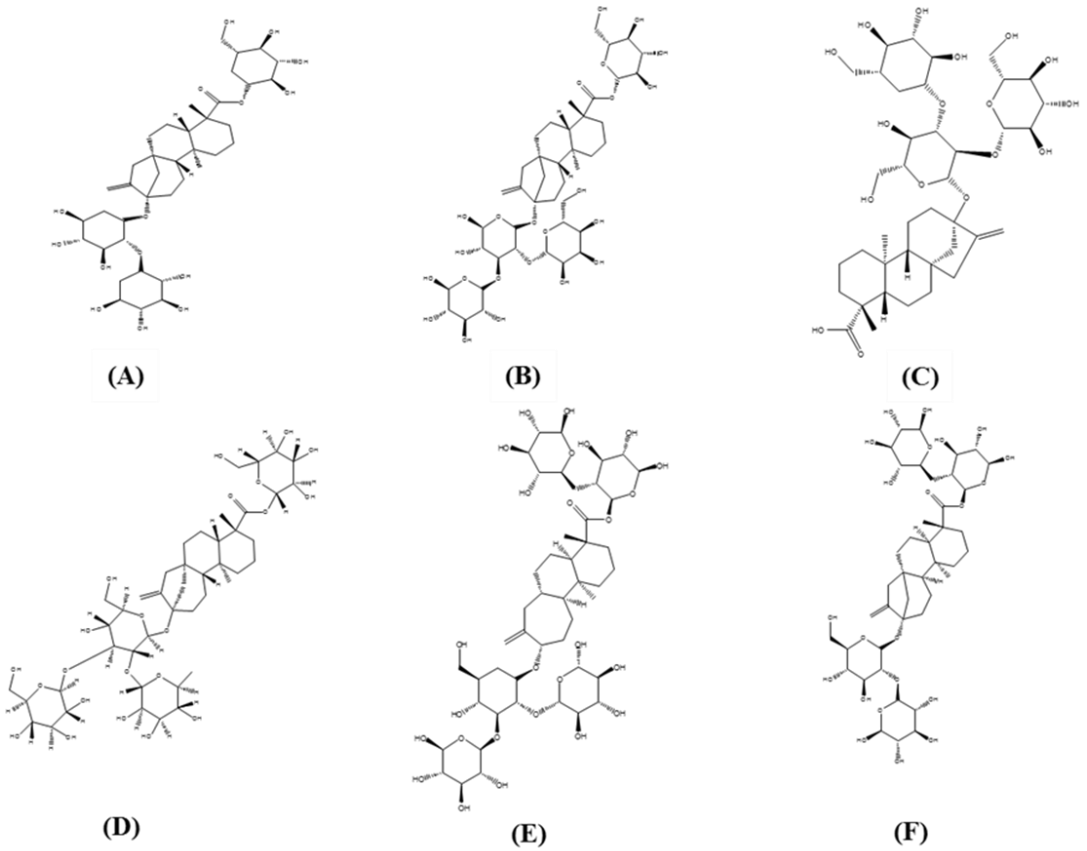


Fig. 2.8 Chemical configuration **(A)** Stevioside, **(B)** Rebaudioside A, **(C)** Rebaudioside B, **(D)** Rebaudioside C, **(E)** Rebaudioside D and **(F)** Rebaudioside E

Several researchers also reported that steviol glycosides are first transported to the liver and then form steviol glucuronide on bonding with glucuronic acid. This steviol glucuronide is then excreted in form of urine (in humans) (Kraemer and Maurer, 1994; Geuns et al., 2006, 2007; Wheeler et al., 2008). Several other studies have concluded that gluconate molecules of steviol glycosides are transferred to kidneys for the filtration process. While, the unfiltered glycosides are removed from the body through feces (Koyama et al., 2003; Muanda et al., 2011).

2.4.4 Pharmacological activities

Herbal remedies are more popular among people all over the world for their health care. Stevia also has a long history in the Ayurvedic system for its medicinal value (Megeji et al., 2005; Taak et al., 2019). Various biochemical compounds such as derivatives of

oxygen and phenols are produced by all the plants. These compounds play a crucial role in plant protection against infectious attacks by certain pathogens (Johnson et al., 2010). Stevia plant also produced certain kinds of flavonoids, volatile oil, triterpenes, sterebins A to H, pigments, and gums (Siddique et al., 2014). Several clinical studies suggest the pharmacological and therapeutic potential of stevia extracts. Besides the sweetness property, stevioside and other related compounds exhibit many healing benefits such as immunomodulatory, anti-hypertensive, diuretic, anti-diabetic, anti-diarrhoeal, anti-inflammatory, anti-tumor, anti-oxidant, anti-bacterial, etc. (Zia-Ul-Haq et al., 2011). Stevia leaves have been reported to cure various ailments such as dental caries, obesity, inflammatory bowel disease, renal diseases, cancer etc. (Gupta et al., 2013). Moreover, certain other studies have been confirmed that the stevia plant has the potential against allergies, genetic defects, teratogenesis, and mutagenesis (Yildiz-Ozturk et al., 2015). Table 2.8 and Fig. 2.9 present the various known pharmacological properties of stevia.

Fig. 2.9 Health benefits of *Stevia rebaudiana*

Table 2.8 Summary of known pharmacological activities of stevia

Pharmacological activity	Methodology	Reference(s)
Anti-inflammatory	Investigated immunomodulatory and anti-inflammatory effects of steviosides on THP-1 cells. It has been found that steviosides inhibits the formation of lipopolysaccharides which augment the production of proinflamatory cytokines: TNF-alpha and IL-beta.	Boonkaewwan et al., 2006
	Staphylococcus aureus was used to infect the mammary gland of mice to induce inflammation. Stevioside inhibits the phosphorylation of JNK, IκBα, ERK, p65 and p38 and also regulate the functioning of MAPK and NF-κB pathway.	Wang et al., 2014
Improves renal function	Steviosides were used to determine their effect on polycystic kidneys in the experimental mouse. Results showed that dose of steviol @200 mg successfully inhibited renal cytogenesis by improving the renal function and decreasing the cyst size. This study showed that an optimal dose of steviol can significantly improve kidney functioning.	Yuajit et al., 2014
Immunomodulatory effects	In vivo trials on experimental wistar rats exhibited that dose of stevioside @ 20mg/kg twice a day reduces the promotion of proinflammatory cytokines i.e. IL-10, IL-17a, IL-6, TNF-α and IL-1β. This study showed that particular dose of steviosides can effectively preserve the functioning of liver.	Casas-Grajales et al., 2019
Oral health-promoting properties	6-month experiment was conducted on 108 girls school children (12-15 years) to determine the success of steviosides as a daily solution for mouth wash. The study displayed that steviosides significantly reduces plaque and other oral diseases thus exhibiting oral health-promoting properties.	Vandana et al., 2017

	2 months study was conducted on 22 people (14 women, 8 men) to determine the efficacy of steviosides as a daily mouth rinse solution. The studies showed that leaf extract of stevia significantly reduce the bacterial count and stabilized the mouth pH.	Siraj et al., 2019
Anti-microbial	Antimicrobial activities of stevia leaf extracts (100mg/ml) were tested against *Epidermophyton species* and *Candida albicans*. Ethyl acetate and acetone extracts were found highly significant against *E. coli*, *S. aureus*, *Aeromonas hydrophila*, *B. subtilis*, *Vibrio cholera* and *Salmonella typhi*.	Jayaraman et al., 2008
	Stevia leaf extracts (100mg/l) prepared in acetone and methanol solvents were found most effective against *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus cereus*. While ethyl acetate, chloroform, acetone and methanol extracts were found efficient against *Asperigillus flavus*, *A. parasiticus*, *A. Fusarium* and *A. ochraceus*.	Abou-Arab and Salem, 2010
	Stevia leaf extracts significantly showed anti-microbial activities against different fungal and bacterial pathogens.	Siddique et al., 2014
	1.25- 2.5 % leaf infusion of stevia was used to study their effect on hemolytic potential of L. monocytogenes. It was found that leaf infusion significantly declined the production of Listeriolysin O, thus exhibiting anti-microbial properties.	Sansano et al., 2017
Cardio-vascular	Set of 168 Chinese women and men of age 20-75 suffering from mild hypertension was separated into 2 groups and treated with stevioside capsules (500 mg) 3 times a day. This treatment efficiently reduced systolic and diastolic pressure, hence exhibited potential cardiovascular properties.	Hsieh et al., 2003
	Effects of isosteviol were examined on ventricular myocytes of hearts of Sprague-Dawley rats. Study showed	Fan et al., 2017

	that isosteviol controlled the level of various factors responsible cardiac hypertrophy.	
Anti-cancer	Study showed that isosteviol, steviol and stevioside significantly inhibited the activation of antigens of Epstein-Barr virus.	Takasaki et al., 2009
	This study revealed that stevioside significantly affects the apoptotic pathway and produced reactive oxygen species which ultimately inhibits the growth of MCF-7 cell line.	Paul et al., 2012
	This study revealed that ethanol and methanol extracts of stevia (25-1000 μg/ml) inhibit the activity of Caco and Ca Ski cell lines.	Deshmukh and Kedari, 2014
	Steviosides significantly hinder the development of HT29 cell line (human colon cancer cells).	Ren et al., 2017
	Steviol significantly inhibit the growth of MCF-7 cell line.	Gupta et al., 2017
	In this study, effect of steviol on apoptotic pathway was determined with an escalation of the ratio of Bax/Bcl-2 markers, cellular tumour antigen p53 and cyclin-dependent kinase inhibitor p21. This study suggests that steviol glycosides exhibited anti-cancer properties.	Chen et al., 2018
Anti-diabetic	The study showed that steviol glycosides elevate the amount of GLUT1 and GLUT4 (glucose transporters) in cells. Steviol glycosides also increased the phosphorylated forms of protein kinases B and phosphoinositide 3-kinases.	Rizzo et al., 2013
	Steviosides (25–150 μM) significantly increased insulin sensitivity and also increase glucose uptake of cells.	Mohd-Radzman et al., 2013
	Steviol glycosides significantly increase the activation of 3T3-L1 adipocytes, GLUT4 transcript and L6 myotubes in model cell line.	Bhasker et al., 2015
	In this study, the mechanism of action of steviosides and rebaudiosides on glucose metabolism pathway were determined. It has been reported that rebaudioside A significantly exhibited an insulinotropic effect.	Abudula et al., 2008

	It has been found in this study that steviosides and steviol significantly augmented the glucose-induced calcium activity in cells of pancreas. This study used HEK293T and pancreatic cells of mice (male and female).	Philippaert et al., 2017
	This study was performed to find the effecacy of rebaudioside A on anti-oxidant activity, levels of insulin and glucose in blood, peroxidation of lipids in diabetic wistar rats. It has been showed that rebaudioside A maintained levels of insulin and glucose in blood of diabetic rats and also regulated peroxidation of lipids.	Saravanan and Ramaan, 2013
	In this study steviosides (1 g) were administered orally to 12 overweight diabetic patients. The results showed that steviosides significantly reduced the levels of blood glucagon and glucose levels in diabetic patients.	Gregersen et al., 2004
	60 day long experiment was conducted on 20 diabetics (Mellitus) patients to check the hypolipidaemic and hypoglycaemic potential of stevia leaf powder (1g/day). The study showed that stevia leaf powder significantly reduced the blood glucose levels in diabetic patients.	Ritu and Nandini, 2016
	This study was conducted to check the difference between sucrose and various other low calorie sweeteners (aspartame, monk fruit and stevia). It has been found that insulin and blood glucose levels increased with the consumption of sucrose while low-calorie sweeteners reduce blood glucose levels.	Tey et al., 2017
Hypotensive	In this study, 106 women (28-75 years aged) suffering from hypertension were administered with stevioside (0.25 g) thrice a day for 1 year. A significant decrease in systolic and diastolic BP was reported in patients after 7 days of treatment.	Chan et al., 2000
	In this study group of patients were administered with a dose of steviosides. Significant arterial tension was reported in these patients as compared to control.	Barriocanal et al., 2008

Hypoglycemic	In this study, 12 patients suffering from type II diabetes were supplemented with steviosides (1 g) daily. A significant escalation in insulin index and postprandial glycemia was reported in these patients.	Gregersen et al., 2004
	Hyperglycemia was induced in rats using Streptozotocin. Intake of steviosides in these rats suppresses glucagon secretion and reduces insulin resistance.	Chen et al., 2005
	In this experiment, type II diabetic rats were administered with dose of steviosides @ 0.025 g/kg. This led to significant increase in insulin secretion and activation of glycolysis genes.	Jeppesen et al., 2003
	In this experiment diabetic rats were supplemented with rebaudisoside A. It has been found that rebaudisoside A significantly reduces the glucose level in blood.	Saravanan et al., 2012
	This study was carried out on insulin-resistant obese mice. This showed that steviol glycosides significantly increase glucose and bile acids metabolism, fat catabolism.	Holvoet et al., 2014

2.4.5 Stevia cultivation

Cultivation of stevia has been initiated in the early 1970s (Mitsuhashi et al., 1975; Miyazaki et al., 1978). European people directed their consideration towards stevia for the 1st time in early 1887, when M.S Bertoni collected this species from Mestizos and Paraguayan Indians (Lewis, 1992). Nowadays stevia is commercially grown in South-East Asia, the USA, Japan, and also in humid and hilly areas of India (Amzad-Hossain et al., 2010; Kumar et al., 2013)

2.4.5.1 Plant propagation

Stem cuttings and seed sowing is the conventional method of stevia propagation. In tropical climates, seed sowing is a successful method while in northern climates glasshouse establishment is necessary for stevia cultivation. Poor seed germination is one of the major problems in stevia cultivation. Some researchers reported that 25ºC is the best temperature for seed germination (Felippe and Lucas, 1971; Rocha and Valio, 1972; Randi, 1980; Randi and Felippe, 1981). Takahashi et al., 1996 reported 90.03 % seed germination at 25ºC after 101.4 h. Seeds can be stored at 4ºC for up to 11 months providing sufficient temperature and humidity. The seed-raised plantlets are transplanted and shoots can be harvested after four to five months (Dwivedi, 1999; Cabanillas and Diaz, 1997). For good seed setting bee cultivation (three to four hives/hectare) is also recommended in some studies (Oddone, 1999). Optimal temperature (20ºC) and light are the two major factors for seed germination. Immature seed led to poor or no germination (Colombus, 1997; Kumar and Sharma, 2012).

2.4.5.2 *In vitro* multiplication

In vitro regeneration of stevia is most promising as compared to conventional methods to fulfill the continuously increasing demand for this natural sweetener plant. Various reports are available in the literature regarding successful *in vitro* regeneration of stevia using different types of explants and concentrations of growth hormones (Koppad et al., 2006; Anbazhagan et al., 2010; Satpathy and Das, 2010; Smitha and Umesha, 2012; Khalil et al., 2014; Singh et al., 2014; Kundu et al., 2014; Suarez and Quintero, 2014) (Table 2.9). Nodal explants were used as explants in a study by Mitra and Pal, 2007. The

highest shoot proliferation and multiplication were observed when these explants were inoculated on MS media augmented with IAA (1.0 mg/l), kinetin (10.0 mg/l), and adenine sulfate (30.0 mg/l). A portion of stem cuttings was also used as explants in some studies (Koppad et al., 2006; Smitha and Umesha, 2012; Khalil et al., 2014). A study reported 39 shoots/explants when incubated on MS media augmented with 2 mg/l of BAP and 1.13 mg/l of IAA (Debnath, 2008). While, 7.82 shoots/explant were reported when incubated on 0.5 mg/l BAP and 0.25 mg/l Kinetin (Razak et al., 2014). Various experiments have been performed to find the callus formation from different explants, like leaves (Swanson et al., 1992; Gupta et al., 2010a), anthers (Flachsland et al., 1996), cell suspension (Ferreira and Handro, 1988), flower (Ahmed et al., 2007), nodes and roots (Gupta et al., 2010b). Leaf discs cultured on MS media augmented with NAA (0.5 mg/l) and BAP (0.5 mg/l) by Swanson et al., (1992) produced friable callus. Bondarev et al., 1998, reported that callus formation was highest with leaf explant than stem explants.

Table 2.9 Previous studies on *in vitro* regeneration of *Stevia rebaudiana*

Explant (s)	Media composition + PGR (mg/L)	Response	Reference(s)
Nodal segment	½ MS + 0.01 NAA (ppm)	Multiple shooting	Acuna et al., 1997
	MS + NAA (0.1)	Rooting	
	MS + Kin (0.5) + IAA (2.0)	Multiple shooting	Hwang, 2006
	MS + IBA (2.0)	Rooting	
	MS + Kin (0.5) + BAP (1.5)	Multiple shooting	Ahmed et al., 2007
	MS + IAA (0.1)	Rooting	
	MS + BAP (2.0)	Multiple shooting	Rafiq et al., 2007
	MS + IAA (0.5)	Rooting	
	MS + BAP (1.0) + IAA (0.25)	Multiple shooting	Laribi et al., 2012
	MS + IAA (0.5)	Rooting	
	MS + Kin (2.0) + BAP (0.5)	Multiple shooting	Mehta et al., 2012
	MS + IBA (1.0)	Rooting	
	MS + Kin (2.0) + BAP (1.0)	Multiple shooting	Modi et al., 2012
	½ MS + IBA (0.1) + Charcoal (100 ppm)	Rooting	
	MS + BAP (1.0)	Multiple shooting	Thiyagarajan and Venkatachalam, 2012
	½ MS + NAA (0.4)	Rooting	
	MS + TDZ (1 µM)	Multiple shooting	Lata et al., 2013
	½ MS	Rooting	
	½ MS + TDZ (0.01)	Multiple shooting	Singh and Dwivedi, 2013
	¼ MS + IBA (1.0)	Rooting	
	MS + BAP (1.0) + NAA (0.05)	Multiple shooting	Soliman et al., 2013
	MS + IAA (0.5)	Rooting	
	MS + BAP (1.0)	Multiple shooting	Nower, 2014
	MS + IAA (0.2)	Rooting	
	MS + Kin (0.5) + IBA (1.0)	Multiple shooting	Singh et al., 2014
	MS + NAA (1.0) + BAP (1.0)	Callus formation	Karim et al., 2008
	MS + IBA (0.1)	Rooting	

	½ MS + 2,4-D (0.5)	Callus formation	Singh et al., 2014
Leaf	MS + NAA (2) + NaCl (0.10 %) + Na_2CO_3 (0.025 %)	Callus formation	Gupta et al., 2014
	MS + BAP (0.2) + 2,4-D (2.0)	Callus formation	Babu et al., 2011
	MS + NAA (2.0) + Kin (0.2)	Callus formation	Preethi et al., 2011
	MS + BAP (2.0)	Shoot Regeneration	
	MS + IBA (0.5)	Rooting	
	MS + 2, 4-D (1.0) + Kin (1.0)	Callus formation	Singh et al., 2011
	MS + BAP (0.5) + NAA (0.1)	Shoot Regeneration	
	MS + BAP (0.5) + NAA (0.3)	Rooting	
	MS + 2,4-D (11.31 µM) + BAP (2.22 µM)	Callus formation	Janarthanam et al., 2009
	MS + BA (4.44 µM) + NAA (1.34 µM)	Shoot Regeneration	
	MS + IBA (2.46 µM)	Rooting	
	MS + 2,4-D (1.5) + BAP (0.5)	Callus formation	Guruchandran and Sasikumar, 2013
	MS + Kin (0.5) + BAP (1.5)	Shoot Regeneration	
	½ MS + NAA (1)	Rooting	
	MS + BAP (1) + NAA (2)	Callus formation	Khan et al., 2013
Shoot tip	MS + Kin (10)	Multiple shoot	Ibrahim et al., 2008b
	MS + IBA (0.01)	Rooting	
	MS + BAP (1.5) + Spermine (10)	Multiple shoot	Guruchandran and Sasikumar, 2013
	MS + IAA (1.5)	Rooting	
	MS + BAP (1.0)	Multiple shoot	Javad et al., 2013
	MS + IBA (0.4)	Rooting	
	MS + IAA (1.0) + BA (1.0)	Multiple Shooting	Taleie et al., 2013
Leaf, shoot tip, nodal sections	MS + IAA (5.71 µM) + BA (8.87 µM)	Multiple shooting	Sivaram and Mukundan, 2003
	MS + IBA (4.90 µM)	Rooting	
Nodal section with axillary bud	MS + BAP (2.0) + IAA (1.13)	Multiple shooting	Debnath, 2008
	MS + IBA (2.0)	Rooting	
Nodal sections and leaf	MS + BAP (2.2 µM) + NAA (2.8 µM) + $CuSO_4.5H_2O$ (5 µM)	Multiple shooting	Jain et al., 2009
Leaf, shoot tip,	MS + BA (1.0) + IAA (0.5)	Multiple shooting	Anbazhagan et al., 2010

nodal sections	½ Nitsch (N6) + IAA (1.0)	Rooting	
Root, leaf, node	MS + NAA (0.75) + 2,4-D (1.0)	Callus formation	Gupta et al., 2010
Nodal and leaf segment	MS + BAP (0.2) + 2,4-D (2) + TDZ (0.2)	Callus formation	Banerjee and Sarkar, 2009
	MS + BAP (0.2) + 2,4-D (1) + TDZ (0.2)	Somatic embryogenesis	
Different explants	MS + BAP or Kin (5.0)	Multiple shooting	Tiwari et al., 2013
	MS + IBA (2.0)	Rooting	
Inter-nodal sections, nodal sections, leaf	MS + 2,4-D (3.0)	Callus formation	Uddin et al., 2006
Nodal segment, shoot tip	MS + BAP (2.0)	Multiple shooting	Hassanen and Khalil, 2013
	MS + IBA (0.5)	Rooting	
Nodal segment, shoot tip	MS + BA (1.0) + Kin (2.0)	Multiple shooting	El-Motaleb et al., 2013
	MS + IBA (0.5)	Rooting	
Microshoots	MS + chlorocholine chloride (3.0) + IBA (3.0)	Multiple shooting	Dey et al., 2013
Nodal segments, shoot tip, axillary bud	MS + Kin (2.0)	Multiple shooting	Das et al., 2011
	MS	Rooting	

2.4.5.3 Planting time

Plantation time has a crucial role in crop yield improvement or total biomass production. It has been reported in stevia that planting time significantly affects the crop biomass (fresh and dry). In a report, stevia seedlings were transplanted on 15th and 30th March and 15th of April in rows. It was found that maximum plant yield was obtained from the plants transplanted on the 15th of March (Taleie et al., 2012a). High temperature, high light intensity, and long days conditions significantly increase the vegetative growth and ultimately the plant yield (Maheshwar, 2005). Stevia is a short-day plant with an average duration of the day is of about 12–13 h and in the Northern Hemisphere of Canada it plantation time is in the month of May (Mizukami et al., 1983; Brandle et al., 1998). There are various reports regarding the most optimal temperature for stevia crops. According to Chalapathi et al., 1997, in subtropical parts of India, stevia is cultivated at a temperature range of 28-39ºC. While, Brandle and Rosa, 1992 stated that the ideal temperature for stevia plants is ranging from -6 to 43ºC.

2.4.5.4 Irrigation

Stevia can be cultivated successfully with suitable irrigation facilities. A field study was carried out to calculate the water requirement by stevia crop grown in clayey or medium black soil. Maximum leaf yield was recorded with irrigation at 1.2 IW/CPE (IW = irrigation water; CPE = cumulative pan evaporation) (10.54 t/ha) (Aladakatti et al., 2012). Various studies have been concluded the water requirement for stevia crops are 100% pan evaporation for 2.74 t/ha (Behera et al., 2013), 5.44 mm per day evapotranspiration for 0.0048 t/ha (Fronza and Folegatti, 2003), 117% evapotranspiration for 4.4 t/ha (Fronza and Folegatti, 2002) and 100% evapotranspiration for 4.6 t/ha (Lavini et al., 2008). A field experiment was conducted by Aladakatti et al., 2012 in clayey medium black soil to find the most effective irrigation schedule. Maximum leaf yield was found in the plots irrigated with irrigation scheduled at 1.2 IW/CPE.

2.4.5.5 Nutrient requirement and harvesting time

For commercial cropping, manuring is necessary for good yield in stevia and it is mandatory to fertilize the crop with basic nutrient amendments (Table 2.10). In the first year of stevia plantation, a high amount of nitrogen and potassium is required in comparison to phosphorus (Luciana and Silvia, 2014). A field experiment was conducted in China to compare the consequences of organic and chemical fertilizers on stevia crops. It has been reported that organic manure significantly enhanced the root activity, rate of photosynthesis; crop biomass as well as steviol glycosides content (Liu et al., 2011b). Yang and co-workers found that decomposed remains of stevia crops fulfilled the nutrient requirements and significantly improved the leaf biomass (Yang et al., 2003). Kumar et al., 2012 reported that the highest dry leaf yield was found in the plots treated with farmyard manure, and the highest steviol glycosides were found in the plots treated with 50:60:50 kg NPK/ha. Augmentation of 60 kg N/ha and 45 t/ha farmyard manure successfully amplified the dry leaf yield, leaf area index, number of leaves per plant, and dry matter accumulation per plant (Rashid et al., 2013). A pot study was carried out by Das et al., 2007 to find the efficacy of bio-fertilizers on stevia biomass and NPK content. It has been reported in this study that crop yield and NPK content were increased initially, and then decreased with the plant growth. A study was conducted by Wu et al., 2013 to compare the effects of purple phototrophic bacteria on yield and stevioside content in stevia crops. For this, PPB was applied to the stevia field for 10 days in form of a foliar spray, spray + irrigation, and rhizosphere irrigation. Among all the methods, foliar spray significantly increased plant growth and spray + irrigation increased the stevioside content in leaves by 69.2 % as compared to control plants.

The harvesting time of the stevia crop varies according to climate as it grows as a perennial crop in tropical climates and as an annual crop in temperate to sub temperate climates (Donalisio et al., 1982; Shuping and Shizhen, 1995). Several studies are available in the literature regarding nutrient requirements and harvesting time of stevia crops. The optimum time of harvesting with maximum leaf yield and glycosides content has been reported in the month of September (Megeji et al., 2005; Luciana et al., 2014). According to Kumar and Sharma, 2012, the optimum harvesting time is 60 days after the

transplantation and 50 % flowering stage. In a report by Li et al., 2012 it has been found that stevia harvesting at 95 days after transplantation was most optimum and contain the highest stevioside content. In a pot trial by Vafadar et al., 2014, stevia plants were augmented with PGPRs (plant growth-promoting rhizobacteria) and AMF (arbuscular mycorrhizal fungus). It has been found that PGPRs and AMF successfully increased the stevioside content, root and shoot biomass, chlorophyll, and NPK content as compared to control plants.

2.4.5.6 Weed management

Stevia is a poor competitor of weed plants during the initial growth period which reduces its yield and also makes harvesting more difficult (Ramesh et al., 2006). The most common weed species in stevia cultivation are Cotula australis, Solanum nigrum, Coronopus didymus, Panicum dichotomiflorum, Trifolium repens, Echinochloa crus-galli, and Setaria verticillate. A study was performed to check the safety of 25 different herbicides (used previously for stevia crop) to stevia plants. Among post-emergence herbicides clethodim, pyridate, haloxyfop, flumetsulam, propyzamide and propyzamide, and among pre-emergence trifluralin, alachlor, pendimethalin, methabenzthiazuron, oryzalin, linuron, bromacil, and terbacil were tolerated by stevia plants (Harrington et al., 2011). Manual (hand weeding) weed control increases the stevia yield up to 30 folds, but the best results were obtained with a spray of pre-emergence herbicide bromacil with 19 folds increase in yield (Santo, 2003).

2.4.5.6.1 Mulching

Other than herbicide spray, mulching is an important agronomic practice that has been used to suppress weed growth as well as to conserve soil moisture and fertility (Yang et al., 2013) (Fig. 2.10). Crop residues (also known as harvested crop remaining) such as maize stalks, straw, and leaf stubble can also be used as mulch material (Bot and Benites, 2005). Other materials which can be used for mulching are newspapers, compost, sawdust, bark clippings, etc (Table 2.11).

Table 2.10 Effect of nutrients and harvesting time on stevia leaf (dry) biomass

Dose	Nutrients	Dry leaf biomass	References
110:45:45 kg/ha	NPK	2.53 t/ha	Behera et al., 2013
60 kg/ha	Nitrogen	1.083 t/ha	Rashid et al., 2013
75:25 ratio	Vermicompost + bone meal	7.52 g/plant	Dushyant et al., 2014
45 t/ha	FYM	1.147 t/ha	Rashid et al., 2013
5 g/l	Mussorie rock phosphate + phosphate solubilizing bacteria	0.79 g/plant	Gupta et al., 2011
50:60:50 kg/ha	NPK	0.67 t/ha	Kumar and Sharma, 2012
Harvesting time			
Harvesting time		**Dry leaf biomass**	**References**
60 DAT and 50% flower bud stage		18.2 g/plant	Kumar and Sharma, 2012
September		3.4 t/ha	Hoyle, 1992
September		8.688 t/ha	Luciana and Silvia, 2014
After 180 DAT		7.659 t/ha	Moraes et al., 2013
September		1.276 t/ha	Megeji et al., 2005

Plastic mulching falls under synthetic mulching as it involves the use of colored plastic sheets as mulch. This type of mulching also helps in increasing the crop yield and retard weed growth. Although the color of plastic sheet vary but most commonly black colored sheet is used for mulching. In synthetic mulch, black sheet of plastic has been used for effective weed suppression in stevia (Basuki and Sumaryono, 1990). Leaf mulches were also used for weed control in stevia. Field studies were performed to evaluate the efficacy of three kinds of mulches i.e. poplar leaf (Populus deltoides), pine needles (Pinus roxburghii), and silver oak (Grevillea robusta) on yield and quality of the crop. It has been found in this study that mulched plots significantly increased the yield than without mulching and maximum rebaudioside A content was found in the plots treated with poplar leaf mulch (Kumar et al., 2014).

Table 2.11 Effect of plastic mulch and herbicide on weed count in stevia cultivation

Year	Work done	Reference
1990	Use of high plant density and black plastic mulch for effective weed control.	Basuki and Sumaryono, 1990
2008	Three postemergence and preemergence herbicides were tested for weed control. Preemergence treatment with pendimethalin and postemergence treatment with aclonifen was found optimal. Symptoms of phytotoxicity were also reported.	Covarelli et al., 2008
2011	Safety evaluation of 25 herbicides (pre and post-emergence). Bromacil and terbacil: effective for pre-emergence weed control and haloxyfop, clethodim, propyzamide: effective for post-emergence treatment. Crop damage to some extent.	Harrington et al., 2011
2013	Four pre-transplant and five post-transplant herbicides were tested. Oxadiazon and pendimethalin provided good results in pretransplant treatment while linuron and oxyfluorfen caused phytotoxicity to stevia. In case of post-transplanting treatment, pendimethalin and fenoxaprop-p-ethyl showed good efficacy. Phytotoxic effects of all the herbicides were reported on stevia plants at initial stage of the treatment.	Angelini et al., 2013
2015	Napronamide, pendimethalin, dimethanamid herbicides were reported to be effective for weed control. Clopirali, prometryn, floumeturon herbicides proved highly toxic for stevia plants.	Zachokostas, 2015
2015	A research was carried out in Australia to assess the effectiveness of 16 weedicides. Pre-emergence treatment with Prolan 500 (6.8 Lha/h) was found effective.	Hopkins and Midmore, 2015

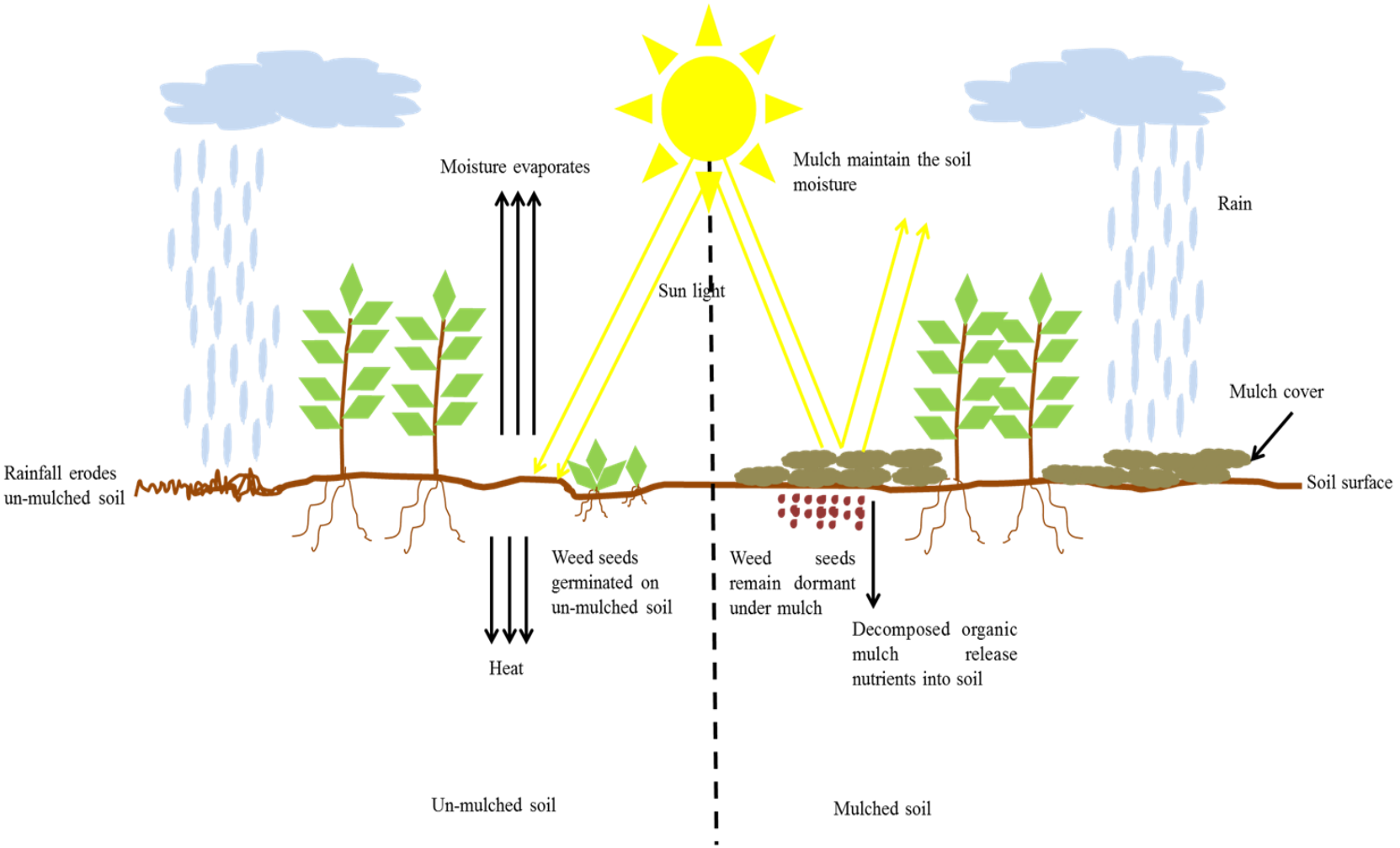


Fig. 2.10 An outline of benefits of mulching

Table 2.12 Applications of different mulches in various crops for weed control and yield improvement

Crop	Mulch (organic/inorganic)	Result	Reference (s)
Lycopersicon esculentum Mill. (tomato)	*Vicia villosa* Roth (hairy vetch) residue and black polyethylene mulch	In this report black polythene mulch respond better in terms of yield and shoot growth as compared to hairy vetch mulch.	Teasdale and Abdul-Baki, 1995
Triticum aestivum (spring wheat)	Plastic film	This study concluded that mulching with plastic film significantly increased the number of tillers, spikelet, number of grain per spike, photosynthesis rate, soluble sugar content and grain yield (8207 kg/ha/year).	Li et al., 1999
Cucumis sativus L (cucumber)	Black polyethylene mulch, grass straw and clear polyethylene mulch	Among all the mulches, clear polythene mulch significantly increased the crop yield by 33% as compared to unmulched plots.	Korir et al., 2006
Allium cepa (onion)	White, black, pink, blue and white polythene mulch	The results of this study concluded that blue colored polythene was most effective to reduce weed population and biomass. The highest yield was reported in plots covered with off-white colored polythene mulch. The maximum mean net production value was reported in blue-colored polythene mulch.	Pramanick et al., 2006
Solanum tuberosum L. (Potato)	Straw mulch	Application of straw mulch to the plots significantly enhanced the available potassium and phosphorus in soil and also tuber yield.	Kar and Kumar, 2007
Triticum aestivum L. (wheat)	Rice husk, transparent and black polyethylene	It has been reported in this study that rice husk mulching respond better in term of water use efficiency and yield attributes.	Chakraborty et al., 2008

Abelmoschus esculentus (okra)	Pea straw	Plots mulched with pea straw were reported with maximum plant height, number of pods and nodes plant^{-1}, fresh pod yield.	Bahadur et al., 2009
Lycopersicon esculentum L. (tomato)	Black and white polyethylene mulch, rice straw mulch	In this experiment, 23-57% increase in crop production was reported in the plots covered with mulch materials than unmulched plots.	Mukherjee et al., 2010
Zea mays (maize)	Black and white plastic mulch, weeds mulch and mungbean (living mulch)	This study concluded that maximum yield (2863 kg/ha) was found in hand weeding followed by living mulch (2145 kg/ha), weeds mulch (2460 kg/ha), black plastic mulch (2813 kg/ha), white plastic (2398 kg/ha).	Gul et al., 2011
Fragaria × *ananassa Duch*. (strawberry)	Black polyethylene mulch and hay mulch	Application of all the mulches significantly increased the crop yield (343%), nutrient uptake (179.20%), water use efficiency (84.40%) and root growth (63%).	Kumar and Dey, 2011
Capsicum annuum L. (Chillies)	Wheat straws and newspaper mulch	It has been found that hand-weeding resulted in a maximum number of fruits/plant (58.1), crop yield (8775 kg/ha) and fruit length (6.8 cm) as compared to all the treatments.	Khan et al., 2012
Dry and wet direct seeded rice	Rice straw mulch	In wet direct-seeded rice, straw mulch significantly increased total root length, plant height, shoot and root biomass.	Devasinghe et al., 2013
Trichosanthes dioica Roxb (pointed gourd)	Paddy straw, water hyacinth, typha and mustand leaves	It has been reported that maximum yield was reported in plots with paddy straw mulch than any other mulch material.	Ram et al., 2013
Pisum sativum L. (pea)	Sugarcane leaves, sawdust, persimmon leaves and wheat straw mulch	In this study saw-dust was found most effective to maximize crop yield (10638.67 kg/ha) in terms of a number of pods per plant.	Sajid et al., 2013
Abelmoschus esculentus (okra), *Cucurbita pepo* L.	Black polyethylene plastic mulch	In this study, late middle and early yield of both crops increased significantly in plots covered with black polythene mulch.	Mahadeen, 2014

(summer squash)			
Cucumis melo L. (melon)	Plastic mulch	Application of plastic mulch significantly increased the plant height, number of fruits, crop water productivity and total crop yield than unmulched plots.	Alenazi et al., 2015
Zea mays (maize)	Chaffed herbage of four crops i.e. sorghum, maize sunflower and rice	In this study, it has been found that chaffed herbage mulch effectively suppress weed growth and enhanced the growth, quality and yield of maize crop.	Mahmood et al., 2015
Arachis hypogaea L (groundnut)	Rice straw mulch	This study reported that rice straw mulch (0.1 m depth) with 1 manual weeding after 6 weeks of sowing responds better in terms of yield attributes.	Olayinka and Etejere, 2015
Solanum lycopersicum L (tomato)	Paddy straw, black polyethylene and clear polyethylene mulch	Among all the mulches, black polythene mulch significantly increased the marketable and total yield in the third week of October under polyhouse conditions.	Dhaliwal et al., 2016
Zingiber officinale Roscoe (ginger)	*Lantana camara* leaves, paddy straw, cowpea plants, *Glycosmis pentaphylla* leaves, black and white plastic mulch, dried coconut leaves and coir pith compost	Maximum weed control efficiency (72%) and maximum plant height were reported in the plots covered with old paddy straw mulch.	Thankamani et al., 2016
Solanum melongena L. c.v Black Beauty (eggplant)	Black plastic mulch	The highest plant growth and crop production were reported in plots covered with black plastic mulch than non-mulched plots.	Abdrabbo et al., 2017
Glycine max L. Merr. (soybean)	Wheat residue mulch	In this study, significant grain and protein yield was reported in the plots treated with wheat residue mulching (5 mg/ha) along with a spray of KNO_3 and $MgCO_3$.	Dass and Bhattacharyya, 2017

Abelmoschus esculentus (okra)	Plastic mulch	Alternate furrow irrigation and every furrow irrigation under plastic mulch ridges increased the crop yield and crop water productivity.	Memon et al., 2017
Stevia rebaudiana Bertoni (candy leaf)	Pine needles	In this study, the highest leaf yield was reported in the plots covered with mulching of pine needles at 15 t/h.	Pal and Mahajan, 2017
Capsicum annuum L. (capsicum)	Black polyethylene mulch	In this study, polythene mulch successfully increased the marketable yield of four varieties by 2.8 and 2.9 folds in 2014 and 2015 respectively.	Angmo et al., 2018
Arachis hypogaea L (groundnut)	Polythene mulch	Polythene mulch successfully increased the kernel yield as compared to unmulched plots.	Mondal et al., 2018
Allium cepa L. (onion)	Water hyacinth, white polythene sheet, saw dust black polythene sheet and rice straw	Among all the mulches, black polythene mulch was found to be most effective for crop yield enhancement as compared to other mulches.	Rachel et al., 2018
Fragaria × annanasa Strawberry	Rice straws and black polythene mulch	It has been found that in Rangpur location black polythene mulch and in Gazipur location rice straw mulch performed better in terms of yield attributes.	Rannu et al., 2018
Arachis hypogaea L. (ground nut)	Wheat straw mulch	Results of this study revealed that wheat straw mulch significantly increased the plant height and crop yield as compared to unmulched plots.	Weldu and Dejene, 2019
Lycopersicum esculentum (L.) H. Karst (tomato)	Sugar-cane peels and rice straw	Both the mulch materials enhanced the fruit appearance, shelf life and fruit yield as compared to unmulched plots.	Ainika et al. 2019
Glycine max (soybean)	Straw mulch + nitrogen fertilizer	It has been reported in this study that application of 6000 kg wheat straw/ha with 27 kg N/h successful increased yield and biomass of soybean by 75% and 67% respectively.	Akhtar et al., 2019

Lycoperscon escullentum Mill. (tomato)	Black oxo degradable mulch, black polyethylene plastic mulch, black biodegradable mulch	This study concludes that biodegradable mulch successfully enhances the production of tomato crop.	Alamro et al., 2019
Solanum tuberosum L. (potato)	Black polyethylene film and maize straw strips	According to this study, maize straw strips can be considered as cost-effective and environmental friendly alternative to plastic mulch for potato production.	Chen et al., 2019
Zea mays (maize)	Straw mulch and plastic film	Straw mulch was found to be most effective for improving the active carbon and organic matter of the soil. While plastic film improved the crop production and water use efficacy of crop plants.	Javed et al., 2019
Cucumis sativus L (cucumber)	*Trifolium pratense* L. (red clover) as living mulch	No direct yield benefits were reported in this study but it has been found that red clover living mulch can reduce herbivorous pests.	Kahl et al., 2019
Oryza sativa (rice)	Rice, maize, mucuna and cymbopogon mulch	Maximum crop yield was observed with mucuna and cymbopogon mulch and rice mulch was found highly significant for weed suppression.	Kaiira et al., 2019
Solanum tuberosum L. (potato)	Black polythene mulch and rice straw mulch	Organic mulching was found most effective for tuber yield improvement.	Sekhon et al., 2019
Cucumis sativus L (cucumber)	Black polyethylene, Clear polyethylene, sugar cane straw mulch	It has been reported that black polyethylene mulch + 1000 mg/l seaweed extract + sugar cane straw mulch + 2 drip irrigation was most significant in yield improvement during both seasons.	Shehata et al., 2019
Lycopersicon esculentum L. (tomato)	Dry neem leaves, pea straw and paddy straw	It has been found that all the organic mulches increased plant growth and fruit yield, control weed growth and maintain soil moisture.	Singh et al., 2019
Vicia unijuga A. Br (perennial vetch)	Straw mulch and plastic-film	Maximum seed yield was reported in plots treated with plastic film.	Tang et al., 2019

Tagetes erecta L (marigold)	Silver-black plastic mulch, black plastic mulch and crop residue mulch	Application of silver black mulch with a dose of gibberellic acid gave the maximum number of flowers, plant height and flower size.	Thakur et al., 2019
Sesamum indicum L. (sesame)	Wheat straw	Application of 7.5 t/ha of straw mulch can be considered as a promising strategy for yield enhancement in water stressed areas.	Behzadnejad et al., 2020
Allium sativum L. (garlic)	Plastic mulch	Application of plastic mulch and organic fertilizer successfully increased the no. of leaves, stem and bulbil diameter, plant height.	Dinda et al., 2020
Zea mays L (summer maize)	Biodegradable transparent and black film mulch and transparent film mulch	Biodegradable black film mulching was found to be most effective to increase the photosynthetic activity and crop yield in the North Plain of China.	Li et al., 2020
Piper nigrum L. (shrub pepper)	Cover crop of *Arachis pintoi*, *Centrosema pubescens*, *Calopogonium mucunoides* and *Pueraria japonica*	Cover crop of *Arachis pintoi* was reported with a maximum number of leaves, the diameter of canopy and fruit panicle.	Hariyadi, 2020

In a study by Coelho et al., 2018, vegetable-compost mulch was used for weed control in stevia cultivation. It was reported that compost treatment significantly reduces weed count and also improves soil properties. Organic mulch treatments have also been used in various other commercial crops such as neem leaves for ginger (*Zingiber officinale* var. Suprabha) crop (Das, 1999), farmyard manure and straw mulch for turmeric (Curcuma longa L.) crop (Gill et al., 1999), paddy straw for aonla (Emblica officinalis) crop (Shukla et al., 2000), straw mulch for potato crop (Kar and Kumar, 2007). In a recent study by Takács-Hájos et al., 2019, the use of two eco-friendly fertilizers Dudarit and Sprintalga in stevia cultivation significantly increased (compared to control plots) the stevia leaf-biomass and did not affect the stevioside and rebaudioside content. Abouziena et al., 2008, conducted a two-year study was for comparative assessment of organic mulch (rice straw), synthetic mulch (plastic sheet), and herbicide (glyphosate) spray-on weed control Mandarin fruit cultivation. All the treatments significantly retarded the weed count as compared to the control plants but, the fruit yield was reported to increase only with rice straw mulch. There are several other reports which recommend the use of mulches for weed-free farming. A field trial was conducted by Ramakrishna and co-workers in 2006 (Northern Vietnam) to study the effect of different mulches (rice straw, polythene) on soil moisture and temperature, weed growth, and groundnut yield. Among them, polythene (synthetic) and straw (organic) mulch was found most effective for weed suppression and promotion of crop yield (Ramakrishna et al., 2006). Field-based experiments were also conducted to find the efficacy of herbicide sprays, mulching with wheat residues, and intercropping with sesbania for weed suppression in dry seeded rice crops. Interestingly, mulching with wheat residues (@4t/h) and 30 days intercropping with sesbania was found most effective for weed suppression (Singh et al., 2007). Similar to stevia, ginger (*Zingiber officinale*) is a slow-growing plant and is susceptible to weeds during the early stages of growth. An experiment was conducted to assess the effect of different types of organic mulches on ginger crop yield and weeds. Maximum weed suppression (72%) and plant height were reordered with the treatment of one-year-old paddy straw with green leaf mulch and Lantana camara leaves. Maximum crop yield was recorded in plots treated with white plastic mulch as compared to other organic mulch treatments (Thankamani et al., 2016). In a similar report, Genger et al., 2018, confirmed that compared to

mechanical weed control methods, the application of straw mulch can effectively control the broad-leaf weeds and increase the yield in a late-season cultivar of potato.

Cover crops are also used in various previous studies to suppress weed growth in commercial crops (Weston, 1996; Lowry and Smith, 2018). These crops cover the empty soil and thus suppress the seed germination of weed plants (Teasdale et al., 2007; Hodgdon et al., 2016; Lowry and Smith, 2018). The growth of cover crops plant has been found to be directly proportional to its weed suppression potential (Liebman and Davis, 2000; Teasdale et al., 2007). Most commonly the crops having high germination rate, rapid growth, and more biomass are selected as cover crops (Hodgdon et al., 2016; Lowry and Smith, 2018).

There are various reports available regarding weed suppression potential of cover crops such as *Medicago sativa* L. suppress the growth of *Stellaria media*, *Poa annua, Chenopodium album, Vicia sativa, Echinochloa crus-galli* and *Polygonum* spp (Kruidhof et al., 2008), *Digitaria* spp, *Amaranthus retroflexus, Portulaca oleracea, S. media* (Hodgdon et al., 2016). *Hordeum vulgare* L. suppress the growth of *Lolium multiflorum, C. album, A. retroflexus* (Singh et al., 2003). *Lathyrus sativus*, *Fagopyrum sagittatum* and *Trifolium incarnatum* suppress the growth of *C. album*, *Abutilon theophrasti*, *Setaria viridis, A. retroflexus* (Wortman et al., 2013; Hodgdon et al., 2016). *Eleucine corocana* suppress the growth of *Isachne globose*, *E. crus-galli*, *Eclipta prostrate* (Samarajeewa et al., 2006). *Raphanus sativus* suppress the growth of *P. oleracea, C. album*, *Digitaria* spp., *S. media, E. crus-galli, V. sativa, A. retroflexus* (Kruidhof et al., 2008; Hodgdon et al., 2016). *Vicia villosa* suppress the growth of *Setaria viridi, A. theophrasti, Panicum capillare, A. retroflexus, E. crus-galli, C. album* (Mohler and Asdale, 1993). *Vicia villosa* suppress the growth of *Lolium temulentum* (Campiglia et al., 2009). *Vicia villosa* suppress the growth of *S. media, A. retroflexus, P. oleracea, Digitaria* spp (Hodgdon et al., 2016). *Avena sativa* L suppress the growth of *L. multiflorum, C. album, A. retroflexus* (Singh et al., 2003). *Pisum sativum* suppress the growth of *S. viridis, A. theophrasti, C. album, A. retroflexus* (Wortman et al., 2013). *Brassica napus* suppress the growth of *L. temulentum, S. media* (Campiglia et al., 2009), *V. sativa, C. album, S. media, E. crus-galli* (Kruidhof et al. 2008), *S. viridis, A. theophrasti, C. album, A. retroflexus* (Wortman et al.,

2013). *Secale cereale* suppress the growth of *P. oleracea*, *Amaranthus* spp (Nagabhushana et al., 2001), *L. multiflorum, C. album, A. retroflexus* (Singh et al., 2003), *V. sativa, C. album, S. media, E. crus-galli* (Kruidhof et al., 2008), *S. media, A. retroflexus, P. oleracea, Digitaria* spp (Hodgdon et al., 2016). *Medicago scutellate* and *Trifolium subterraneum* suppress the growth of *S. media, L. temulentum* (Campiglia et al., 2009). *Trifolium repens* suppress the growth of *S. media, A. retroflexus, P. oleracea, Digitaria* spp (Hodgdon et al., 2016). *Lupinus albus* suppress the growth of *V. sativa, C. album, S. media, E. crus-galli* (Kruidhof et al., 2008). *Sinapis alba* suppress the growth of *S. viridis, A. theophrastis, C. album, A. retroflexus* (Wortman et al., 2013).

CHAPTER 3
HYPOTHESIS

With an increase in climatic deviations, crop plants also encounter increased biotic and abiotic stresses, which directly or indirectly hit the economic value of commercial crops. The agricultural production system faces heavy losses due to biotic (anthropogenic activities or pathogen/insect attack) and abiotic agencies of stress. Hence agricultural sustainability depends upon the implementation of such kinds of approaches which can reduce the opposing effects of these constraints. Weeds are considered as unwanted and most demanding guests who are detrimental to agricultural production. Weed problems directly hit the cost of crops by interfering or competing with crop plants for water, nutrition, resources, light, etc. which ultimately reduce crop yield. About 8000 plant species among the total plants are considered weeds in the World. In India, the weeds-mediated crop-yield reduction is about 36.5% during the summer and rainy season while 22.7% during the winter season, which accounts for an economic loss of 1050 billion annually INR (NRCWS, 2007). Losses caused by weeds may be from 5 to 10 % in the agriculture of developed countries, and 20 to 30 % in emerging or developing countries. Generally, it can be concluded that reduction in plant yield is done by a group of weed plants species rather than single weed species.

The present study aims to find the different weed plant species present in stevia cultivation and to find the most suitable strategy for weed control. Another aim is to produce herbicide-resistant transgenic stevia plants for resistance against glufosinate herbicide.

CHAPTER 4

AIM and OBJECTIVES

4.1 Background

Due to extensive harmful effects caused by weed plants on stevia crops, this problem needs an implementation of an effective strategy to cope with this. Several mulching and herbicides treatment were used earlier in this field of study. For the production of herbicide-resistant transgenic stevia *bar* gene was incorporated into the stevia genome using gene gun method. This study was designed in such a way so as to find the most effective weed control strategy for the stevia crop. A highly efficient method of gene transfer i.e *Agrobacterium*-mediated transformation was used in this study to incorporate *bar* gene into the stevia genome.

We have visited various stevia fields of Punjab at different locations. We have done an extensive literature review and found that mulching is one of the most commonly used conventional strategies for weed control. However, these conventional strategies were not providing sufficient weed control to the farmers as well as are laborious. Hence, to check their efficacy we have done the comparative evaluation of mulching and herbicide treatment. The present study aims to find the different weed plant species present in stevia cultivation and to find the most suitable strategy for successful weed control. Another aim is to produce herbicide-resistant transgenic stevia plants for resistance against glufosinate.

4.2 Objectives

The objectives of the present study are following:

(A) Identification and collection of weeds found in stevia fields from different locations in Punjab.

i) Field visit to stevia fields of different locations in Punjab

ii) Discuss with farmers about the weed problems and collection of weed samples

(B) Comparative evaluation of different weed management approaches including mulching and soil treatments.

i) Two year field trials to check the efficacy of three commercially available herbicides

ii) Synthetic and organic mulch treatments for weed control

(C) Optimization of tissue culture conditions of stevia using different explants (shoot tip, node and leaf).

i) Optimization of direct and indirect regeneration and callus induction

iii) Multiplication and rooting optimization

(D) *Agrobacterium*-mediated transformation and development of herbicide tolerant transgenic stevia lines.

i) Optimization of *Agrobacterium*-mediated genetic transformation of callus and nodal sections with *bar* gene

ii) Generation of putative transgenic lines

iii) Molecular characterization of transgenic lines

(E) Limited field trials using transgenic stevia for weed management.

i) Transgenic plants were subjected to herbicide tolerance assay

ii) Determination of residual phyto-toxicity effect of herbicide on soil

CHAPTER 5
MATERIALS AND METHODS

A detail account of the materials used and the methods followed in this study is presented below:

5.1 Materials

5.1.1 Stevia seedlings, mulches and herbicides

Two months old healthy saplings of stevia were procured from 'Green Valley Stevia Farm' located at Pojewal, Nawanshehar, Punjab, India. The plastic sheet, rice straw and eucalyptus leaves were procured from the local market and the herbicides were procured from Bayer Crop science Ltd Mumbai, India.

5.1.2 Chemicals, glass and plastic wares

Table 5.1 enlisted the chemicals used in this study. **Table 5.2** enlisted the glasswares and plastic wares used in this study.

Table 5.1 List of chemicals used in this study

Chemicals	Company
6-(γ, γ-dimethylallylamino) purine (2-iP)	Sigma
6-benzylaminopurine (BAP)	Sigma
Acetone	Qualigens
Acetosyringone	Sigma
Agar	Sigma
Agarose	Sigma
Ammonium nitrate	HiMedia
Amplification grade DNase	Sigma
Autoclavable bags (Hi Dispo bag)	HiMedia
Boric acid	Sigma
Calcium chloride	HiMedia
Cefotaxime	Sigma
Chloroform	Qualigens
Cobalt chloride	HiMedia
Copper sulphate	HiMedia
Dimethyl sulphoxide (DMSO)	Sigma
Disodium EDTA	HiMedia
Enhanced Avian HS-RT PCR kit	Sigma
Ethidium bromide (EtBr)	Sigma
Ethylenediamine-N,N,N',N'-tetraaceticacid	Sigma

Ferrous sulphate	HiMedia
Formaldehyde	Qualigens
Gibberellic acid (GA_3)	HiMedia
Glacial acetic acid	Qualigens
Glucose	Sigma
Glycerol	Sigma
Glycine	Sigma
HEPES sodium salt	Sigma
Indole-3-acetic acid (IAA)	Sigma
Indole-3-butyric acid (IBA)	Sigma
Inositol	Sigma
Isoamyl alcohol	Sigma
Isopropanol	Qualigens
Kanamycin	Sigma
Kinetin	Sigma
Luria agar	HiMedia
Luria broth	HiMedia
Magnesium sulphate	HiMedia
Maltose	HiMedia
Mannitol	HiMedia
Mercaptoethanol	Sigma
Mercuric chloride	HiMedia
MES hydrate	HiMedia
Phenol solution	Sigma
Polyethylene glycol	Sigma
Potassium chloride	HiMedia
Potassium dihydrogen phosphate	HiMedia
Potassium iodide	HiMedia
Potassium nitrate	HiMedia
Potassium permanganate	HiMedia
Pyridoxine	Sigma
Rifampicin	Sigma
RNase A	Sigma
Sodium bicarbonate	HiMedia
Sodium carbonate	HiMedia
Sodium hydroxide	HiMedia
Sodium molybdate	HiMedia
Sodium thiosulphate	HiMedia
Streptomycin	Sigma
Sucrose	HiMedia
Zeatin	Sigma
Zinc sulphate	Sigma

Table 5.2 List of glasswares and plasticwares used in this study

Material	Company
Glasswares	Borosil Glass Works Ltd. (Mumbai, India) Tarsons Products Pvt Ltd. (Kolkata, India) Glassco Laboratory Equipments Pvt. Ltd. (Haryana, India)
Falcon tubes, cell scrappers pipettes, PCR tubes, tips and micro centrifuge tubes	Tarsons Products Pvt. Ltd. (Kolkata, India) Agilent (Santa Clara, CA, USA) Genetix Biotech. Asia Pvt. Ltd. (New Delhi, India) Eppendorf (Hamburg, Germany)
Plates and cover for Real-time PCR	Applied Biosystems (California, USA)

5.1.3 Instruments

Table 5.3 List of instruments used in the study

Instrument	Company	Use
37ºC incubator	Bacteriological incubator, York Scientific Industries, India	For incubation of culture plates
Agarose gel electrophoresis unit	Hoefer, Holliston, MA	Gel electrophoresis of DNA and RNA
Autoclave	Astell, United Kingdom	For sterilization of nutrient media glass wares, decontamination of biological waste
Camera for photomicrographs	Leica, MPS32, Germany	For documentation of morphogenetic structures
Gel doc	Azure Biosystems, CA, USA	For imaging and analyzing 1-D electrophoresis gels, dot blots, arrays and colonies

High-speed centrifuges	5417R and 5810R	For pelletizing bacterial culture and protein samples, precipitation of DNA, RNA etc
Horizontal laminar hoods	ESCO Global (Singapore)	For providing contamination free work environment
Hot air oven	N-Biotech, Gyeonggi-do, Korea	For DNA-DNA and DNA-RNA hybridization
Infinite® 200 PRO NanoQuant	Tecan, Switzerland	Nucleic acid concentration and purity; Purified protein analysis (A280), Cell density measurements
Low temperature freezer (–80ºC)	Ultima II, Revco, USA	For cryopreservation of bacterial samples and plant samples, Xray cassettes
Magnetic stirrer	Thermo Fisher Scientific, MA, USA	For preparation of culture media, reagents etc
Mastercycler® PCR	Eppendorf, Hamburg, Germany	For DNA amplification
Microwave oven	Sizzlet Convection, LG electronics South Korea	For melting agarose
pH meter	Sartorius AG, Goettingen, Germany	For adjusting the pH of culture media components, reagents etc
Plant growth chambers	Conviron, Manitoba, Canada	Hardening of young plantlets
Real-time PCR System	Applied Biosystems, CA, USA	Real time applications
Stereomicroscope	Leica M3Z, Germany	For observation of regenerating explants
Water bath	Jeiotech, Seoul, Korea	For heat shock treatment at 42 ºC during bacterial transformation, for denaturing radiolabelled probes
Water purification system	Merck Millipore, Darmstadt, Germany	Deionized water used for molecular biology
Weighing machine	Denver Instruments, Bohemia, NY	For weighing

5.2 Methods

5.2.1 Field experiment

5.2.1.1 Collection and weed identification

Weeds from stevia fields were collected and identified with the help of taxonomist and Handbook on weed identification (Naidu, 2012). Following are the locations:

a) Green valley Stevia farm, Pojewal, Nawansheher, Punjab, India (Fig. 5.1) (Farmer – Mr. Gandhi)

b) Stevia farms, Jainpur, Ludhiana, Punjab (Farmer – Mr. Ravi Sharma)

c) Stevia farms, Sayan Khurd and Kuhli Khurd Ludhiana, Punjab (Farmer- Mr. Manjinder Singh)

d) Stevia farms Pathankot, Punjab (Farmer- Mr. Bhagwan Das Sharma) (Fig. 5.2)

e) Stevia farms, Rupnagar, Punjab (Farmer- Mr. Manu Grag)

f) Stevia farms, Pathankot, Punjab (Farmer- Mr. Lakhbir Singh) (Fig. 5.3)

5.2.1.2 Site of field experiment

The field experiments were conducted for two subsequent years (2017 and 2018) on two different locations in the agricultural field of Lovely Professional University, Phagwara (300 m above sea level) Punjab (latitude 29.30°-32.32° North and longitude 73.55°-76.50° East), India (Fig. 5.6). The field soil was tested in the Soil Testing Laboratory of Punjab Agriculture University (PAU), Ludhiana, Punjab, India. In the first week of February, prior to planting, decomposed farmyard manure was added to the field and the soil was ploughed, harrowed uniformly and fertilized with NPK fertilizer @ 100:50:50 kg/h. Field irrigation was done after every three weeks except during the rains.

5.2.1.3 Field design and treatment

Plantation was done in the last week of February. The experiment was designed in a RBD (randomized block design) with seven different treatments in triplicate. The treatments included untreated control, plastic mulch [PM: black colored plastic sheet], organic

Fig. 5.1 Weed plant sample collection from Green Valley Stevia Farm, Pojewal, Nawanshehar, Punjab, India.

Fig. 5.2 Weed plant sample collection from Pathankot (site 1) Punjab India.

Fig. 5.3 Weed plant sample collection from Pathankot (site 2) Punjab India.

mulch-1[OM1: rice straws (15t/h)], organic mulch-2 [OM2: eucalyptus leaves (15t/h)], herbicide-1[H1: Prolan (6.8 l/h)], herbicide-2 [H2: Pendimethalin (2.2 l/h)] and herbicide-3[H3:Glufosinate ammonium/Basta (5 l/h)]. In the experimental layout, the area of each plot was 1.89 m^2, and the area of the total experimental plot was 85.49 m^2. The distance between each plot and the outer boundary was kept 50 cm, while plant to plant distance was kept at 30 cm. Twelve stevia plants were planted in each plot (Fig. 5.5). The plastic sheet, rice straw, and eucalyptus leaves were procured from the local market while the herbicides were procured from Bayer Crop science Ltd Mumbai, India. The rice straw and eucalyptus leaves were spread uniformly on the soil-bed after 1 week of plantation while the foliar spray of herbicides was done in the month of July and September, with the aid of an agricultural sprayer.

5.2.1.4 Growth characteristics and yield analysis

Plant growth parameters like plant height, number of branches, leaves per plant, leaf area, chlorophyll content, and dry biomass (after harvest) were recorded by randomly selecting six plants per plot. Plant height was determined by using a meter ruler. For chlorophyll estimation, 100 mg leaf tissue was ground in 80% acetone, followed by centrifugation at 10,000 rpm, at 4°C, for 10 min. The supernatant was diluted with 80% acetone and the absorbance was read at 663 nm for chlorophyll a, and 645 nm for chlorophyll b using a spectrophotometer (UV-2700 UV-VIS Spectrophotometer, Shimadzu, Japan) (Arnon, 1949). For determining the leaf area, stevia leaf lamina was traced on the Whatman 1 MM paper, cut, and weighed. Leaf area was calculated by dividing the weight of paper leaf by the weight of one cm^2 Whatman paper. This exercise was done with randomly selected large and small leaves from a plant and the individual leaf area was multiplied to the total leaf count of that particular plant to get the total leaf area of a plant. Leaf area index (LAI) was calculated by dividing leaf area by ground area per plot. For determining the dry biomass, plants were uprooted and dried (stem and root at 60°C and leaves at 40°C) in a hot air oven (N-Biotek, South Korea) for two days and then weighed using a weighing balance (Mettler Toledo, USA) (Kumar et al., 2014).

5.2.1.5 Weed count

For estimation of weed density quadrat method was used wherein the Quadrat frame of $1m^2$ (Fig. 5.7) was placed randomly three times in each plot before harvesting, during both the years. To estimate the dry weed weight, weeds were washed, dried in hot air oven at 70°C and weighed (Kumar et al., 2014).

5.2.1.6 Statistical analysis

Each experiment was conducted in triplicates and the analysis of variance (ANOVA) of the data was carried out using IBM SPSS (Statistics 22 software, USA). Different parameters such as plant height, number of branches per plant, number of leaves per plant, chlorophyll (mg/g), leaf area index, weed count ($n°/m^2$), dry weed weight (g/m^2), root dry biomass (g/plant), and stem dry biomass (g/plant) in the year 2017 and 2018 were considered as the dependent variable while all the treatments (PM, OM1, OM2, H1, H2, H3) were considered as experimental factors which effect these variables.

5.2.2 Plant Tissue Culture Techniques

5.2.2.1 Callus development, direct regeneration, shoot and root induction

The composition of the MS medium is shown in Table 5.4. Rooted plantlets were washed with autoclaved Milli-Q® water to remove phytagel media from roots. The plantlets were planted in small plastic pots containing sterile soilrite (soil-conditioning mixture) and regularly irrigated with Hoagland solution. The pots were shielded with transparent perforated polythene sheets to maintain moisture (80-90%) and kept in a plant growth chamber (Conviron, USA) set at 22±2°C and 16 light /8h dark photo-period with PFD of 80 µmol/m^2/s. After 22−25 days of acclimatization, polythene bags were removed from the plants.

5.2.2.2 ISSR-PCR analysis

DNA amplification was performed in a Prima-96™ Thermal Cycler 96 wells block. Out of twenty different primers initially tested (Eurofins Genomics), only four primers no. 4, 6, 8, and 9 showed clear PCR products were used for ISSR analysis (Table 5.6). PCR

reaction for amplification of ISSR was performed in a total volume of 20 µl containing 10 µl of IX Emerald master mix (Takara Bio Inc.), 2 µl of primer, I µl of DNA template (200ng) and 7 µl of MQ. DNA was initially denatured for 3 min at 94°C, at 94°C for 1 min (35 cycles). The annealing temperature was 50°C for 1 min and the final extension was performed at 72°C for 7 min. Amplified PCR products were then resolved on 1.5% (w/v) agarose gel by gel electrophoresis, under-voltage of 80 V for 90 min. 100 bp DNA ladder (Generuler, Sigma) was used and bands were observed under UV trans-illuminator at 300 nm and images were captured using gel documentation equipment system (Bio-Rad).

5.2.2.3 *Agrobacterium tumefaciens* culture preparation for plant transformation

The plant expression vector pPZP200 35sde-*bar*-loxp having *bar* gene (herbicide tolerant) driven by DECaMv35s and loxp, promoter and terminator respectively were used in the study (Fig. 5.4). Phosphinothricin acetyl transferase enzyme encoded by the *bar* gene inactivates the activity of phosphinothricin. This construct was transformed into *Agrobacterium tumefaciens* (GV1301).

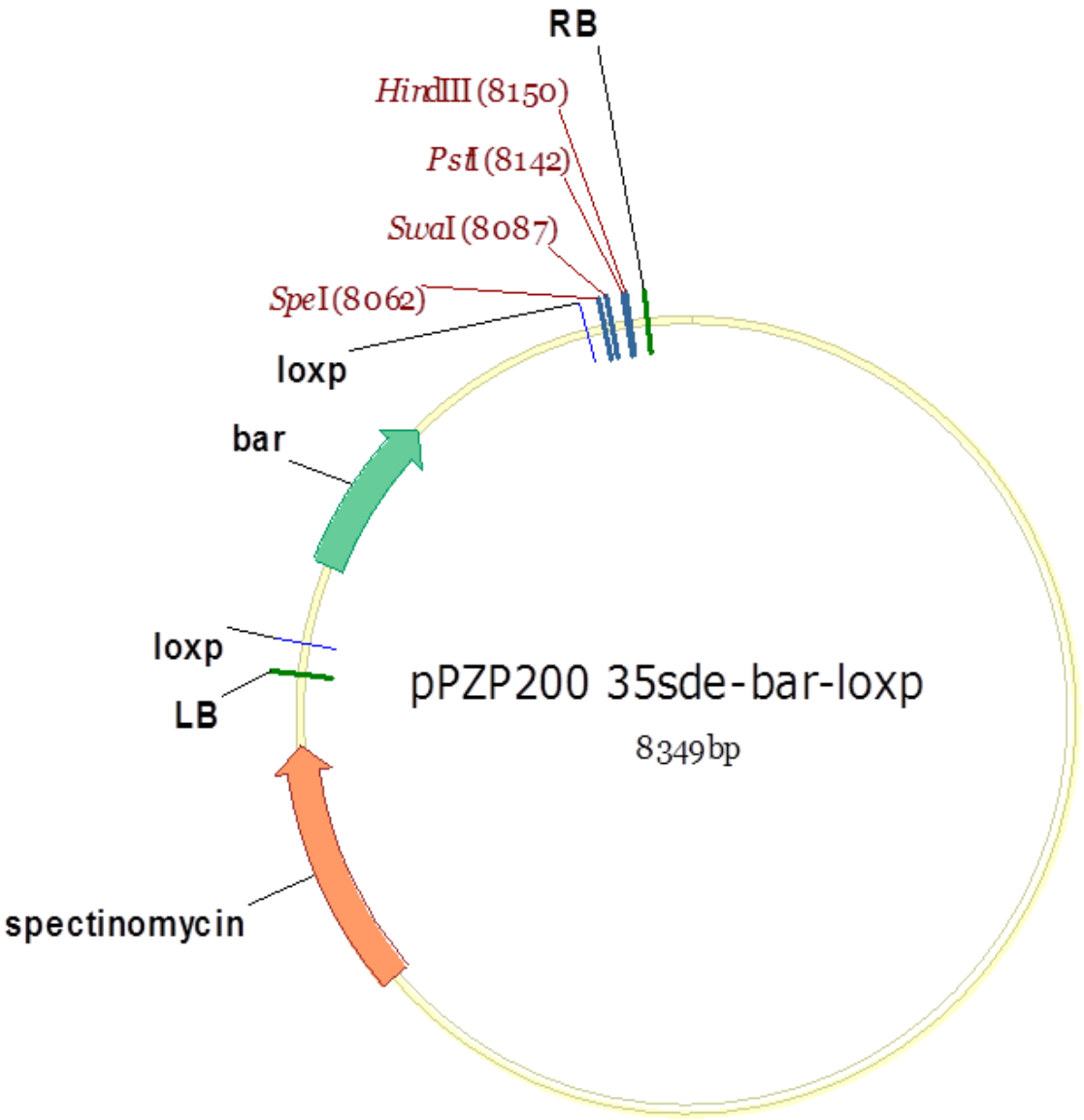


Fig. 5.4 Gene map of plant expression vector pPZP200

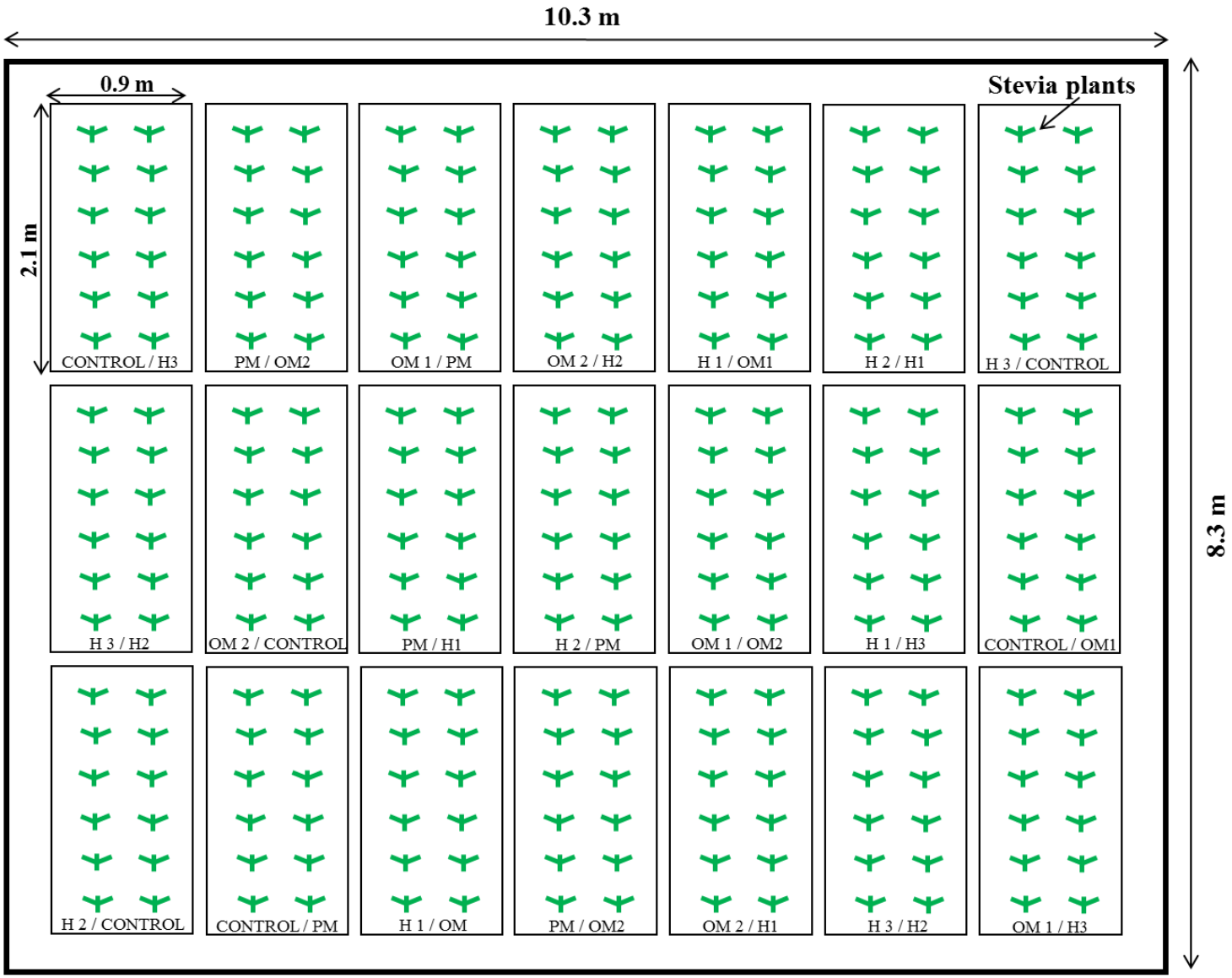


Fig. 5.5 Field layout (not to scale) for stevia plantation during the year 2017 / 2018. (PM = Plastic mulch; OM1 = Rice straws (15 t/h); OM2 = Eucalyptus leaves (15 t/h); H1 = Prolan (6.8 l/h); H2 = Pendimethalin (2.2 l/h); H3 = Glufosinate ammonium (5 l/h)

Fig. 5.6 Field view **(A)** Experimental site, **(B)** Three months old stevia seedlings, **(C)** Plot with plastic mulch, **(D)** Plot with rice straw mulch and **(E)** Control (plot with untreated stevia plants)

Fig. 5.7 Field view **(A)** Weed density in plastic mulch plot, **(B)** Weed density in organic mulch plot and **(C)** Effect of pendimathalin on Stevia plant

5.2.2.4 *Agrobacterium*-mediated transformation of stevia

Reagent setup

Stock solution	Preparation
IBA (Sigma I5386-5G)	Weigh 5 mg IBA in 1.5 ml microcentrifuge tube ↓ Dissolve in 70 % ethanol ↓ Filter sterile (under laminar air flow) ↓ Stored at -20^{o}C
GA_3 (Sigma G1025-1G)	Weigh 5 mg GA_3 in 1.5 ml microcentrifuge tube ↓ Dissolve in 70 % ethanol ↓ Filter sterile (under laminar air flow) ↓ Stored at 4^{o}C
BAP (Sigma B3408-5G)	Weigh 25 mg BAP in 1.5 ml microcentrifuge tube ↓ Dissolve in 1N NaOH ↓ Filter sterile (under laminar air flow) ↓ Stored at 4^{o}C
Acetosyringone (Sigma D134406-5G)	Weigh 20 mg acetosyringone in 1.5 ml microcentrifuge tube ↓ Dissolve in DMSO ↓ Filter sterile (under laminar air flow) ↓ Stored at 4^{o}C
Spectinomycin (Sigma S0774-25G)	Weigh 50 mg Spectinomycin in 1.5 ml microcentrifuge tube ↓ Dissolve in sterile water ↓ Filter sterile (under laminar air flow) ↓ Stored at 4^{o}C
Rifampicin (Sigma R7382-1G)	Weigh 20 mg rifampicin in 1.5 ml microcentrifuge tube ↓ Dissolve in DMSO ↓ Filter sterile (under laminar air flow)

	↓ Stored at 4°C
Cefotaxime (Sigma C7039-1G)	Weigh 1g cefotaxime in 1.5 ml microcentrifuge tube ↓ Dissolve in sterile water ↓ Filter sterile (under laminar air flow) ↓ Stored at 4°C

Table 5.4 Composition of stock solution of MS media

Component	For 100 ml
MS (Major) - stock 50X	
NH_4NO_3	8.25gm
KNO_3	9.5gm
$MgSO_4.7H_2O$	1.85gm
KH_2PO_4	0.85gm
MS (Minor) - stock 1000X	
$MgSO_4.4H_2O$	1.51gm
$ZnSO_4.7H_2O$	0.86gm
H_3BO_4	0.62gm
KI	0.0831gm
$NaMoO_4.2H_2O$	0.025gm
$CuSO_4.5H_2O$	0.0025gm
$CoCl_2.6H_2O$	0.0025gm
MS ($CaCl_2$) - stock 100X	
$CaCl_2.2H_2O$	4.4gm
MS (MS Vit) - stock 1000X	
Glycine	0.2gm
Thiamine HCl	0.01gm
Nicotinic acid	0.05gm
Pyridoxine HCl	0.05gm
MS (Fe-EDTA) - stock 200X	
$Na_2EDTA.2H_2O$	0.746gm
$FeSO_4.7H_2O$	0.556gm

5.2.2.5 Surface sterilization of explants

Leaves, nodal segments, and shoot tips from mother plants were taken as explants for in vitro callus regeneration. Explants were surface sterilized with 5% (v/v) Tween® 20 for

20 min, washed with tap water, and further treated with 0.1% (w/v) bavistin for 15 min to remove fungal spores and rinsed 3−4 times with autoclaved Milli-Q® water (Merck Millipore, USA). Thereafter, the explants were treated with 0.1 % (w/v) $HgCl_2$ for 5 min and rinsed with autoclaved Milli-Q® water, 3−4 times to remove any traces of $HgCl_2$. The explants were then treated with ethanol (70%) for 60 s, followed by several rinses with autoclaved Milli-Q® water. The sterilized explants were kept on MS media [24] containing 0.3 % Phytagel™ (Sigma, USA), and different combinations and concentrations of PGRs (2,4-D, BAP, Kin, and NAA). Prior to autoclaving (at 121^{oC} for 15 min), the pH of the MS medium was adjusted to 5.7. After autoclaving, the sterile medium was dispensed into sterile jam bottles. All the cultures were kept at 22 ± 2°C under white light (PFD: ~52 μmol $m^{-2}s^{-1}$) for a photo period of 16/8h light/darkness. All experiments were repeated at least thrice.

5.2.2.6 Pre-incubation of explants

Nodal sections and leaf-originated callus from *in vitro* regenerated plants were dissected and incubated for 2 days on pre-incubation media containing MS salts + BAP (2 mg/l). These acclimatized explants were used for *Agrobacterium*-mediated transformation with pPZP200-*bar*-loxp construct.

5.2.2.7 Preparation of bacterial inoculum

The culture inoculum from the glycerol stock of transformed GV strain of Agrobacterium was streaked on LA (Luria Agar) plates containing gentamycin (50 mg/l), rifampicin (50 mg/l), and spectinomycin (50 mg/l) as the selection antibiotics and incubated at 28ºC. For primary culture, a single colony from a freshly streaked plate was inoculated in 50 ml culture tubes containing LB (Luria Broth) medium with the same antibiotics. The culture was incubated at 28ºC for 48 h at 220 rpm under dark conditions. For secondary culture, 9 ml of the primary culture was inoculated in 41 ml of LB medium having the same antibiotics. Incubate the secondary culture at 28ºC for 48 h at 220 rpm. The next day secondary culture was centrifuged at 3500 rpm at 4ºC for 10 min. Discard the supernatant and resuspend the pellet in LB medium containing 200 μM Acetosyringone without any antibiotics. Incubate the culture at 25ºC for 2-3 h at 70-90 rpm. Centrifuge the culture

again at 4ºC (10 min) at 3500 rpm. Pellet was suspended in fresh LB medium and O.D was adjusted to 0.6 at 600 nm wavelength.

5.2.2.8 Explant preparation and agro-inoculation

Explants were wounded with a sterile needle so as to increase the transformation efficiency. Leaf segments were prepared by cutting the leaves with a sterile knife or blade. Leaf segments of (~2–3mm) were suspended in liquid co-cultivation medium supplemented with 100 μM As, with the help of sterilized forceps, for 15 min, with gentle agitation.

5.2.2.9 Co-cultivation, cefotaxime supplemented media and first selection

After agro-inoculation, the explants were incubated on a co-cultivation medium containing MS salts + BAP (2 mg/l) + acetosyringone (As), at 22°C, in dark for 3 days. Thereafter, the explants were washed two times with liquid MS media fortified with cefotaxime (250 mg/l), followed by incubation on MS media containing 250 mg/l cefotaxime. After 8-10 days, explants were subjected to first selection media (SIM-1) having MS salts + BAP (1 mg/l) + NAA (0.5 mg/l), cefotaxime (250 mg/l) + glufosinate-ammonium (2 mg/l) for 22−30 days. The detailed procedure for stevia transformation is outlined in Fig. 5.8.

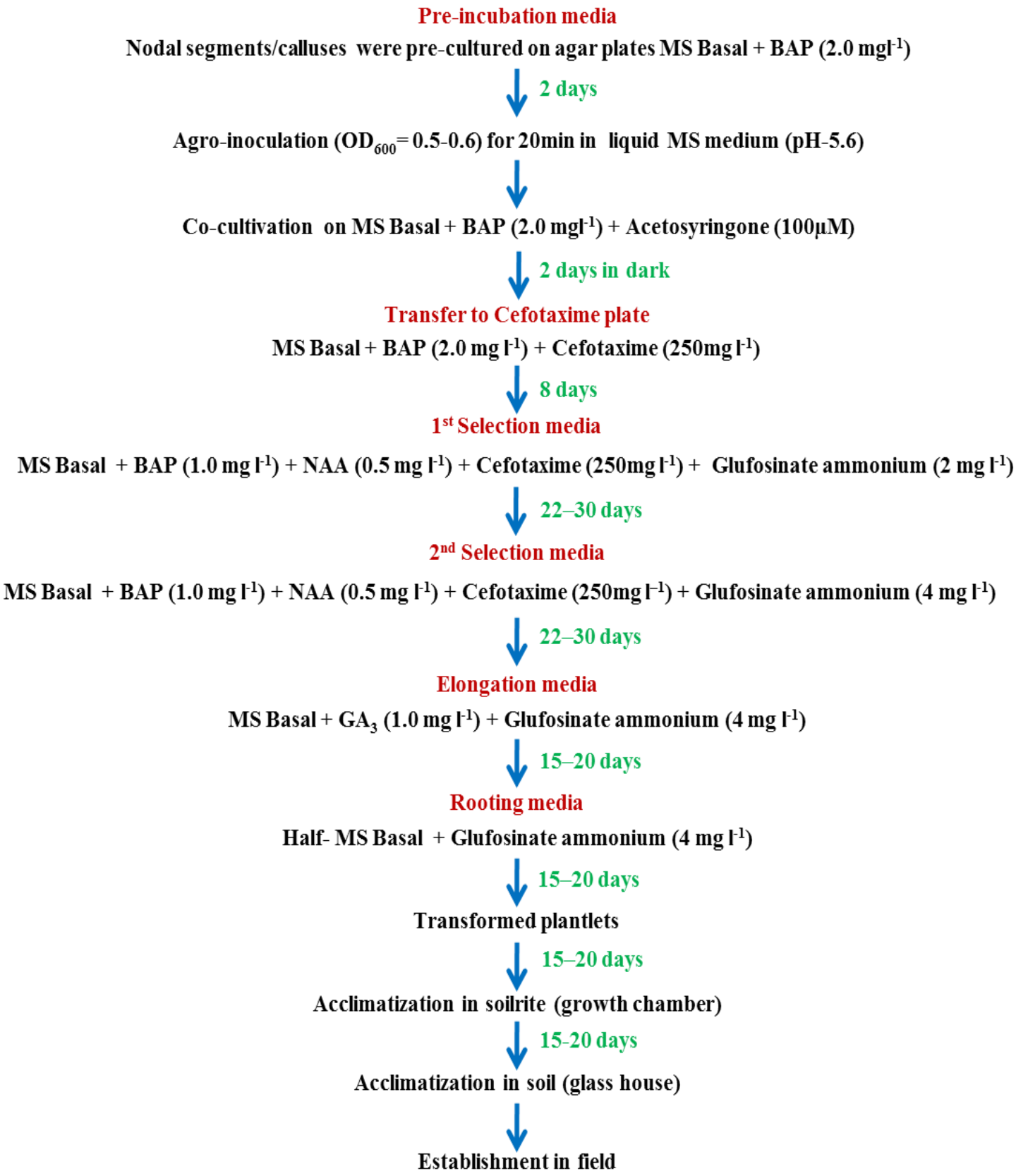


Fig. 5.8 Detailed procedure of *Agrobacterium*-mediated transformation of stevia

5.2.2.9.1 Second selection

The regenerated shoots with a pair of vegetative leaves were identified, excised into segments, and were placed on the second selection medium (SIM–2) having the same constituents as SIM–1, except 4 mg/l of glufosinate-ammonium, and incubated for 22–30 days.

5.2.2.9.2 Transfer to shoot elongation media

The independent shoots that regenerated on SIM-2 were transferred to culture-tubes containing elongation medium (SEM) having MS salts + GA_3 (1 mg/l) and kept for 15−20 days.

5.2.2.9.3 Root initiation media

The elongated shoots were incubated on a rooting medium (RIM) containing ½ strength MS medium, for 15−20 days.

5.2.2.9.4 Hardening of in vitro developed plantlets

The rooted plants were shifted to plastic pots containing sterile soil rite mixture augmented with Hoagland solution. The plastic pots were then covered with polythene bags (perforated) and placed inside the plant growth chamber (Conviron, USA) for 15-22 days at 805 of relative humidity.

5.2.2.9.5 Transfer to pots and seed collection

The acclimatized plantlets were planted in plastic pots (12 inches) filled with soil: sand: farmyard manure (3:1:1) and transferred to a glasshouse maintained at 24 ± 1°C under natural light, for normal vegetative and reproductive growth phases. Seeds were collected from control and transgenic plants after 3 months of hardening.

5.2.3 Molecular techniques

5.2.3.1 DNA Elution from agarose gel

Agarose gel was prepared and DNA samples (plasmid DNA) were electrophoresed to resolve its fragments. Desired size band of DNA was then cut from the gel with the help of a sharp blade. Elute gel extraction kit from Sigma was used to recover the DNA fragment as per the instructions provided by the manufacturer.

5.2.3.2 Restriction digestion of DNA samples

For restriction digestion, a buffer provided by Sigma was used to make the final volume of 30 µl of a reaction. This reaction contains: 2-5 µl of plasmid DNA, 4 µl of 10 X buffer solution, 1 µl of BSA (10 mg/l), 1 µl of restriction enzyme, and sterile MQ water was added to make the final volume 30 µl.

5.2.3.3 Ligation of promoter and *bar* gene

For ligation reaction, insert and pPZP200 vector DNA containing DECaMv35s promoter were mixed in a ratio of 3:1, and the reaction was performed at 16ºC for 16 h. The reaction was performed as 100-200 ng of vector DNA, 3 ratios of insert DNA, 1.5 µl of 10X ligation buffer, 1.5 µl of 10 mM ATP, 1.0 µl of DNA ligase, and sterile MQ water was added to make the final volume 15 µl.

5.2.3.4 Agrobacterium competent cell preparation

Glycerol stock of Agrobacterium tumefaciens strain GV was streaked on Luria Agar (LA) plates containing rifampicin (50 mg/ml) antibiotic and incubated for 2 days at 28ºC. After 2 days isolated colony was inoculated in Luria Broth (5 ml) as primary culture and kept at 28ºC overnight with continuous shaking. From primary culture, 200 µl was added in Luria Broth (100 ml) and kept at 28ºC overnight with continuous shaking until O.D600 = 0.4 - 0.5 was achieved. The secondary culture was placed on ice for thirty min and centrifuged (5 min) at 5000 rpm at 4ºC. The supernatant was discarded and the pellet was resuspended in 20 mM $CaCl_2$ (40 ml) and kept on ice for 30 min. The culture was again centrifuged at 5000 rpm at 4ºC for 5 min. Pellet was resuspended in 20 mM $CaCl_2$ (4 ml)

+ 10 % glycerol and kept on ice for 30 min. Cells were then transferred to pre-chilled, sterile 1.5 ml centrifuge tubes and were immediately stored at -80ºC for future use.

5.2.3.5 *Agrobacterium* transformation with plasmid by heat shock method

Competent cells of *Agrobacterium tumefaciens* strain GV were thawed on ice for 30 min. 10 µl of plasmid DNA was added into 100 µl of competent cells (under laminar) and kept on ice for 15 min. After that cells were dipped in liquid nitrogen for 3 min and immediately kept at 37ºC (for heat shock). 600 µl LA was added into it and incubated at 28ºC for 3 h with continuous shaking. Cells were centrifuged for 5 min at 5000 rpm and the supernatant was rejected. Pellet was suspended with a pipette and then spread on LA plates containing gentamicin (50 mg/ml), rifampicin (50 mg/ml), and spectinomycin (50 mg/ml). Plates were then incubated at 28ºC for 2 days for growth check.

5.2.3.6 Colony PCR

For colony PCR, 9 ul of MQ water was added to each PCR vial placed on ice. A single isolated colony was picked with the help of a toothpick or pointed tip and then mixed with MQ in a PCR vial. Gently mixed for 15 sec and then incubated at 95ºC for 10 min. Vials were then centrifuged (5 min) at 10,000 rpm. After that, 8 ul of supernatant was taken from this vial and transferred to fresh sterile PCR vials. The supernatant was discarded. 1 ul of primers (forward and reverse) and 10 ul of Emerald master mix were added to each vial. PCR was performed: denaturation for 5 min at 95ºC, annealing for 30 sec at 55ºC, and extension for 2 min at 72º C and final extension for 5 min at 72º C. Bands were visualized under gel documentation system.

5.2.3.7 DNA and RNA isolation

Young leaf samples (500 mg) from transgenic stevia plants were cleaned with MQ water and crushed in liquid nitrogen to make a fine powder. The leaf powder was shifted to a centrifuge tube (2 ml) and 1 ml of DNA extraction buffer was added to it. The composition of the DNA extraction buffer was 2 M NaCl, 2% CTAB, 20 mM EDTA (pH-8.0), 4% PVP, 100 mM Tris HCL (pH-8.0), and 1.4% β-mercaptoethanol. Samples were then incubated at 65ºC for 30 min and mixed occasionally. Samples were

centrifuged for 5 min at 13000 rpm at room temperature. Pellet was discarded and the supernatant was mixed with chloroform: isoamyl alcohol (24:1) and vortexed vigorously. Samples were then centrifuged at 12000 rpm for 10 min and the supernatant was shifted to a fresh vial. 2 µl of RNase A (20 mg/ml) was added to each vial and placed at 37 ºC for 2 h. After incubation, Phenol:Chloroform: Isoamyl alcohol (1:1) was added to it and centrifuged at 4 ºC for 15 min at 12000 rpm. The supernatant was collected, C: I was added to it and mixed by inverting. Samples were centrifuged at 12000 rpm for 10 min and the supernatant was shifted to a fresh 1.5 ml vial. Now, 300 µl of chilled isopropanol was added to it and mixed gently by inverting. Vials were placed for 20 min at RT and centrifuged for 15 min (4ºC) at 12000 rpm. The supernatant was rejected and 300 µl of 70 % molecular grade ethanol was added to the pellet. Again centrifuged for 10 min at 4ºC at 12000 rpm. The supernatant was discarded, the pellet was dried on thermo-mixer at 37 ºC for 1 h and 30 µl of autoclaved MQ water was added to each vial. The purity and yield of genomic DNA were checked with nanoquant and integrity was checked on 0.8 % agarose gel with gel electrophoresis.

Total RNA was extracted from young leaf samples of transgenic and control plants as per the protocol described by Ghawanan et al. 2011. Young leaf tissues (100 mg for each sample) were ground in liquid nitrogen to make a fine powder. Now, 2 ml of solution I have added into leaf powder and ground again. After that 800 µl of DEPC treated water was added to it. The content was then shifted to 2 ml sterile vials and kept at room temperature for 5 min. 2 µl of chloroform was added into each vial, vortex for 10s, and kept at RT (10 min). Samples were centrifuged (4ºC) for 10 min at 13000 rpm and the upper aqueous layer was shifted to fresh vials (sterile). 600 µl of isopropanol was added into each vial, vortex for 10s, and kept at RT for 10 min. Samples were again centrifuged at 13,000 rpm for 10 min at 4ºC. The supernatant was discarded and the RNA pellet was washed with molecular grade ethanol, air-dried, and then dissolved in DEPC treated water. RNA was checked on 1 % Agarose gel electrophoresis for integrity. Quantification of RNA was checked with NanoQuant. For removal of DNA contamination, good quality RNA samples were treated with DNase using a DNA-free™ kit as per the manufacturer's instructions (Ambion Thermo Scientific, USA). 1 µl of DNase and 1 µl of 10X DNase buffer was added into RNA and mixed with a pipette. The reaction mixture was

incubated at 37ºC for 30 min and 2 µl of inactivation reagent was added into it. Kept at room temperature for 2 min and then centrifuged for 2 min at 10,000 rpm. The supernatant was collected and transferred to the fresh sterile vial, quantified, and checked for quality.

5.2.3.8 Polymerase chain reaction (PCR)

Genomic DNA isolated from leaves of non-transformed control plants and putative T_0 transformants were subjected to PCR amplification with a particular set of primers. The set of primers used for PCR were given in Table 5.5. For 20 µl PCR reaction mixture, 1 µl of genomic DNA, 1 µl of each primer (forward and reverse), 10 µl PCR master mix, and 7 µl of nuclease-free water was added. The reaction was then carried out for amplification: initial denaturation at 95ºC (5 min), denaturation at 95ºC (30 s), annealing (30 s), extension at 72ºC (1 min) and final extension at 72ºC (5-7 min).

5.2.3.9 Complementary deoxyribonucleic acids (cDNA) synthesis

For the synthesis of cDNA RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific, USA) was used as per the manufacturer's instructions. Nuclease-free water, oligo (dTs) primer, and DNA-free RNA were mixed together to make the final volume 12 µl. The reaction mixture was then incubated for 5 min at 65ºC. After incubation, vials were placed on ice for 5 min. After that, RiboLock RNase inhibitor (20U), 5× reaction buffer (1×), RevertAid RT (200U), and 10 mM dNTP mix (1 mM) was added into the vial to make the final volume 20 µl and mixed properly. The reaction mixture was incubated for 1 hr at 42ºC and then at 70ºC for 5 min.

5.2.3.10 Quantitative real-time PCR

qPCR was done with syber green RT master mix to check the expression of the candidate gene. The actin gene was used as a housekeeping gene as an internal control. A list of the primers used for desired genes is given in Table 5.5. The total reaction volume was kept at 10 µl consisted of 0.5 µl of cDNA (1/20 times diluted), 1 µl of each primer (5 pM, forward and reverse), autoclaved MQ water, and 1X SYBR green master mix. $2^{-\Delta\Delta Ct}$

method was used to check the relative expression level of genes (Schmittgen and Livak, 2008). The experiments were conducted in technical and biological replicates.

Table 5.5 List of gene specific primers

Gene	Forward	Reverse
bar (for PCR)	5′CGCCGATGGTTTCTACAAAGA3′	5′TCAATGACCGCTGTTATGCG3′
bar (for qRT-PCR)	5′GTTTCACCACGTCATCAACG3	5′TGCCAATTTCCATGTTTGAA3′
β-actin	5′TCTTGATCTTGCTGGTCGTG 3′	5′GCGGTTTCAAGTTCTTGCTC3′

5.2.3.11 Southern blotting

For Southern blotting, ~ 10 µg genomic DNA of T_0 transgenic stevia leaves was digested overnight (12-14h) with EcoRI (Cat # R0101L, New England Biolabs, USA) run on 0.7% gel and transferred on BioBond-plusTM nylon membrane (Sigma, USA). After pretreatment, the Southern-blot was hybridized for 24h at 58°C with α[32P] dCTP labeled 552 bp *bar* gene-probe (BRIT, Mumbai India), followed by 3 rigorous washings. The blots were then exposed under the Fuji screen for 48 h and wewre observed under phosphoimager (TyphoonTM Trio+, Sweden).

5.2.4 Stevioside and rebaudioside analysis using HPLC

Leaves were collected from the same node from 2 control plants and T1-T10 plants. Leaves were shade dried for 7 days and crushed uniformly to make powder. 10 ml methanol was added into each 50 mg powdered leaf sample and then filtered using Whatman filter paper. The mixture was then evaporated in a rotary evaporator system so as to evaporate the methanol. 100 ml of acetonitrile: water (80:20) was used to dissolve the pellet and then again filtered using nylon membrane (pore size 0.45 mm). This dissolved mixture was poured into sterile HPLC tubes (Kabiri et al., 2017). Rebaudioside A and stevioside standards were prepared by dissolving 10 mg of each standard in 50 ml of acetonitrile: water (80:20).

%age of standard in sample =

$$= \frac{\text{Area of sample}}{\text{Area of standard}} \times \frac{\text{Weight of standard}}{\text{Weight of sample}} \times \frac{\text{Dilution of sample}}{\text{Dilution of standard}} \times \%\ \text{purity of standard}$$

HPLC analysis was done in three technical repeats, Student's t-test was applied to find the statistical significance.

5.2.5 Agronomic data collection

5.2.5.1 Comparison of morphological characters and chlorophyll content

After shifting the transgenic plants to green-house, morphological characters (height of the plant, no. of leaves and branches) and chlorophyll content of transgenic and control plants were recorded. For chlorophyll estimation fresh leaves (100 mg) were crushed in acetone (80%), centrifuged at 10000 rpm for 10 min, at 4°C. The absorbance of the supernatant was recorded spectrophotometrically (UV-2700 UV-VIS Spectrophotometer, Shimadzu, Japan) at 663 and 645 nm for chlorophyll a and b respectively. The experiments were performed three times and in triplicates.

Table 5.6 List of ISSR primers used in this study

No.	Name	Sequence	No.	Name	Sequence
1.	UBC-808	AGAGAGAGAGAGAGAGC	**11.**	UBC 856	ACACACACACACACACYA
2.	UBC 807	AGAGAGAGAGAGAGAGT	**12.**	HB 8	GAGAGAGAGAGAGG
3.	UBC 811	GAGAGAGAGAGAGAGAC	**13.**	HB 9	GTGTGTGTGTGTGG
4.	UBC 812	GAGAGAGAGAGAGAGAA	**14.**	HB 11	GTGTGTGTGTGTCC
5.	UBC 817	CACACACACACACACAA	**15.**	HB 12	CACCACCACGC
6.	UBC 826	ACACACACACACACACC	**16.**	HB 15	GTGGTGGTGGC
7.	UBC 834	AGAGAGAGAGAGAGAGYT	**17.**	844B	CTCTCTCTCTCTCTCTGC
8.	UBC 836	AGAGAGAGAGAGAGAGYA	**18.**	HB 14	CTCCTCCTCGC
9.	UBC 842	GAGAGAGAGAGAGAGAYG	**19.**	HB 13	GAGGAGGAGGC
10.	UBC 845	CTCTCTCTCTCTCTCTRG	**20.**	HB 10	GAGAGAGAGAGACC

5.2.5.2 Herbicide tolerance assay

Herbicide tolerance (glufosinate) assay was performed to check the efficacy of transgenic stevia plants for herbicide tolerance as compared to non-transgenic plants. To perform this assay, the wild-type (non-transgenic control) stevia plants were divided into 5 groups, each having 5 plants (4 test + 1 control). Each group was sprayed (using a hand sprayer) with different concentrations of glufosinate (50 ml of 2, 4, 6, and 8 mg/l) under green-house conditions to find the minimum lethal dose for stevia plants. It was observed that 8 mg/l of glufosinate was the minimum lethal dose for stevia plants. Thereafter, five healthy T_0 transgenic and non-transgenic control (wild type) stevia plants each were sprayed separately with 8 mg/l glufosinate, in green-house. The experiment was repeated three times and the observations were recorded after 12 days of spray (Taak et al., 2021).

5.2.5.3 Residual phytotoxic effect

The residual phytotoxic effect of glufosinate on soil was studied by spraying the potting soil with three different concentrations of glufosinate i.e. 0.25, 0.50, and 1.0% (v/v) under greenhouse conditions. After five days of spray, 10 seeds each of indicator plants i.e. corn and cucumber were sown into these pots. The experiment was performed in triplicates and the soil of the control plant was sprayed with water as an experimental control. Seed germination percentage was recorded after 10 days of sowing. The plantlet height was recorded with the help of a measuring scale after 20 days of sowing.

CHAPTER 6

IDENTIFICATION AND COLLECTION OF WEEDS FOUND IN STEVIA CULTIVATION AND COMPARATIVE EVALUATION OF DIFFERENT WEED MANAGEMENT APPROACHES

6.1. Growth parameters

Treatments of both the organic mulches had a significant effect on plant height. In the year 2017, OM1 plot was recorded with the highest average plant height (98.2 ± 2.81 cm; $P < 0.0001$) while in the year 2018, OM2 plot had the highest average plant height (98.1 ± 1.73 cm; $P < 0.0001$) as shown in Fig. 6.4 A, B and Table 6.2. Trend observed for plant height in the year 2017 and 2018 was H3 < Control < H2 < PM < H1 < OM2 < OM1 and H2 < H3 < PM < Control < OM1 < H1 < OM2 respectively. OM2 plot was recorded with the maximum number of branches (6.0 ± 2.23 / plant) during the year 2018 which differs significantly from the control plot. Highest chlorophyll content was observed in OM1 plot during the both the years, 8.12 ± 0.07 ($P < 0.0001$) and 9.42 ± 0.06 mg/g ($P < 0.0001$) respectively, which differs significantly from control plots. During both the years 2017 and 2018, the plants of OM1 plots were observed with the highest leaf area index. Leaf area index of plastic mulch plot plants was not significantly different from control plants during the year 2018 (Taak et al., 2020). But, herbicide-treated plants had a low leaf area index as compared to other plots.

6.2. Weed count and dry matter accumulation

Various weed plants belonging to families Poaceae, Cyperaceae, Commelinaceae, Primulaceae, Asteraceae, Caryophyllaceae, Fabaceae, Plantaginaceae, Malvaceae, Solanaceae, Amaranthaceae, Cucurbitaceae, Caesalpinioideae etc. were observed in stevia fields during both the years 2017 and 2018. Maximum numbers of weeds were reported from Poaceae family, followed by caryophyllaceae and fabaceae. Major weed species which were found during the rainy season (end of June to end of August) are shown in Fig. 6.1 and enlisted in Table 6.1. Graphical representation in Fig. 6.2 C and D shows that during both the years maximum numbers of weeds were observed in control plots while, the other treatments significantly reduced the weed count (Table 6.3).

Table 6.1 Weeds reported in stevia field during the year 2017 and 2018

Weed plant (Botanical name)	Common name(s)	Family	Rank
Cynodon dactylon (L.) Pers.	Bermuda grass	Poaceae	Monocotyledon
Dactyloctenium aegyptium (L.) Willd	Crowfoot grass	Poaceae	
Panicum dichotomiflorum Michx.	Fall panic grass, autumn millet, fall panicum	Poaceae	
Echinochloa crus-galli (L.) P.Beauv.	Cockspur	Poaceae	
Setaria verticillata (L.) P.Beauv	Hooked bristle grass, rough bristle-grass and bristly foxtail	Poaceae	
Cyperus rotundus L.	Purple nuts edge	Cyperaceae	
Commelina diffusa Burm.f.	Climbing day flower	Commelinaceae	
Anagallis arvensis L.	Scarlet pimpernel	Primulaceae	Dicotyledon
Parthenium hysterophorus L.	Congress grass	Asteraceae	
Spergula arvensis L.	Corn spurry	Caryophyllaceae	
Stellaria media (L.) Vill.	Common chickweed	Caryophyllaceae	
Medicago polymorpha L.	California burclover	Fabaceae	
Veronica persica Poir.	Bird eye speedwell	Plantaginaceae	
Vicia hirsute (L.) Gray	Hairy tare	Fabaceae	
Ageratum houstonianum Mill.	Flossflower, bluemink, blueweed, pussy foot, Mexican paintbrush	Asteraceae	
Sida rhombifolia L.	Arrowleaf sida	Malvaceae	
Solanum nigrum L.	Black nightshade, duscle, garden nightshade, garden huckle berry, hound's berry, petty morel, wonder berry, small-fruited black nightshade, or popolo	Solanaceae	
Chenopodium album L.	Common lambsquarter	Amaranthaceae	
Cucumis melo agrestis	Muskmelon, cantaloupe, honeydew	Cucurbitaceae	
Senna tora(L.) Roxb	Sicklepod	Caesalpinioideae	

A lesser number of weeds (0.4 ± 0.15 n°/m^2) was observed in plots treated with H3 in July 2017. At the time of harvest minimum (0.2 ± 0.07 n°/m^2), weed count was observed in H3 treated plots, while weed number was not reduced significantly by OM treatments. Minimum dry weight (12.7 ± 1.22 g/m^2, $P < 0.0001$) of weeds was recorded in the plots with H2 treatment during the year 2017. On the contrary maximum dry weight of weeds was recorded in untreated (control) plots (132.5 ± 1.80 g/m^2) during the year 2018 while all the other treatments significantly affected it. To sum up, the trend observed for dry weight of weeds in both the years was H2 < H3 < H1 < PM < OM2 < OM1 < Control (Fig. 6.2 E).

6.3. Dry matter accumulation in the stevia plant

All the treatments had significant effects on root, stem, and leaf biomass of stevia plants except PM treatment, while leaf biomass got increased with PM treatment (Fig. 6.2 F). Maximum average root dry biomass of crop plant was observed in OM2 plots during the year 2017 (13.18 ± 1.05 g/plant; $P < 0.0001$) and 2018 (12.11 ± 0.75 g/plant; $P < 0.0001$). Except for PM treatment, all other treatments had a significant effect on root dry biomass.

Stem dry biomass was significantly higher in the plot treated with OM2 and OM1 in the year 2017 (23.12 ± 1.74 g/plant; $P < 0.0001$) and 2018 (21.75 ± 2.16 g/plant; $P < 0.0001$) respectively. Significant increase (2 folds) in leaf dry biomass has been observed with OM2 treatments as compared to control plants during both the year 2017 (10.22 ± 1.35 g /plant; $P < 0.0001$) (2 folds) and 2018 (12.78 ± 1.42 g/plant; $P < 0.0001$) (more than 2 folds) (Table 6.4, Table 6.5). Before beginning with our experiments, a rigorous review of the extant literature was done. In our study, all the herbicides treatments were effective in controlling stevia-weed(s) density.

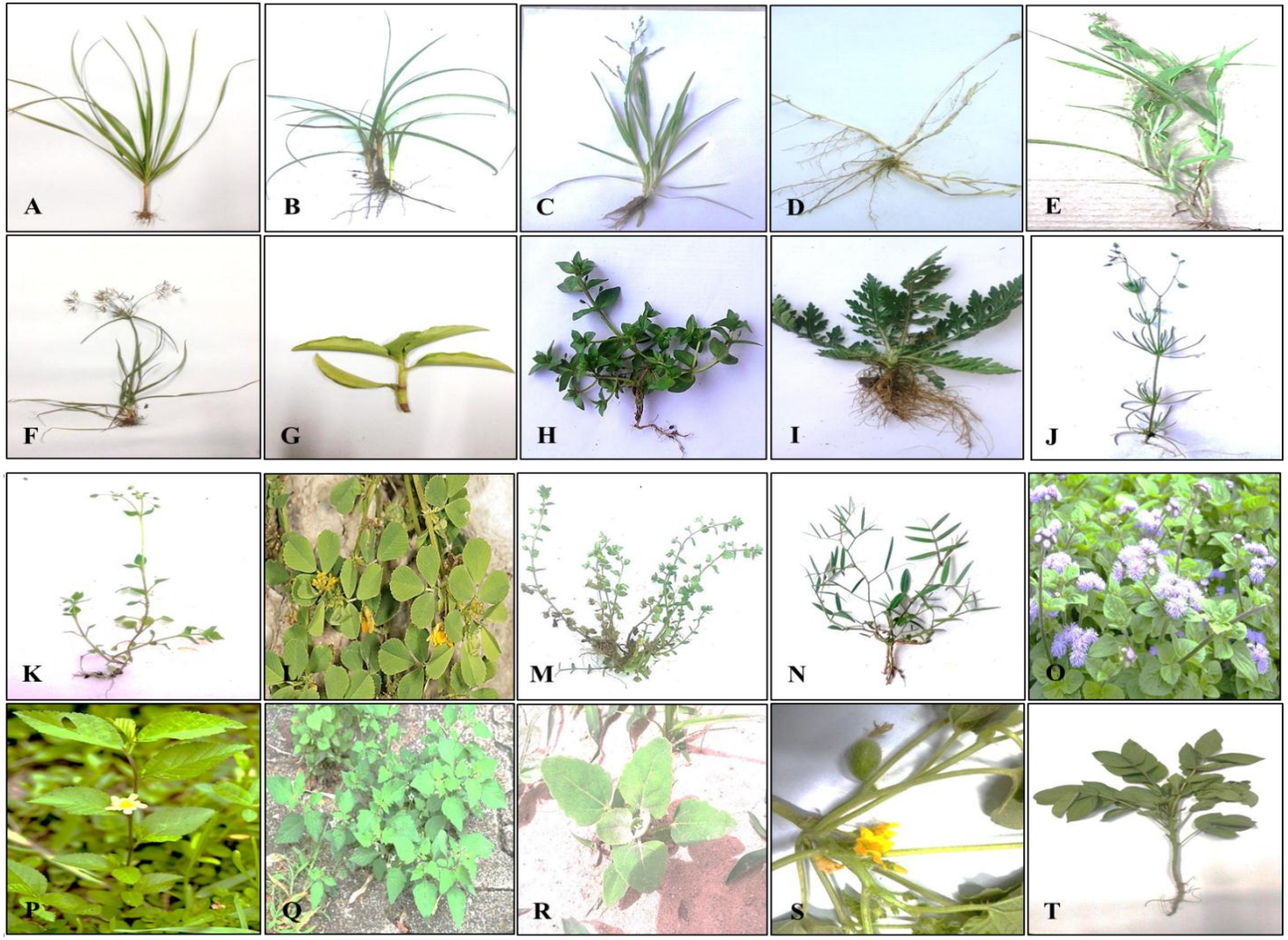


Fig. 6.1 Major weed species observed in stevia field. **(A)** *Cynodon dactylon*, **(B)** *Dactyloctenium aegyptium*, **(C)** *Panicum dichotomiflorum*, **(D)** *Echinochloa crus-galli*, **(E)** *Setaria verticillata*, **(F)** *Cyperus rotundus*, **(G)** *Commelina diffusa*, **(H)** *Anagallis arvensis*, **(I)** *Parthenium hysterophorus*, **(J)** *Spergula arvensis*, **(K)** *Stellaria media*, **(L)** *Medicago polymorpha*, **(M)** *Veronica persica*, **(N)** *Vicia hirsute*, **(O)** *Ageratum houstonianum*, **(P)** *Sida rhombifolia*, **(Q)** *Solanum nigrum*, **(R)** *Chenopodium album*, **(S)** *Cucumis melo* and **(T)** *Senna tora*

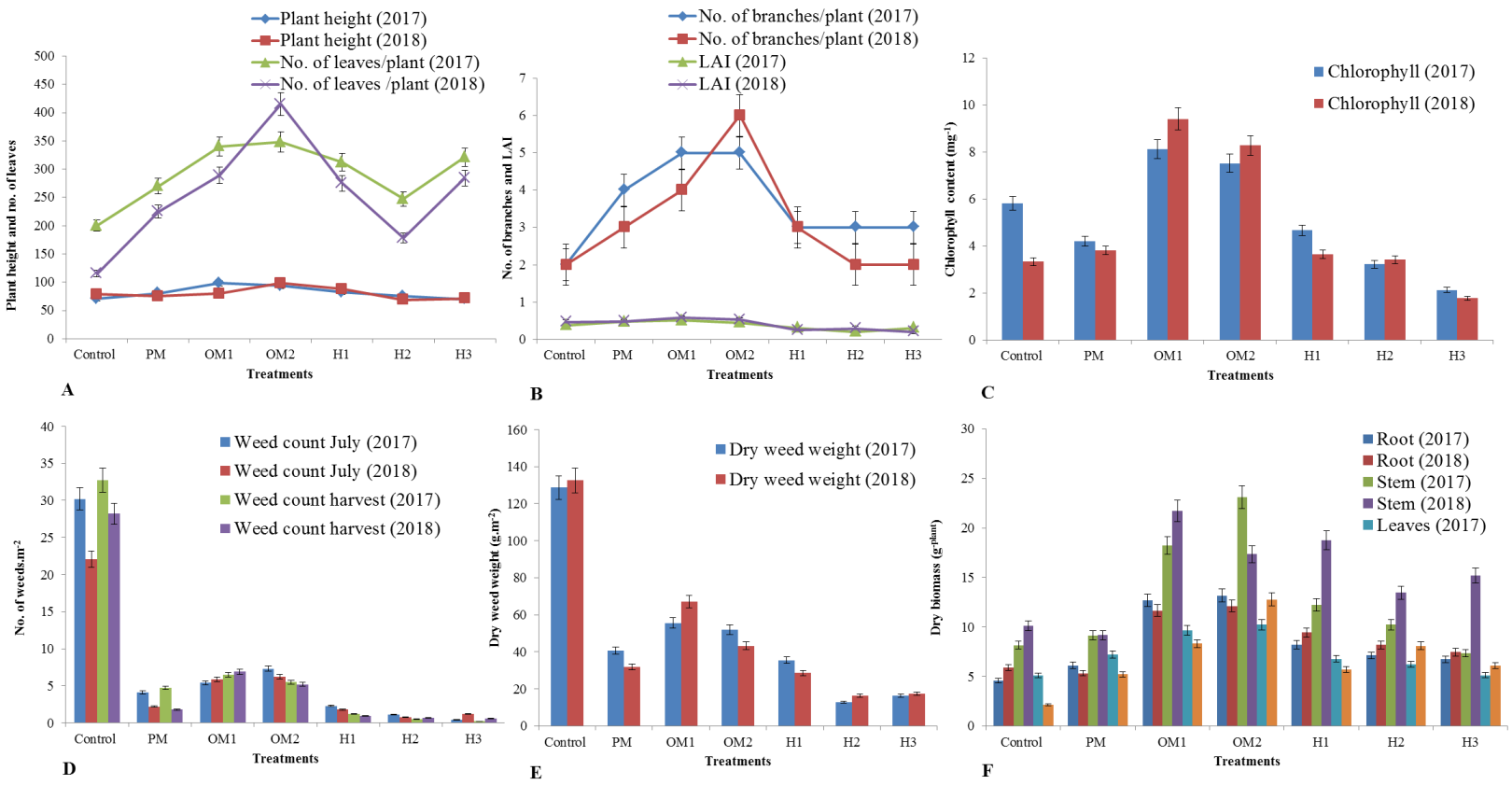


Fig. 6.2 Effect of various treatments on plant growth during year 2017 and 2018 **(A)** on plant height, number of leaves per plant, **(B)** Number of branches per plant and leaf area index (LAI), **(C)** Chlorophyll content (mg/g), **(D)** Number of weeds/m^2, **(E)** Dry weed weight and **(F)** Dry biomass of stevia plants

6.4 Discussion

In the early months when the temperature is not too high, rainfall is less weed density is also much less but as the plants start growing, the temperature started rising and heavy rainfall occurs, weeds in the plots started growing enormously. In a study by Covarelli et al. (2008), three post-emergence and pre-emergence herbicides were used. It was observed that post-emergence treatment with aclonifen and pre-emergence treatment with pendimethalin was highly effective for maximum weed control. Besides that, symptoms of phytotoxicity were also observed in stevia plants with the treatment of quizalofop-ethyl. In another study by Harrington et al. (2011), two field trials and the one-pot trial was conducted to evaluate the herbicide tolerance potential of stevia plants. Among the eight pre-emergence and eleven post-emergence herbicides tested, terbacil (960g a.i/h) and bromacil (970g a.i/h) were found to be most effective for weed suppression but, some symptoms of phytotoxicity was also observed. Moreover, 19 folds increase in crop yield was found with a spray of bromacil before stevia transplantation and 30 fold increase with frequent manual weeding. In a similar study by Zachokostas (2015), among the nine herbicides (aclonifen, clopyralid, fluoeturon, prometryn, dimethenamid, imazamoz, acetochlor, napronamide and pendimethalin) tested only pendimethalin was found most effective followed by dimethenamid and napronamide. In a recent study, Hopkins and Midmore (2015), tested sixteen different herbicides for weed management in stevia fields. It was reported that Prolan 500 (6.8l/h) i.e. 500 g/l was most effective for weed control as compared to other herbicides. Reviewing the aforementioned studies, we selected one preemergence herbicide ‘prolan’, one post-emergence herbicide ‘pendimethalin’, and one broad-spectrum herbicide ‘glufosinate ammonium’. In our study, herbicide treatments were found more promising for efficient weed control as minimum weeds count and dry weed weight was observed in herbicides treated plots, during both experiments. The lowest dry weed weight was found in H2 treated plot during the year 2017. On the contrary, the highest weeds count was observed in untreated or control plots during the initial growth period and at the time of harvest.

Table 6.2 Effect of different treatments on plant morphology and physiology

Treatments	Plant height (cm)		Number of branches Branches/plant		Number of leaves/plant		Chlorophyll (mg/g)		Leaf area index	
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
Control	70.3 ± 0.81	78.7 ± 0.71	2.0 ± 0.70	2.0 ± 0.70	200 ± 2.34	115 ± 1.0	5.82 ± 0.08	3.34 ± 0.04	0.38 ± 0.10	0.47 ± 0.09
Plastic mulch (PM)	80.2 ± 1.23*	75.3 ± 2.56*	4.0 ± 1.22*	3.0 ± 0.70*	270 ± 3.0*	225 ± 1.58*	4.21 ± 0.05*	3.82 ± 0.04*	0.48 ± 0.03	0.49 ± 0.02
Organic mulch 1 (PM1)	98.2 ± 2.81*	80.1 ± 1.79	5.0 ± 0.83*	4.0 ± 1.87*	348 ± 1.58*	415 ± 1.87*	8.12 ± 0.07*	9.42± 0.06*	0.51 ± 0.11*	0.58 ± 0.03*
Organic mulch 2 (PM2)	93.7 ± 3.58*	98.1 ± 1.73*	5.0 ± 1.09*	6.0 ± 2.23*	349 ± 1.58*	418 ± 1.87*	7.53 ± 0.04*	8.29 ± 0.03*	0.45 ± 0.06	0.53 ± 0.02*
Herbicide 1 (H1)	82.0 ± 2.86*	88.0 ± 0.66*	3.0 ± 0.70*	3.0 ± 1.0	312 ± 0.70*	275 ± 1.22*	4.67 ± 0.06*	3.65 ± 0.02*	0.31 ± 0.01	0.25 ± 0.09*
Herbicide 2 (H2)	75.1 ± 2.79*	68.7 ± 1.80*	3.0 ± 0.70*	2.0 ± 0.70	247 ± 1.58*	178±2.34*	3.23 ± 0.07*	3.42 ± 0.07*	0.21 ± 0.08*	0.29 ± 0.05*
Herbicide 3 (H3)	70.0 ± 2.68	71.4 ± 1.38*	3.0 ± 1.73*	2.0 ± 1.0	321 ± 1.41*	284 ± 1.73*	2.14 ± 0.02*	1.78 ± 0.06*	0.31 ± 0.09	0.21 ± 0.10*

*There is a statistically significant difference observed between control and treated plots (P≤0.05).

Table 6.3 Effect of different treatments on weed dynamics

Treatments	Weed count (n°/m^{-2}) (July)		Weed count (n°/m^{-2}) (Harvest)		Dry weed weight (g/m^{-2}) (Harvest)	
	2017	2018	2017	2018	2017	2018
Control	30.2 ± 1.91	22.1 ± 1.93	32.7 ±1.29	28.2 ± 2.02	128.6 ± 2.03	132.5 ± 1.80
PM	4.1 ± 1.05*	2.2 ± 0.22*	4.7 ± 0.57*	1.8 ± 0.45*	40.6 ± 3.04*	31.9 ± 1.54*
OM1	5.4 ± 0.43*	5.9 ± 0.36*	6.5 ± 0.96*	6.9 ± 0.36*	52.0 ± 2.65*	67.0 ± 3.22*
OM2	7.3 ± 0.83*	6.2 ± 0.36*	5.5 ± 0.28*	5.2 ± 0.27*	55.6 ± 2.49*	43.2 ± 4.44*
H1	2.3 ± 0.51*	1.8 ± 0.4*	1.2 ± 0.56*	0.9 ± 0.25*	35.4 ± 0.90*	28.5 ± 1.46*
H2	1.1 ± 0.27*	0.8 ± 0.25*	0.5 ± 0.33*	0.7 ± 0.31*	12.7 ± 1.22*	16.2 ± 1.60*
H3	0.4 ± 0.15*	1.2 ± 0.36*	0.2 ± 0.07*	0.6 ± 0.18*	16.4 ± 2.08*	17.3 ± 1.70*

*There is a statistically significant difference observed between control and treated plots (P≤0.05).

Table 6.4 Effect of different treatments on dry matter accumulation in different plant parts of stevia

Treatments	Dry biomass (g/plant)					
	Root		Stem		Leaf	
	2017	2018	2017	2018	2017	2018
Control	4.56 ± 0.23	5.89 ± 0.32	8.17 ± 0.70	10.13 ± 1.34	5.10 ± 0.53	2.16 ± 0.97*
PM	6.13 ± 0.20*	5.32 ± 0.45	9.16 ± 1.51*	9.18 ± 0.73	7.21 ± 1.30*	5.23 ± 1.01*
OM1	13.18 ± 1.05*	12.11 ± 0.75*	18.23 ± 1.75*	18.76 ± 0.28*	9.65 ± 2.45*	8.31 ± 0.82*
OM2	12.71 ± 0.49*	11.65 ± 0.56*	23.12 ± 1.74*	21.75 ± 2.16*	10.22 ± 1.35*	12.78 ± 1.42*
H1	8.21 ± 0.69*	9.43 ± 0.478*	12.21 ± 0.93*	17.34 ± 2.04*	6.78 ± 0.61*	5.72 ± 1.02*
H2	7.12 ± 0.25*	8.19 ± 0.68*	10.23 ± 1.36*	13.45 ± 1.09*	6.23 ± 0.84*	8.10 ± 1.07*
H3	6.74 ± 0.30*	7.45 ± 0.72*	7.35 ± 2.28*	15.18 ± 1.71*	5.13 ± 0.86	6.12 ± 2.55*

*There is a statistically significant difference observed between control and treated plots ($P \leq 0.05$).

Table 6.5 Observed trends of various parameters during both the years

Parameters	Year	Trend
Plant height	2017	H3 < Control < H2 < PM < H1 < OM2 < OM1
	2018	H2 < H3 < PM < Control < OM1 <H1 < OM2
Number of branches/plant	2017	Control < H3 < H2 = H1 < PM < OM1 < OM2
	2018	Control = H2 < H3 < PM < H1 < OM1 < OM2
Number of leaves/plant	2017	Control < H2 < PM < H1 < H3 < OM1 < OM2
	2018	Control < H2 < PM < H1 < H3 < OM1 < OM2
Chlorophyll (mg/g)	2017	H3 < H2 < PM < H1 < Control < OM2 < OM1
	2018	H3 < Control < H2 < H1 < PM < OM2 < OM1
Leaf area index	2017	H2 < H1 < H3 < Control < OM2 < PM < OM1
	2018	H3 < H1 < H2 < Control < PM < OM2 < OM1
Weed count (n^o/m)	2017	H3 < H2 < H1 < PM < OM1 < OM2 < Control
	2018	H2 < H3 < H1 < PM < OM1 < OM2 < Control
Dry weed weight (g/m^2)	2017	H2 < H3 < H1 < PM < OM2 < OM1 < Control
	2018	H2 < H3 < H1 < PM < OM2 < OM1 < Control
Root dry biomass (g/plant)	2017	Control < PM < H3 < H2 < H1 < OM2 < OM1
	2018	PM < Control < H3 < H2 < H1 < OM2 < OM1
Stem dry biomass (g/plant)	2017	H3 < Control < PM < H2 < H1 < OM1 < OM2
	2018	PM < Control < H2 < H3 < H1 < OM1 < OM2
Leaf dry biomass (g/plant)	2017	Control < H3 < H2 < H1 < PM < OM1 < OM2
	2018	Control < PM < H1 < H3 < H2 < OM1 < OM2

Mulching (synthetic and organic) is one of the substitutes for herbicide treatments to suppress weed growth and increase crop yield. In a study by Basuki and Sumaryon, 1990 it was reported that black plastic mulch sheets can efficiently retard weed growth in stevia cultivation. An experiment was conducted by Kumar et al., 2014 to assess the effect of various types of organic mulches on the growth, quality, and production of stevia crops. These mulch treatments included leaves of silver oak (*Grevillea robusta*), poplar (*Populus deltoides*), and pine needles (*Pinus roxburghii*). The organic mulch treatment significantly increased the crop yield, stevioside and rebaudioside content in stevia leaves as well as soil fertility and micro-flora (fungal and bacterial populations). Our results are in consonance with the report of Kumar et al., 2014. In another study by Coelho et al., 2018, vegetable-compost mulch was used for weed control in stevia cultivation. It was reported that compost treatment significantly reduces the weed count and also improves the soil properties. In the present study, the highest root, stem, and leaf biomass of stevia plants was observed in mulched plots because leaf biomass (organic matter) also improves soil properties (Gupta et al., 2009). Organic mulch treatments have also been used in various other commercial crops such as neem leaves for ginger (*Zingiber officinale* var. Suprabha) crop (Das, 1999), farmyard manure and straw mulch for turmeric (*Curcuma longa* L.) crop (Gill, et al., 1999), paddy straw for aonla (*Emblica officinalis*) crop (Shukla et al., 2000), straw mulch for potato crop (Kar and Kumar, 2007). In a recent study by Takacs-Hajos et al., 2019, the use of two ecofriendly fertilizers Dudarit and Sprintalga in stevia cultivation significantly increased (compared to control plots) the stevia leaf-biomass and did not affect the stevioside and rebaudioside content. Abouziena et al., 2008, conducted a two-year study for comparative assessment of organic mulch (rice straw), synthetic mulch (plastic sheet), and herbicide (glyphosate) spray-on weed control in Mandarin fruit cultivation. All the treatments significantly retarded the weed count as compared to the control plants but, the fruit yield increased only with rice straw mulch. Our results are also in agreement with this study because all the treatments significantly reduced the weed count and dry weed weight but the plant biomass was only increased with mulching treatment. There are several other reports which recommend the use of mulches for weed-free farming. A field trial was conducted by Ramakrishna and co-workers in 2005 (Northern Vietnam) to study the effect of different mulches (rice straw, polythene) on soil moisture and temperature, weed growth, and groundnut yield. Among them, polythene (synthetic) and straw (organic) mulch was found most effective for weed suppression and promotion of crop yield (Ramakrishna et al., 2006). Field-based experiments were also conducted to find the efficacy of herbicide sprays,

mulching with wheat residues, and intercropping with sesbania for weed suppression in dry seeded rice crops. Interestingly, mulching with wheat residues (@4t/h) and 30 days intercropping with sesbania was found most effective for weed suppression (Singh et al., 2007). Similar to stevia, ginger (*Zingiber officinale*) is a slow-growing plant and is susceptible to weeds during the early stages of growth. An experiment was conducted to assess the effect of different types of organic mulches on ginger crop yield and weeds. Maximum weed suppression (72%) and plant height were reordered with the treatment of one-year-old paddy straw with green leaf mulch and Lantana camara leaves. Maximum crop yield was recorded in plots treated with white plastic mulch as compared to other organic mulch treatments (Thankamani et al., 2016). In a similar report on potato cultivation, Genger et al., 2018 confirmed that compared to mechanical weed control methods, the application of straw mulch can effectively control the broad-leaf weeds and increase the yield in a late-season cultivar of potato.

CHAPTER 7

Optimization of *in vitro* regeneration and establishment of *Agrobacterium*-mediated transformation of stevia and molecular characterization

7.1. Callus induction

In this study, we used different concentrations of 2, 4-D (1−3 mg/l), Kin (1−2 mg/l), and BAP (1-3 mg/l) to obtain callus from different explants viz. leaf, nodes and shoot tips of in vitro raised stevia plants. Callus was initiated from leaf discs after 4-5 weeks on culture media while the other explants responded after 6−7 weeks. Hence, leaf discs were most efficient in callus formation and maximum callus induction was achieved on MS2 medium [2,4-D (2 mg/l) and Kin (1 mg/l)] (Table 7.1, Fig. 7.1). Significantly higher callus induction was reported with leaf explants cultured on MS1, MS2, and MS3 media as compared to nodes and shoot tips. The trend observed for callus induction in different media was MS2 > MS3 > MS1 > MS6 > MS5 > MS4 > MS9 > MS7 > MS8. Leaf discs were found most efficient for callus formation while shoot tips were found least effective.

Table 7.1 Percentage callus induction using different explants

Explant	Treatment ID	2,4-D (mg/l)	Kin (mg/l)	BAP (mg/l)	Callus induction (%)
Leaf	MS1	1	0	0	70* ± 5.00
	MS2	2	1	0	82* ± 5.29
	MS3	3	2	0	71* ± 5.56
Node	MS4	1	0	1	12 ± 1.73
	MS5	2	0	2	20* ± 1.00
	MS6	3	0	3	24* ± 1.73
Shoot tip	MS7	1	0	0	10 ± 0.50
	MS8	2	1	1	9 ± 1.10
	MS9	3	2	2	12 ± 3.60

The values are means of three replicates ± SD

7.2. Shoot regeneration

In this study, it has been found that less number of shoots was produced from callus in comparison to direct shoot regeneration from explants (Table 7.2) (Fig. 7.1). Although, leaf explants derived-callus cultured on MS3 media [BAP (1 mg/l) and NAA (0.5 mg/l)] showed maximum shoot regeneration (4 ± 1.00) while, the nodal sections cultured on MS6 media [BAP (1 mg/l) and NAA (0.5 mg/l)] exhibited maximum direct shoot regeneration (25 ± 3.2).

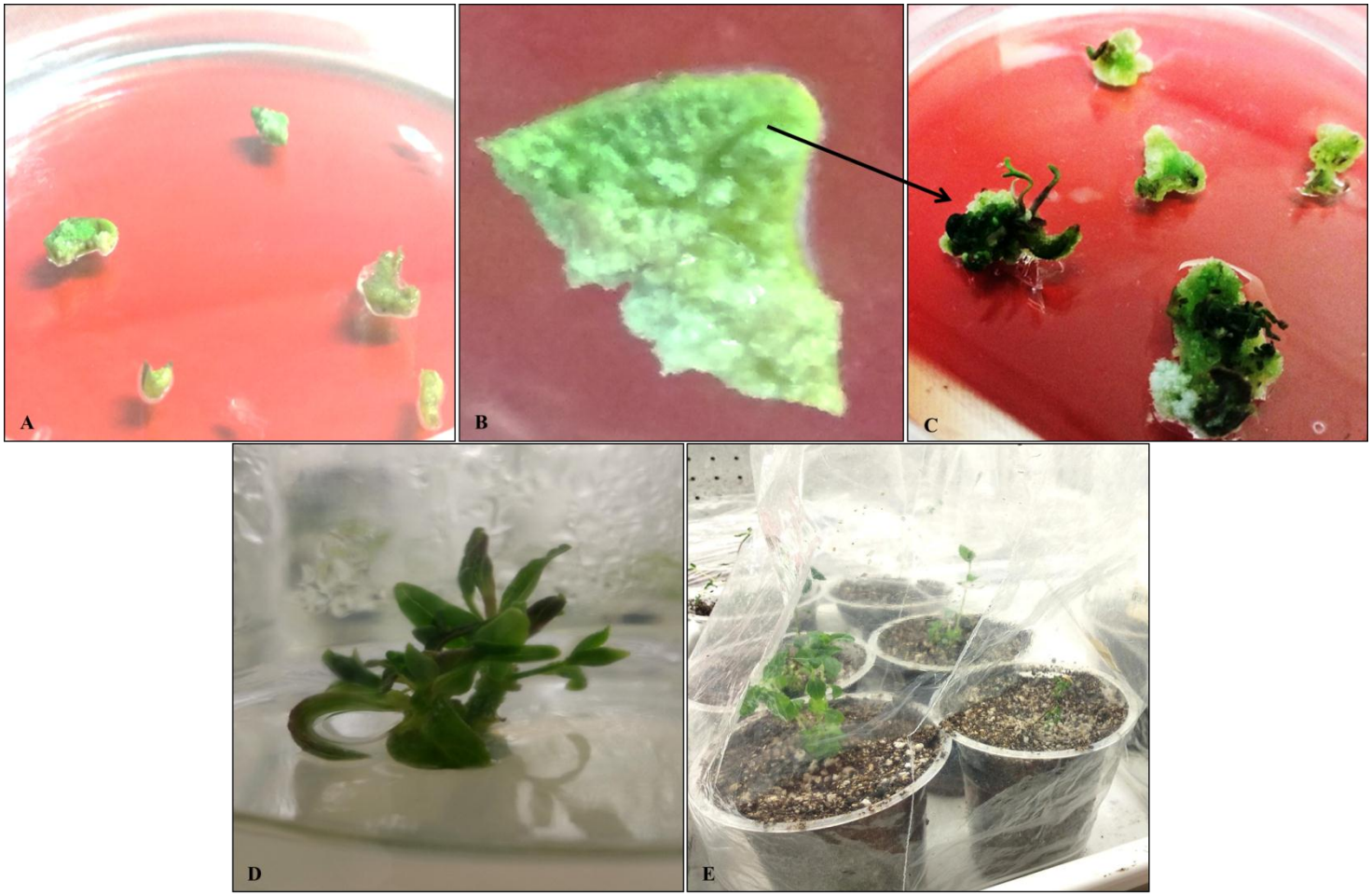


Fig. 7.1 *In vitro* regeneration of callus explants **(A)** Leaf discs for callus formation (bar = 1.8 cm), **(B)** Callus initiation (bar = 0.25 cm), **(C)** Shoot regeneration from callus (bar = 1.8 cm), **(D)** Shoot elongation (bar = 2.0 cm) and **(E)** Hardening of *in vitro* raised plantlets (bar = 4.0 cm)

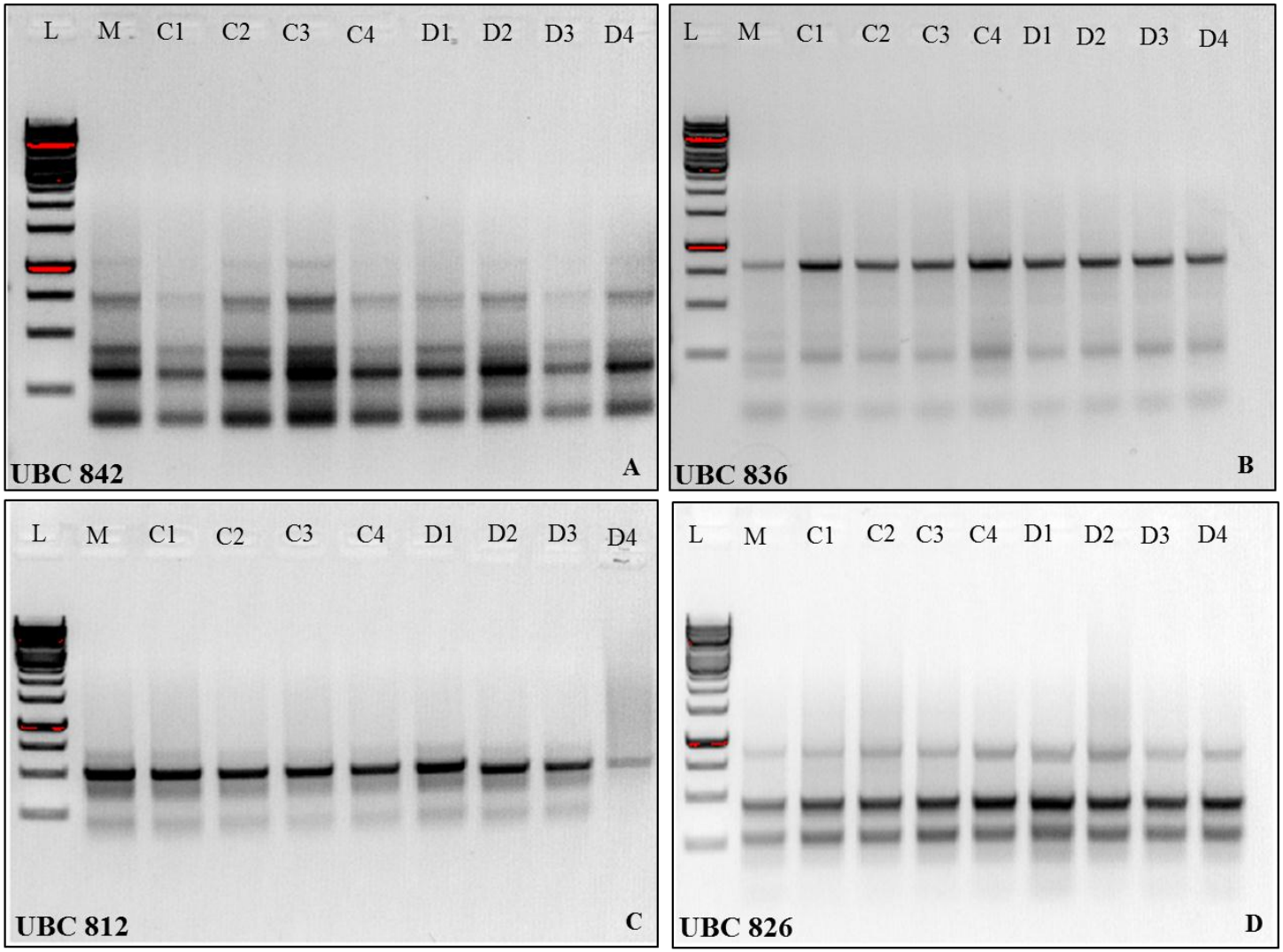


Fig. 7.2 ISSR-PCR profiles generated by primers **(A)** UBC 842, **(B)** UBC 836, **(C)** UBC 812 and **(D)** UBC 826. Lane L, 100 bp DNA Ladder; Lane M, field grown mother plant; Lanes C1 to C4, Indirectly regenerated plants; Lanes D1 to D4, Directly regenerated plants

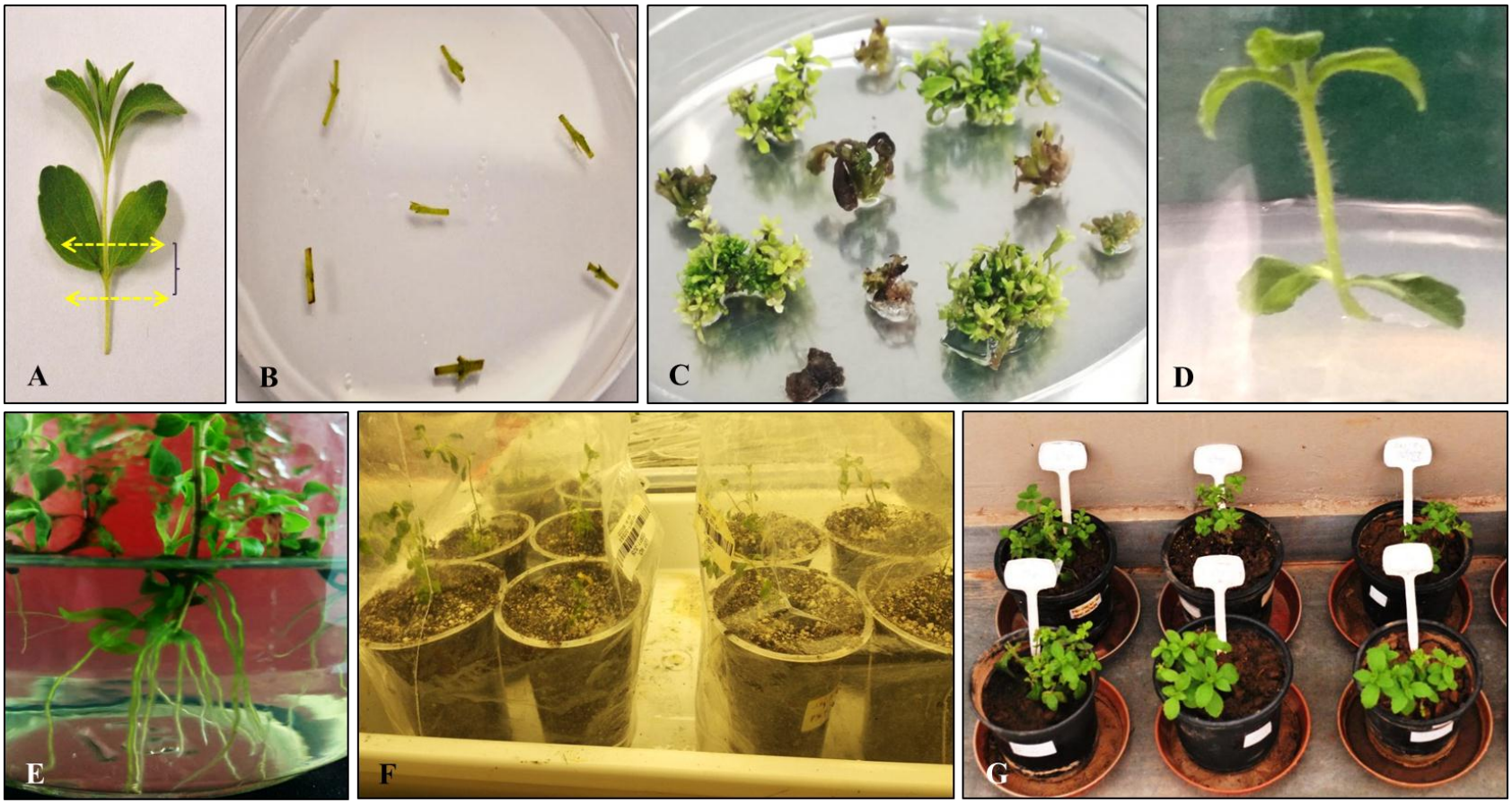


Fig. 7.3 Stevia transformation and *in vitro* regeneration of nodal-explants, **(A)** Explant (nodal segments) preparation for Agro-inoculation (bar = 1.0 cm), **(B)** Explants incubated on co-cultivation media (bar = 1.0 cm), **(C)** Regenerated explants subjected to anti-biotic selection (bar = 1.8 cm), **(D)** Shoot elongation in SEM (bar = 1.8 cm), **(E)** Rooting in RIM (bar = 1.0 cm), **(F)** Hardening of *in vitro* raised plantlets (bar = 2.5 cm) and **(G)** Green-house grown acclimatized transgenic stevia plants (bar = 4.5 cm).

Direct shoot regeneration is more efficient than indirect regeneration of shoots without callus formation. Callus-mediated shoots are asynchronous but directly regenerated shoots are homogenous. A maximum number of shoots was regenerated from nodal segments cultured on MS4, 5, and 6 media.

Table 7.2 Direct and indirect *in vitro* regeneration of stevia shoots

Explant	Treatments	BAP (mg/l)	NAA (mg/l)	No. of shoots regenerated directly	No. of shoots regenerated from callus
Leaf	MS1	0.1	0.0	12 ± 1.0	1 ± 1.00
	MS2	0.5	0.1	9 ± 3.40	0.00
	MS3	1.0	0.5	8 ± 1.00	4* ± 1.00
Node	MS4	0.1	0.0	18* ± 1.73	1 ± 2.00
	MS5	0.5	0.1	25* ± 5.56	0.00
	MS6	1.0	0.5	25* ± 3.2	1 ± 1.00
Shoot tip	MS7	0.1	0.0	5 ± 2.00	1 ± 1.00
	MS8	0.5	0.1	9 ± 2.00	0.00
	MS9	1.0	0.5	13 ± 3.00	0.00

The values are means of three replicates ± SD

7.3. Shoot elongation and rooting

The regenerated shoots were cut and further sub-cultured on SEM containing MS media supplemented with various concentrations of GA_3 (0.5 to 3.0 mg/l). It has been observed that 1.0 mg/l of GA_3 exhibited maximum significant shoot elongation as compared to other concentrations of GA_3 within 15 days of incubation (Fig. 7.4). The elongated shoots (~2 cm) were transferred to the root-induction medium (RIM). In our study, a maximum number of roots (9 ± 2.0) was reported from shoots (5−7 cm) regenerated from the nodal section, cultured on half-strength MS media devoid of PGR (Table 7.3). No. of roots and root length was found significantly higher in directly regenerated shoots from nodal sections cultured on MS5 and ½ MS media. A comparison between the number of shoots and roots originated from directly regenerated shoots and callus regenerated shoots are presented in Tables 7.2 and 7.3 respectively.

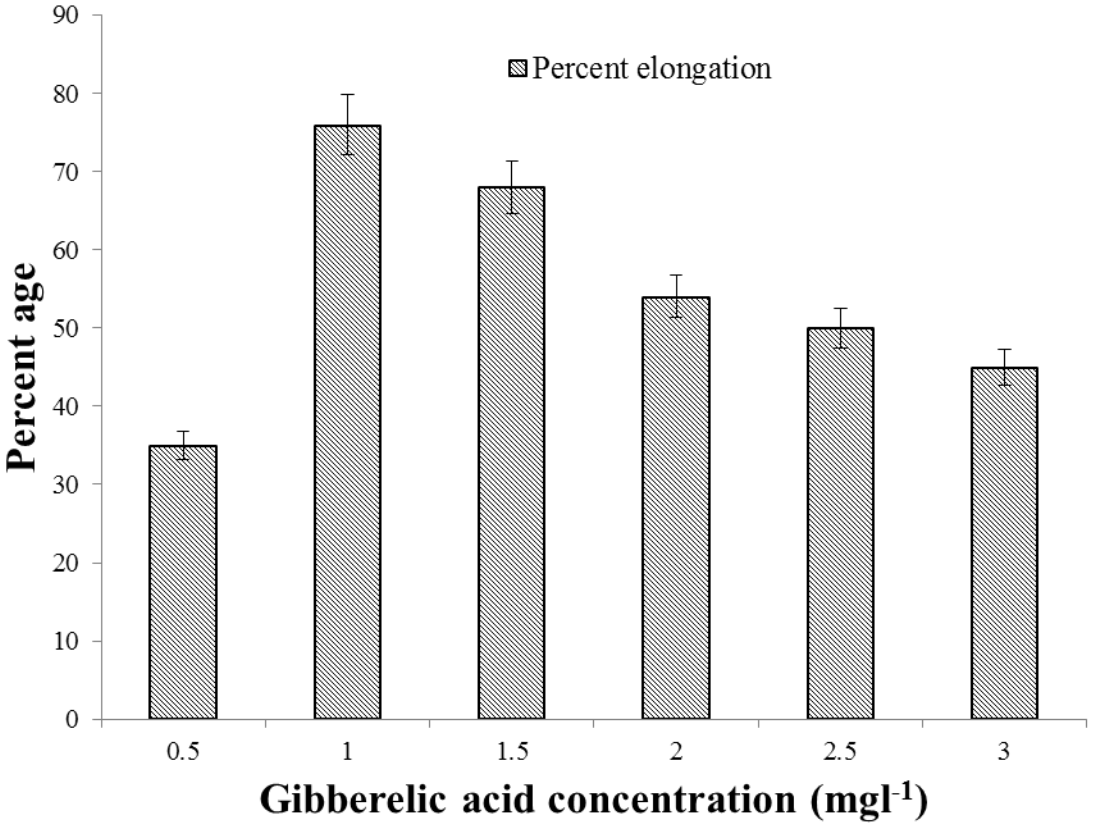


Fig. 7.4 Effect of various concentrations of GA_3 on shoot elongation of *in vitro* regenerated shoots of stevia

7.4. ISSR-PCR analysis

In the present study genetic stability of directly and indirectly regenerated plants was screened with ISSR markers. A total of 20 primers was tested, among them, only four were responded for amplification of genomic DNA of stevia. Amplification patterns of the mother plants and in vitro regenerated plants were shown by gel profiles of primers UBC 842, UBC 836, UBC 812, and UBC 826 (Fig. 7.2). All the fragments obtained from these primers were monomorphic and showed a similar DNA profile as that of the mother plant. A very low percentage of polymorphism was detected with UBC 812 primer. Various studies are available regarding the genetic stability of in vitro regenerated plantlets with ISSR markers. In a study by Thiyagarajan and Venkatachalam (2012), it has been reported that plantlets regenerated from nodal sections of stevia do not show any kind of genetic variations. It has been reported in a study that the genetic makeup of in

vitro regenerated plantlets showed variations in their DNA profile with an upsurge in the sub-culturing. The plants showed DNA profiles similar to the mother plant, while after the fifth subculturing polymorphism was detected in the DNA profile of some plantlets (Soliman et al. 2013). In a study by Ramírez-Mosqueda et al. 2016, an automated temporary immersion system (fortified with growth hormones) was used for the production of stevia seedlings at the commercial level. The plants regenerated from this system were checked for their genetic profile using ISSR markers and it has been detected that a very low percentage of genetic variations was there. In our study, very little polymorphism was detected in the case of indirectly regenerated plantlets, while all the plants which were raised from nodal sections showed a similar genetic profile as that of the mother plant. These results are in support with Singh et al. 2014, in which they have reported that no polymorphism was detected in plants raised from nodal sections while a low percentage of polymorphism was detected in plants regenerated through callus formation. Our results are also in agreement with ISSR analysis of *in vitro* regenerated plants of *Dictyospermum ovalifolium* (Chandrika et al., 2008), cauliflower (Leroy et al., 2000), *Cannabis sativa* (Lata et al., 2010, 2011), *Swertia chirayita* (Joshi and Dhawan 2007), *Nothapodytes foetida* (Chandrika et al., 2010), *Populus tremuloides* (Rahman and Rajora, 2001), *Hydrangea macrophylla* (Liu et al., 2011), *Musa* spp. (Ying et al., 2011),), *Anoectochilus formosanus* (Zhang et al., 2010) and *Gentiana stramina* (Tao et al., 2011).

7.5. Optimization of *Agrobacterium*-mediated transformation

To find the effect of the explants type on the genetic transformation of stevia, two types of explants (nodal sections and callus) were subjected to Agro-inoculation (O.D600 = 0.6). Young nodal sections (0.5 cm) exhibited a high regeneration response of 69.92%, in comparison to low response of 31.43% with callus explants (Fig.1A). Different concentrations (50, 100, 200, and 300 µM) of acetosyringone (As) were used to find their effect on transformation efficiency. Supplementation of 100 µm acetosyringone (As) gradually increased the percentage of responding explants to 72.5% while, a lower or a higher concentration (than 100 µM) resulted in a reduction of percentage regeneration response (Fig. 1C). Different cell densities (0.2, 0.4, 0.6 and 0.8 O.D600 of

Table 7.3 Number of roots and root length from callus derived shoots and directly regenerated shoots

Explant	Treatments	NAA (mg/l)	IAA (mg/l)	Callus-derived shoots		Directly regenerated shoots	
				Number of roots per plants	Root length (cm)	Number of roots per plants	Root length (cm)
Leaf	MS1	0	1	1 ± 1.0	2 ± 1.0	2 ± 1.0	4 ± 1.70
	MS2	1	2	0.00	0.00	3 ± 2.0	3 ± 1.00
	MS3	2	0	0.00	0.00	2 ± 1.0	4 ± 1.00
	1/2 MS	0	0	2 ± 1.0	2 ± 0.5	4 ± 1.1	5 ± 1.73
Node	MS4	0	1	3 ± 1.0	2 ± 1.0	5 ± 1.0	4 ± 1.00
	MS5	1	2	4* ± 1.0	4* ± 1.73	6 ± 1.0	4 ± 2.00
	MS6	2	0	2 ± 1.0	3 ± 1.0	7* ± 1.7	3 ± 1.00
	1/2 MS	0	0	7* ± 1.0	6* ± 2.0	9* ± 2.0	7.2* ± 2.90
Shoot tip	MS7	0	1	0.00	0.00	5 ± 2.0	4.2 ± 0.7
	MS8	1	2	2 ± 1.0	2 ± 1.1	7 ± 2.0	5.4 ± 2.1
	MS9	2	0	1 ± 0.5	2 ± 0.5	5 ± 1.0	4.4 ± 0.5
	1/2 MS	0	0	0.00	0.00	6 ± 1.0	6.3 ± 2.4

The values are means of three replicates ± SD

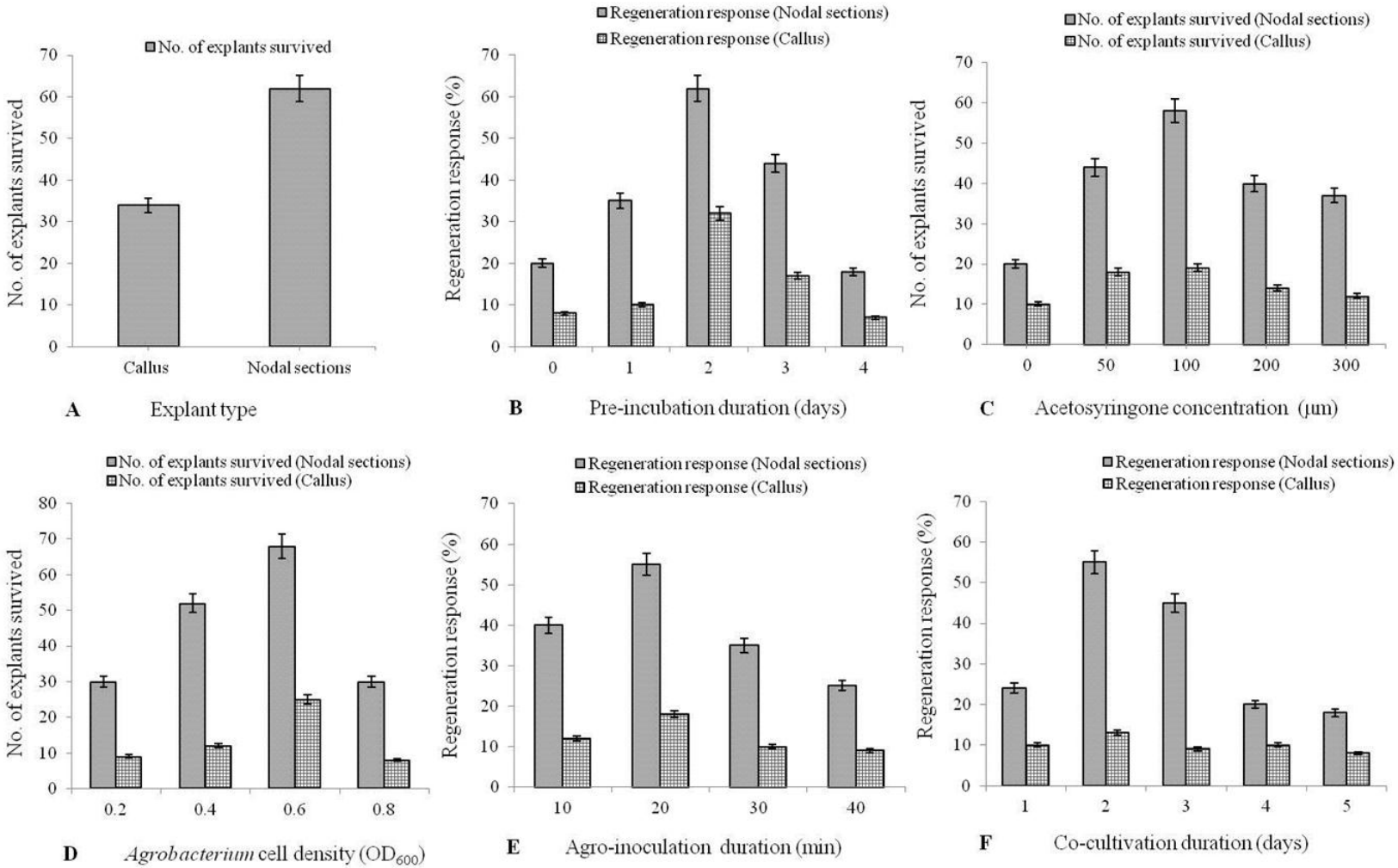


Fig. 7.5 Optimization of *Agrobacterium*-mediated nuclear transformation of stevia **(A)** Effect of explant type, **(B)** Concentration of acetosyringone (µm), **(C)** $O.D_{600}$ of *Agrobacterium* co-cultivation medium, **(D)** Pre-incubation duration (days), **(E)** Agro-inoculation duration (min) and **(F)** Co-cultivation duration (days).

Agrobacterium culture) were used to evaluate their effect on transformation efficiency Maximum regeneration response observed at $O.D_{600}$ was 0.6, while at higher $O.D_{600}$, (Fig. 7.5 C) *Agrobacterium* contamination was observed on explants (Fig. 7.5D). Maximum regeneration response (62%) was achieved with a pre-incubation duration of 2 days (Fig. 7.5B), Agro-inoculation duration of 20 min (55 % regeneration response) (Fig. 7.5E) and co-cultivation duration of 2 days (55 % regeneration response) (Fig. 7.5F) (Taak et al., 2020b). Incubation with *Agrobacterium* enhances the transformation process due to active cell division and formation of vir-inducing compounds which enhance the binding of *Agrobacterium* cells on the surface of newly synthesized cell wall.

The optimizations for *Agrobacterium*-mediated transformation and regeneration of stevia are (i) preferred explant type: nodal sections; (ii) acetosyringone (As) concentration: 100 µm; preincubation duration: 2 days; (iii) *Agrobacterium* cell density (OD600): 0.5-0.6; (iv) Agro-inoculation duration: 20 min; and (v) Co-cultivation duration: 2 days; (vi) shoot-induction medium (SIM): [MS Basal + BAP (1.0 mg/l) + NAA (0.5 mg/l)] ; (vii) shoot elongation medium (SEM): [MS Basal + GA_3 (1.0 mg/l)]; and (viii) root-induction medium (RIM): half-strength MS Basal. After co-cultivation, the explants were cultured on two step selection regime (MS media containing 2 and 4 mg/l of glufosinate ammonium). A high transformation efficiency of 40.48 ± 0.72% was achieved with nodal sections as compared to 27.94 ± 5.75% with the callus explants (Table 7.4). The parameters (for shoot regeneration, elongation and rooting) that were optimized for *in vitro* regeneration of stevia were in consonance with the regeneration after transformation.

7.6. Molecular characterization of putative transformants

Genomic DNA and total RNA from randomly selected nine putative transformants (TR1-TR9) were used for bar gene integration and expression analyses by PCR, RT-PCR, Southern-blot hybridization, and qRT-PCR. PCR result of the nine putative transformants showed amplification of anticipated 146 bp region of bar gene which was similar to a positive control (plasmid DNA developed with gene-specific primers) (Fig. 7.6A). However, no such amplification was observed with untransformed control plantlets. RT-PCR analysis of the nine promising transformants also revealed amplification of the

expected fragment of 200 and 150 bp, which verify the formation of bar and actin gene transcript in the transgenic plants (Fig. 7.6 B, C, D). However, the band intensities of the cDNA amplification product differed in each plant. The TR1 exhibited the highest band intensity while the TR9 exhibited the lowest. The nine T_0 transformants were also subjected to qRT-PCR analysis. The bar gene expression levels of the nine T_0 transgenic plants were in consonance with the respective band intensities obtained during RT-PCR analysis. TR9 having the lowest expression level was taken as a reference control for qRT-PCR result analysis. The fold change in expression of the bar gene was calculated in terms of the $2^{-\Delta\Delta CT}$ method and plotted on a graph. Fig. 7.6 E shows a near about 47-fold rise in the expression of the bar gene in the TR1 stevia plant than the control plant (TR9).

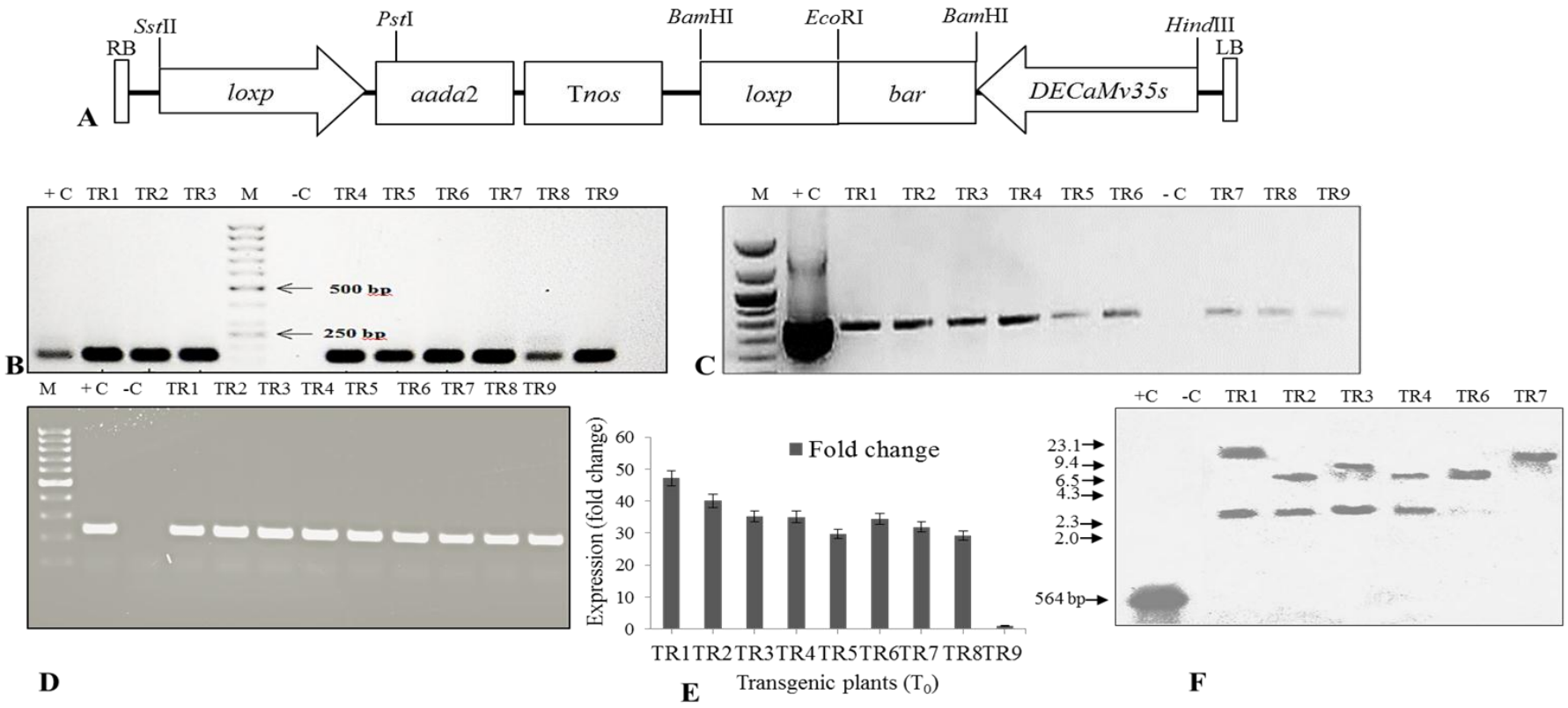


Fig. 7.6 Molecular characterization of T_0 transgenic stevia plants. **(A)** T-DNA region of pPZP200 vector harbouring *bar* gene driven by DECaMv35s promoter, **(B)** PCR amplification of 146 bp of *bar* gene, **(C)** RT-PCR analysis of nine randomly selected transformants showing an amplicon size of 200 bp (bar gene), **(D)** RT-PCR analysis of nine randomly selected transformants showing an amplicon size of 150 bp (actin gene), **(E)** relative fold change in expression of bar gene in T_0 transgenic plants with respect to TR9 (low expressing transgenic plant taken as reference). C control/wild type, TR-T_0 transgenic plants, M 100 bp ladder and **(F)** southern hybridization analysis of six T_0 transgenic plants probed with 552 bp bar gene. + C: 552 bp bar gene (positive control); − C: wild type (negative control)

Table 7.4 Stevia transformation (callus and nodal explant) and selection on glufosinate supplemented medium

Ist selection cycle					IInd selection cycle					
Explant	**Number of explants used (A)**	**Number of respondin g explants (B)**	**% response (B/A)**	**Number of shoots produced**	**Number of explants used (C)**	**Number of responding explants (D)**	**% respo nse (D/C)**	**Herbicide resistant plants produced**	**% transformation efficiency**	
Callus	80	15	18.75	50	80	22	27.50	06	27.27	27.94± 5.75
	80	10	12.50	58	98	39	39.79	12	31.42	
	80	20	25.00	45	74	20	27.02	04	21.42	
Nodal sections	80	34	42.00	128	187	87	46.52	35	40.81	40.48± 0.72
	80	23	28.75	98	145	112	77.24	44	39.65	
	80	28	35.00	112	143	123	86.01	50	40.98	

Table 7.5 Comparative analysis of morphological characters and chlorophyll content of control and transgenic plants

Parameters	Control plants	Transgenic plants
Plant height (cm)	73 ± 2	71 ± 1.73^{ns}
No. of leaves	215 ± 1	211 ± 2^{ns}
Total chlorophyll	7.85 ± 0.23	7.32 ± 0.04^{ns}

ns = Non-significant

Southern hybridization analysis of six highly expressing T_0 transgenic events revealed the transgene copy number. Genomic DNA from non-transgenic and T_0 transgenic plants (TR1, TR2, TR3, TR4, TR6 and TR7) was digested with EcoRI and subsequently hybridized with 552 bp of *bar*-gene-probe. The hybridization pattern of six T_0 transgenic plants revealed single and double copy integration that ranged in sizes from 3.5 to 20.5 kb, but the non-transgenic plant (control) did not show hybridization with the gene probe (Fig. 7.6 F).

7.7. Comparison of morphological characters and chlorophyll content

The transgenic plants did not exhibit any significant difference from the control in terms of morphological characters and chlorophyll content (Table 7.5). The average plant height observed in wild type was 73 cm while it was 71 cm in transgenic plants. The leaf count in wild-type and transgenic plants was 215 and 211 respectively. Moreover, the average chlorophyll content was 7.85 mg/g and 7.32 mg/g in wild-type and transgenic plants respectively.

7.8. Herbicide tolerance assay

Herbicide tolerance assay was performed by spraying the wild-type stevia (non-transformed) and T_0 transgenic plants with 8mg/l glufosinate-ammonium, in green-house. The wild-type stevia plants showed symptoms of chlorosis (Fig.7.7A), phytotoxicity, and defoliation after the 4th day of herbicide spray and even death after the 12th day. On the other hand, the transgenic plants did not show such symptoms and remained healthy (Fig. 7.7 B, C, D, E).

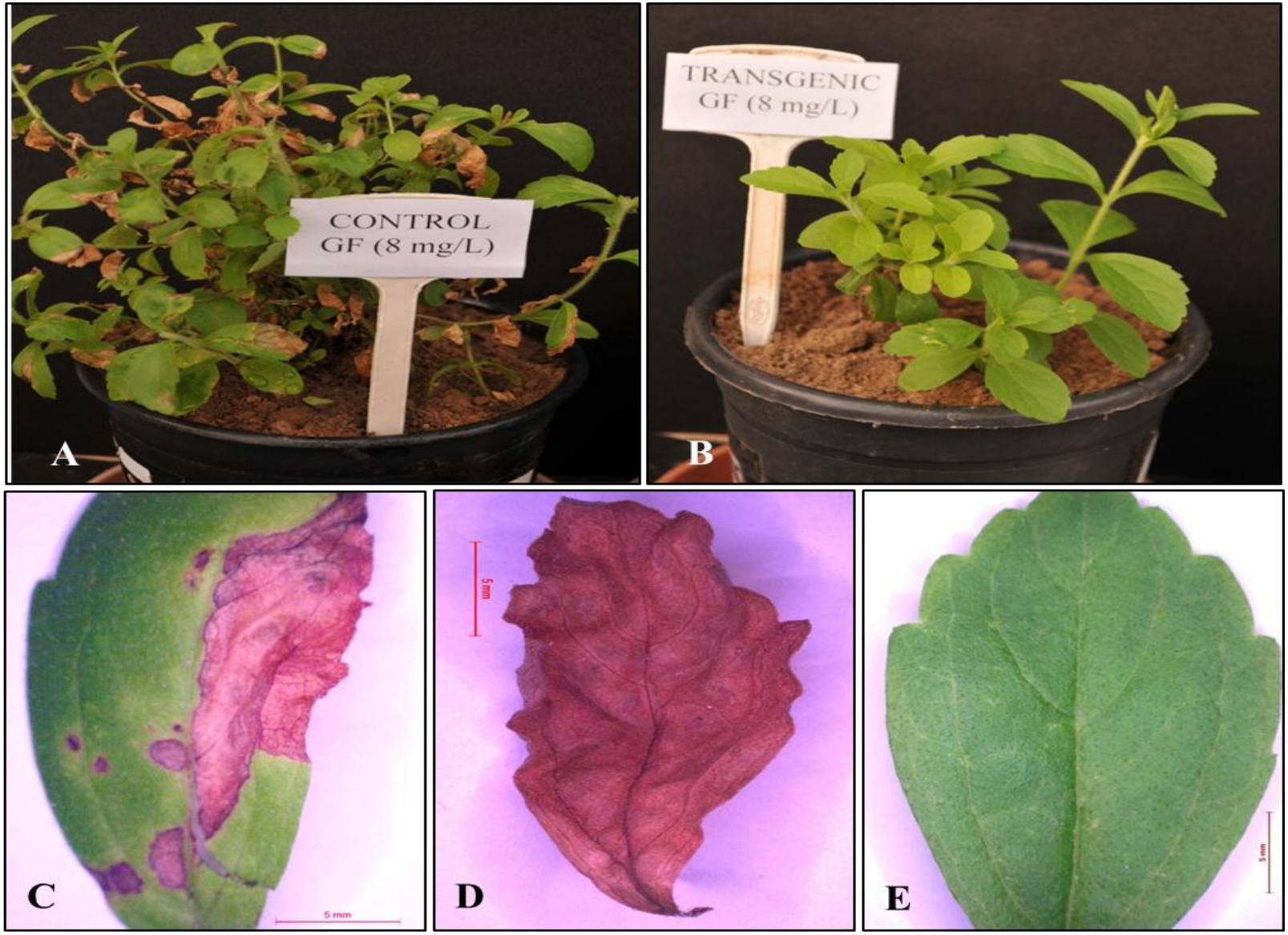


Fig. 7.7 Herbicide tolerance assay of T_0 transgenic stevia plants **(A)** Control (wild type) plants sprayed with glufosinate ammonium (8mg/l), **(B)** T_0 transgenic plants sprayed with glufosinate ammonium (8mg/l). Leaf morphology after glufosinate spray **(C)** Control leaf after four days of spray, **(D)** Control leaf after twelve days of spray and **(E)** Transgenic leaf after twelve days of spray. bar = 5mm

7.9. Residual phytotoxic effect

No harmful phytotoxic effect of glufosinate was found on seed germination of both the indicator plants. The difference between parameters (seed germination and seedling length) of control and treated pots was found non-significant. The corn seed germination percentage of 92.78 % was observed in water-treated pots (control) and 91−92 % in glufosinate-treated pots. Similarly, with cucumber seed germination percentage of 93.28 % was observed in water treated pots and 90−91 % in glufosinate treated pots. The corn seedling length of 33.43 cm was recorded in water-treated pots and 32−33 cm in glufosinate-treated pots. The cucumber seedling length of 7.34 cm was recorded in water-treated pots and 6−7cm in glufosinate-treated pots. Moreover, no phytotoxic effect was observed on seedlings of both the indicator plants (Table 7.6).

7.10. HPLC analysis

Major peak was shown at retention time 7.067 and 5.253 for steviosides and rebaudioside A respectively (Fig. 7.9, 7.10, 7.11 and 7.12). Rebaudioside A content in TR1 to TR10 plants was found in the order of TR9 (2.04 ± 0.05) < TR2 (2.053 ± 0.01) < TR4 (2.056 ± 0.005) < TR3 (2.063 ± 0.02) < TR10 (2.063 ± 0.03) = TR5 (2.063 ± 0.02) < TR7 (2.066 ± 0.01) < TR8 (2.07 ± 0.01) < TR1 (2.08 ± 0.03) < TR6 (2.083 ± 0.01) (Fig. 7.8). Maximum rebaudioside content was found in TR6 while minimum in TR9. Stevioside percentage in TR1 to TR10 plants was found in the order of TR2 (7.106 ± 0.01) < TR3 (7.13 ± 0.08) < TR7 (7.146 ± 0.01) < TR9 (7.15 ± 0.04) < TR4 (7.153 ± 0.04) < TR10 (7.16 ± 0.03) < TR8 (7.163 ± 0.01) < TR6 (7.166 ± 0.01) < TR1 (7.183 ± 0.05) < TR5 (7.193 ± 0.02). Highest stevioside content was found in TR5 while lowest in TR2. Maximum rebaudioside content was reported in TR6 plant while maximum stevioside content was reported in TR5 plant. No significant difference was reported between transgenic and control plants in terms of stevioside and rebaudioside content.

Table 7.6 Effect of basta herbicide residues (in soil) on germination percentage of indicator plants

Basta % (v/v)	Germination percentage (10 days after spray)		Plant height (cm)	
	Corn	Cucumber	Corn	Cucumber
Control (water)	92.78 ± 0.15	93.28 ± 1.03	33.43 ± 0.43	7.34 ± 0.35
0.25	91.00^{ns} ± 1.23	90.17^{ns} ± 0.88	32.23^{ns} ± 0.60	6.81^{ns} ± 0.71
0.50	92.34^{ns} ± 0.66	91.22^{ns} ± 0.28	32.00^{ns} ± 0.98	7.00^{ns} ± 0.23
1.00	91.87^{ns} ± 1.15	90.00^{ns} ± 1.06	33.15^{ns} ± 0.43	6.73^{ns} ± 0.40

The values are means of three replicates ± SD; ns = Non-significant

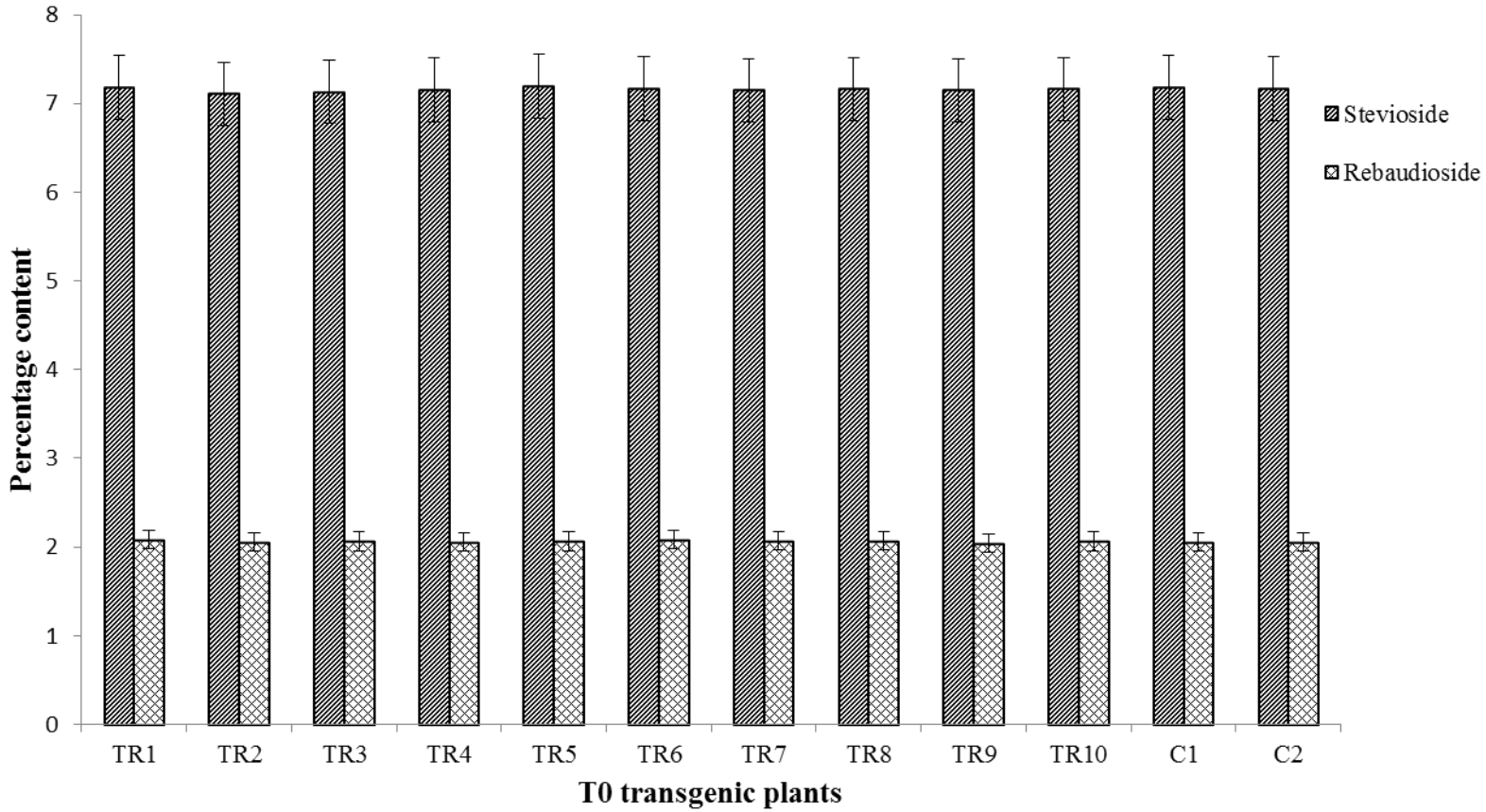


Fig. 7.8 Steviosides and rebaudiosides content in control and transgenic plants. TR1-10 (Transgenic); C1-2 (Control)

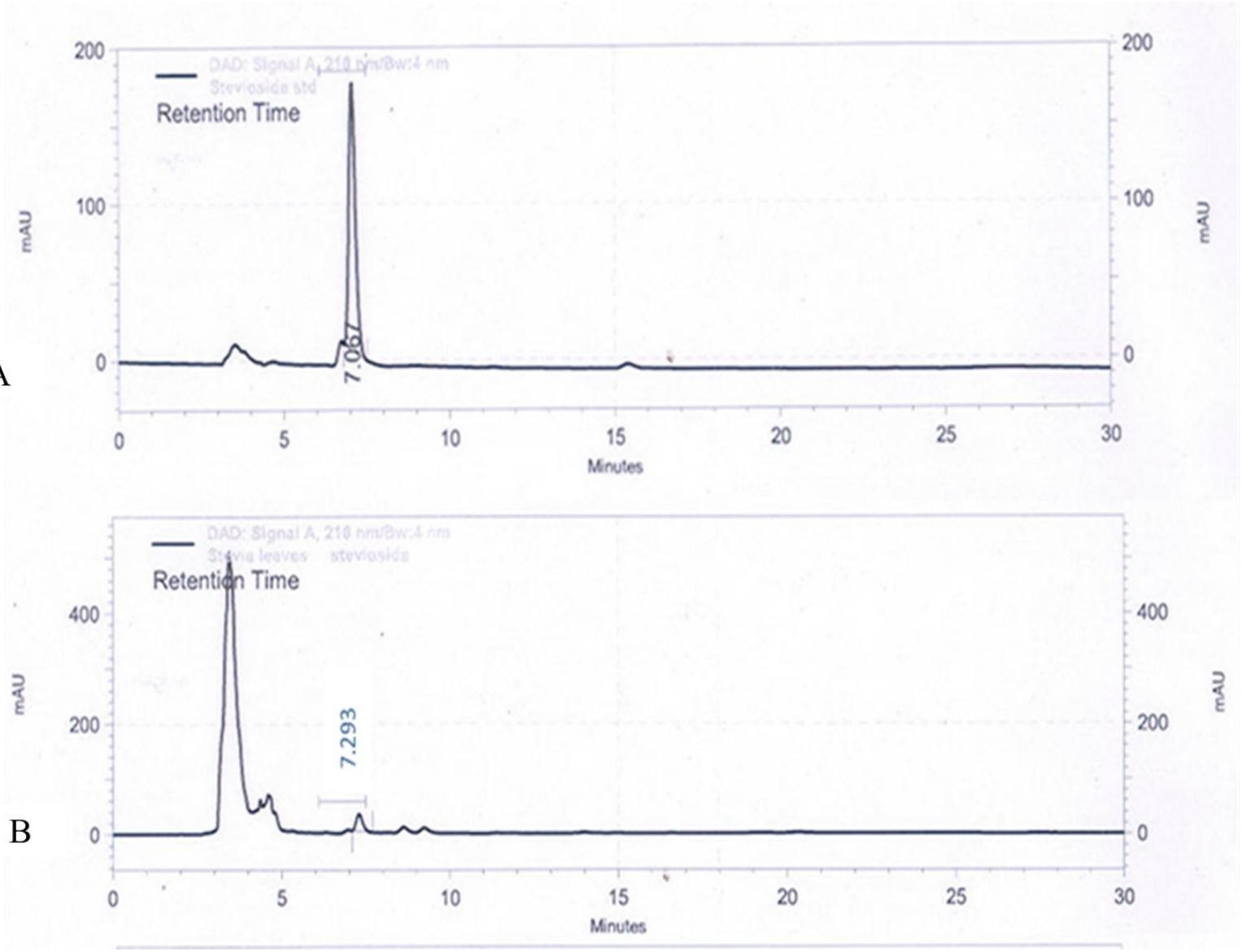


Fig. 7.9 HPLC chromatograms of transgenic and control plants **(A)** Stevioside standard (rt = 7.067), **(B)** Steviosides in control plant C1 (rt = 7.293)

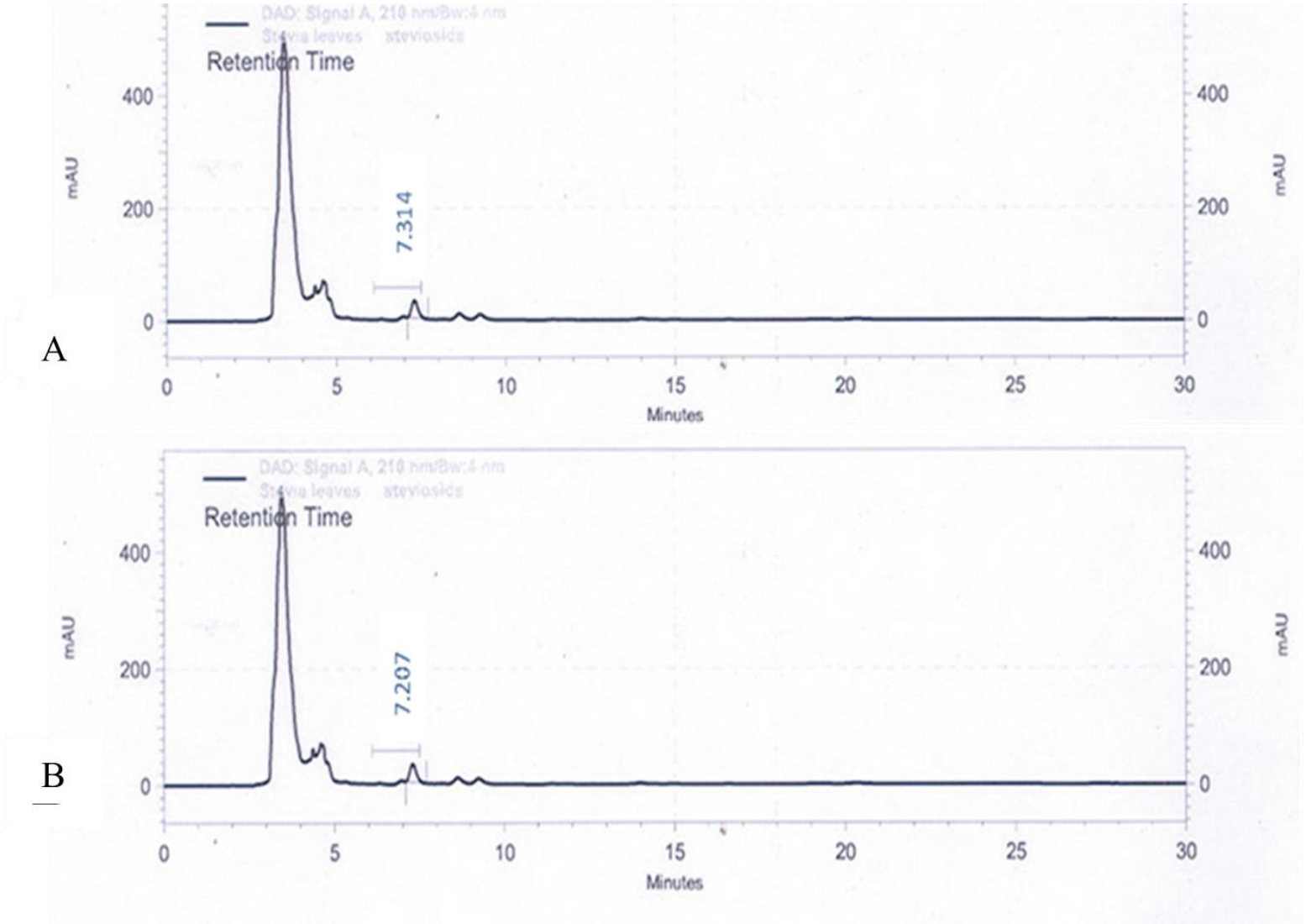


Fig. 7.10 HPLC chromatograms of transgenic plants **(A)** Steviosides in TR5 (rt = 7.314), **(B)** Steviosides in TR1 (rt = 7.207)

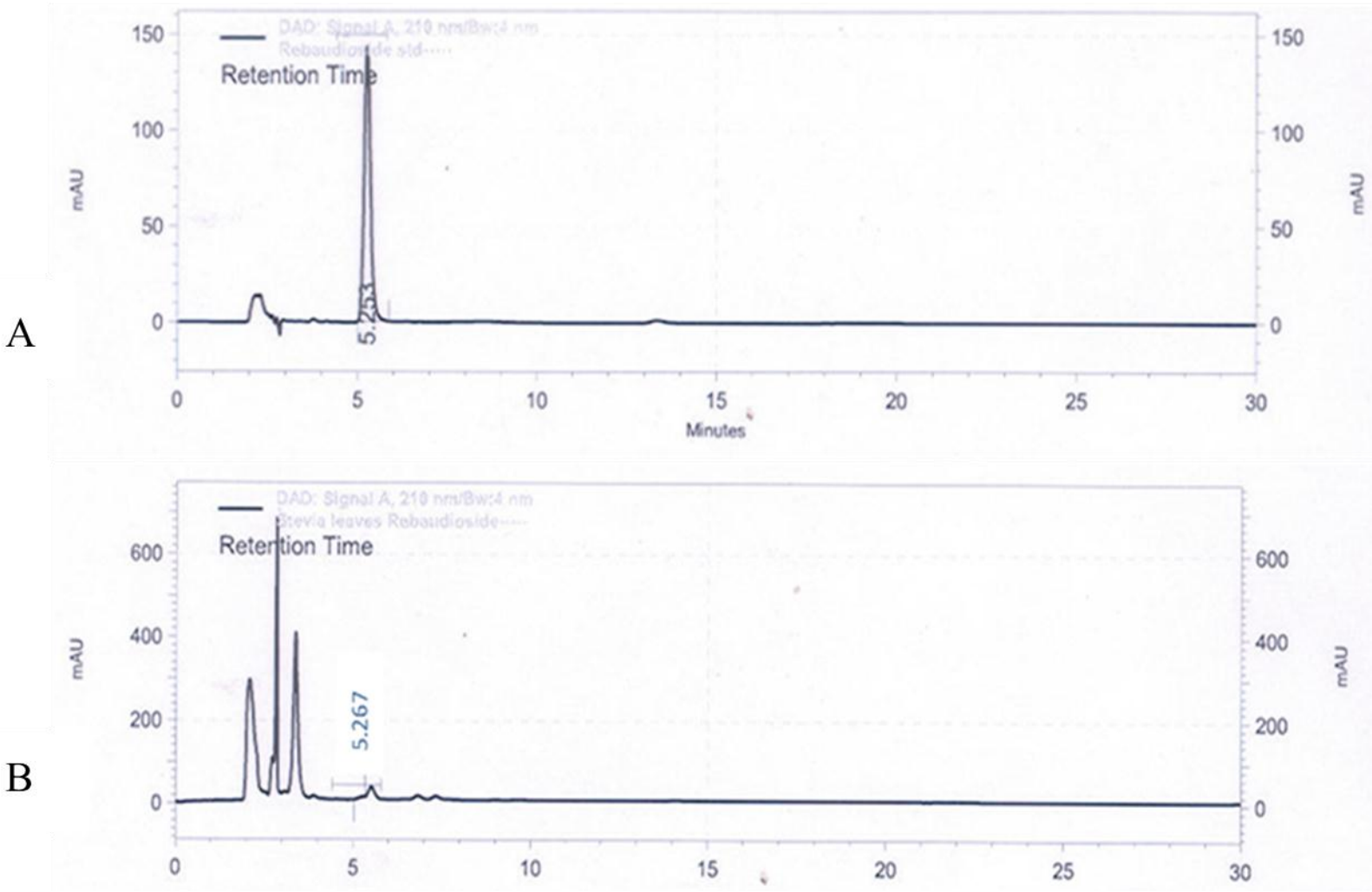


Fig. 7.11 HPLC chromatograms of transgenic and control plants **(A)** Rebaudioside A standard (rt = 5.253), **(B)** Rebaudioside A in control plant C1 (rt = 5.267)

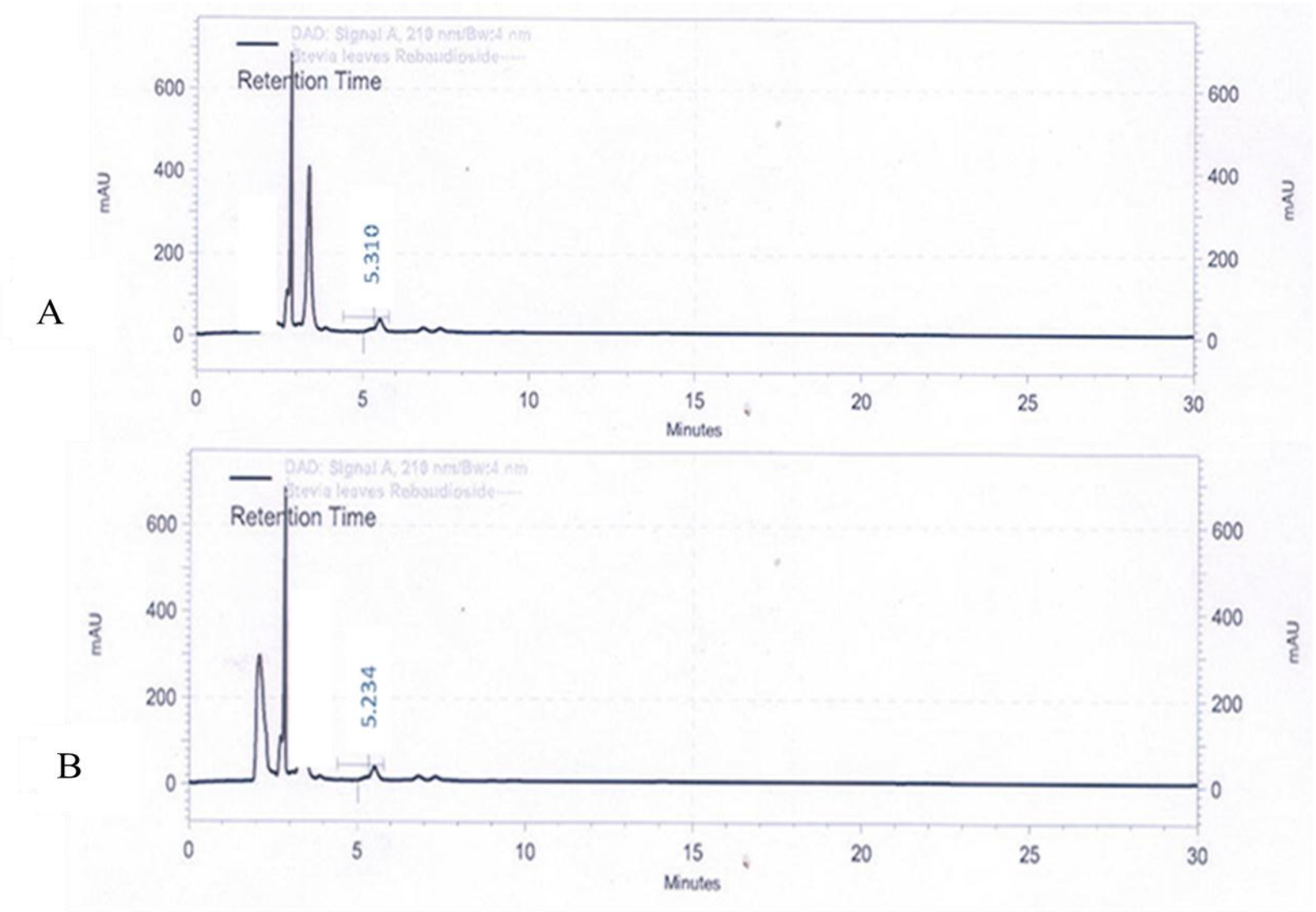


Fig. 7.12 HPLC chromatograms of transgenic plants **(A)** Rebaudioside A in TR5 (rt = 5.310) and **(B)** Rebaudioside A in TR1 (rt = 5.234). rt = retention time

7.11. Discussion

Various studies have been conducted to find the potential of callus formation from different stevia explants including leaves (Gupta et al., 2010a), anthers, cell suspension, flower (Ahmed et al., 2007), nodes, and roots (Gupta et al., 2010a). Our results are in consonance with that of Janarthanam et al., who found that leaf (juvenile) explants respond better with respect to callus formation (MS media fortified with 2.22 µM BAP and11.31 µM 2, 4-D) as compared to nodal explants 29.80 % of callus formation was achieved with leaf segments as compared to 60 % with nodal segments.

Patel and Shah have reported that maximum regeneration was found in nodal explants than leaf explants. Cent percent regeneration response was reported from nodal segments when placed on MS media fortified with 0.2 to 2 mg/l of NAA and 1 to 2 mg/l of BAP. 58.58% regeneration was reported from leaf segments when cultured on MS media fortified with 2 mg/l of NAA and 1 mg/l of BAP (Patel and Shah, 2009).

In a report by Gupta et al., 2010b, it has been reported that leaf sections produced the maximum amount of callus in a short time period when incubated on MS medium containing 2,4-D and NAA 1.0 mg/l and 0.75 mg/l respectively. Shiny green colored callus was obtained from root and leaf explant while brown and hard callus was obtained from nodal sections. An experiment was conducted by Sikdar and co-workers to find the most efficient explant for callus formation and direct regeneration of shoots from nodal sections. Among all the explants used nodal sections performed best in terms of callus formation (93.33 ± 6.67%) when cultured on MS media fortified with 2.0 mg/l of BAP and NAA. While inter-nodal sections showed 73.33 ± 6.67% of callus formation. In their study leaf explants showed poor callus formation (Sikdar et al., 2012). Nodal segments of stevia were first time cultured for shoot regeneration on MS media fortified with De Fossard vitamins, and different concentrations of NH4NO3 and 11 uM of IBA (Bespalhok-Filho et al. 1993). Directly regenerated plants are generally true to type or homogenous while callus mediated plants are asynchronous (Bhojwani and Razdan 1996). In a report by Sairkar et al., leaf discs were found highly efficient for callus formation as compared to nodal segments when incubated on MS media fortified with 1 mg/l Kin and 2 mg/l 2,4-D. Subculturing of callus was done after intervals of 25-30 days. In a study, a

combination of 1.5 mg/l BAP with 0.5 mg/l Kin was reported to efficiently induce multiple shoot regeneration from nodal explants (Ahmed et al., 2007). In a study by Debnath, maximum shoot formation was observed with nodal sections and shoot tips cultured on MS media fortified with IAA (1.13 mg/l) and BAP (2.0 mg/l) (Debnath, 2008). Singh and Dwivedi reported that nodal sections of stevia presented the highest (98%) shoot formation than 55% in shoot tips and 15% in inter-nodal sections. Regeneration from bud was reported prior (5.50 days) in nodal section than from other explants (Singh and Dwivedi, 2013). Undoubtedly, nodal sections have been the explants of choice for direct shoot regeneration using MS medium fortified with different concentrations of PGRs viz. 1.0 mg/l BAP and 0.25 mg/l IAA (Laribi et al., 2012), 0.5 mg/l of BAP and 2.0 mg/l of Kin (Mehta et al., 2012). According to Sreedhar et al. MS medium fortified with IBA 4.92 µM and 30 gm sucrose was most efficient for shoot elongation (Sreedhar et al., 2008). GA_3 was used for shoot elongation in stevia regeneration by Giridhar et al. 2010. It has been reported in their study that 0.05 µM of GA_3 was most efficient for maximum shoot elongation (Giridhar et al., 2010). Sivaram and Mukundan reported that rooting medium (MS media fortified with 4.90 µM IBA) also acted as shoot elongation medium (Sivaram and Mukundan, 2003). Various research groups have reported root-induction in *in vitro* regenerated shoots, using different combinations of growth hormones. Singh and Dwivedi, 2003 reported the maximum rooting response with ¼ MS media augmented with 1.0 mg/l of IBA + 50 mg/l of activated charcoal. This media combination produced an average 11 number of roots per shoot. Sreedhar et al. reported the best rooting response in shoots incubated on ½ MS fortified with 4.92 µM IBA and 15 g dm-3 sucrose (Sreedhar et al., 2008). Bespalhok-Filho and coworkers transferred *in vitro* regenerated shoots (of size 5 cm) into ½ and full-strength MS medium augmented with 90 uM of NAA. It has been reported in their study that ½ strength MS media was highly efficient for root formation (Bespalhok-Filho et al., 1993). In our study also it was found that ½ MS media composition was efficient in root formation in comparison to full strength media fortified with growth hormones.

Numerous studies have been performed regarding the glufosinate application on weed and crop plants. Manickavasagam and team made certain trails for herbicide resistance on two transgenic (herbicide-resistant) sugarcane cultivars Co671 and Co92061 (Manickavasagam et al., 2003). In this study *in vitro* regenerated non-

transformed sugarcane plants were sprayed with different concentrations (0.5, 2.5 and 5.0 g/l) of glufosinate-ammonium to find the lethal dose of herbicide. Glufosinate @ 2.5 g/l with an average dose of 6.25 mg/plant was observed as a lethal dose. This lethal dosage was then sprayed on transgenic plants under greenhouse conditions. Observations were recorded after 30 days to select the transgenic plants. Herbicide-resistant sweet potato (Ipomoea batatas L.) cultivar "Yulmi" was sprayed and painted with 0.5 % glufosinate herbicide (@ 900 mg/l) under greenhouse conditions to estimate their efficacy for herbicide resistance. It was found that control plants showed extensive leaf necrosis while transgenic plants remained green without any symptoms of leaf necrosis (Choi et al., 2007). In a study by Abdeen and Miki, 2009, it has been reported that glufosinate spray-on Arabidopsis plants led to inhibition of photosynthesis and ultimately plant death, after 6−48 h of spray. While the transgenic Arabidopsis harboring *bar* gene survived under the experimental conditions. Two Chinese rice cultivars (HD297T-31, HD297T-523) were also transformed with *bar* gene through *Agrobacterium*-mediated transformation, making them resistant to glufosinate herbicide. Transgenic HD297T-31 exhibited almost 100% resistance to glufosinate while, HD297T-523 showed moderate resistance (Tian et al., 2015). In our study, glufosinate adversely affected the wild type (non-transgenic plants), while the transgenic shoots survived on 4 mg/l glufosinate concentration. Herbicide tolerance assay with T1 transgenic jute (Corchorus sp.) plants was carried out to analyze their herbicide resistance potential. It was found that control plants died after 12 h of glufosinate spray (0.25%) while transgenic plants successfully recovered from herbicide stress after the 7th day of spray (Yang et al., 2016).

Zheng and coworkers successfully produced transgenic stevia lines by overexpressing the two important genes DXS1 (1-deoxy-d-xylulose-5-phosphate synthase 1) and KAH (kaurenoic acid hydroxylase). These genes play an important role in steviol glycoside synthesis pathway. A significant increase in steviol glycoside concentration was reported in SrKAH (42-54%) and SrDXS1 (67-88%) overexpressing lines. Similar to our study, no morphological alterations were observed in transgenic stevia plants (Zheng et al., 2019). *Agrobacterium*-mediated transformation system was also used by Wu et al., for the production of transgenic stevia lines using axillary young shoots of stevia as an explant. These transgenic plants overexpressed the gene

UGT76G1 (Protein=UDP-glycosyltransferase 76G1) which plays an important in the steviol glycoside synthesis pathway (Wu et al., 2020).

CHAPTER 8

SUMMARY

Stevia rebaudiana is now cultivated in many developed and developing countries as a main commercial crop due to its zero-calorie or natural sweetener properties. Weed management in stevia cultivation is an important factor for the yield sustainability of this crop. Weed emergence significantly reduced the crop yield, quality, and harvesting cost. The first and main objective of this study was the identification of the most common weed species which were found in stevia fields of different locations and evaluate the most efficient weed management strategy including the use of different herbicides and mulches. The second objective was an optimization of *in vitro* regeneration of stevia from different explants and includes the detailed procedure of *Agrobacterium*-mediated transformation of stevia and molecular characterization for selection of stable transgenic lines.

The present study has revealed that the application of glufosinate-ammonium (5l/h) herbicide offers the highest weed control efficiency as compared to other treatments. Minimum dry weed weight has been observed with the application of pendimethalin (2.2l/h) herbicide. The highest plant height and leaf count per plant were observed in the plots treated with rice straws mulch (15t/h). As expected, higher plant growth was observed in the plots treated with organic mulch as compared to herbicide treatments, and maximum weed suppression was recorded with herbicide treatments. Although, herbicide applications can successfully control weed growth their harmful impact on the crop yield and the environment raises new challenges to the farmers and scientists. Moreover, the high cost of herbicides and lack of knowledge among the farmers about their optimal dose and usage is also a matter of concern. Hence, an amalgamation of both chemical and cultural practices (integrated weed management strategies) is a prerequisite for effective weed management in a commercial crop like stevia where leaves are the main economic part.

Production of herbicide-resistant transgenic stevia is a convenient solution to address the weed problems in stevia cultivation. The efficient regeneration and transformation protocols are prerequisites for the generation of transgenic events with the desirable trait(s). The stevia stransformation protocol was successfully optimized in this study from nodal explants of stevia. In our study, much higher transformation efficiency (40.48 ± 0.72) was achieved with nodal sections of stevia plants rather than callus explants. Molecular characterization of transgenic plants through PCR, RT-PCR, and

Southern hybridization has successfully confirmed the integration of the *bar* gene into the stevia genome.

To date, this study is the first report on the production of herbicide-resistant stevia using the *Agrobacterium* tumefaciens-mediated transformation method. This study provides a strong recommendation on using this method for foreign gene incorporation into the stevia genome. Furthermore, for successful weed management, it is necessary to understand the concepts regarding weed identification, selection and dosage of herbicides as well as the residual effects of herbicides on the soil.

Although transgenic technology has a great contribution in reducing the usage of herbicides in the fields and increase the farmer's income to great extent. But, before their commercialization, various regulatory approvals from the government should be addressed. Furthermore, mulching and herbicide application both can be used in an integrated way so as to retard the weed growth and intensify the crop yield. The biotechnological method developed for stevia transformation is a scientific advancement in the area of plant biotechnology.

This work has already performed the successful gene transfer into the stevia genome. Therefore, further work should be conducted that how we can overexpress that particular gene. The present work can be elaborated further by using other elite genes driven by different promoters such as FMV (Figwort Mosaic Virus) and MMV (Mirabilis Mosaic Virus) and the best promoters can be isolated in terms of their efficiency. Various synthetic promoters can be produced so as to enhance the efficiency of the foreign genes. Furthermore, protoplast transformation can be performed in stevia for gene transfer. The future work should be focused on the topic that how we can extend the stevioside contents in the plant without affecting its yield. Stable expression of the herbicide tolerance gene can be checked in T1 generation after segregation. Thereafter, the expression and function can be studied till T8 generation so as to develop a stable transgenic stevia variety.

CHAPTER 9
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